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Research report

# Overexpression of muscle specific kinase increases the transcription and aggregation of acetylcholine receptors in Xenopus embryos

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**Abstract**

Muscle specific kinase (MuSK) mediates agrin-induced acetylcholine receptor (AChR) aggregation on muscle membrane at the neuromuscular junction (NMJ). To examine whether MuSK enhances NMJ formation during embryonic development in vivo, the level of expression of MuSK was manipulated in Xenopus embryos and the functional consequence at the NMJ was assessed. We found that overexpression of MuSK enhanced the formation of NMJ by increasing the aggregation of AChRs at innervated regions in developing embryos. The area of AChR aggregation increased by ~2-fold in MuSK injected embryos during the critical stages of NMJ formation. Interestingly, overexpression of MuSK in Xenopus embryos was found to induce the level of AChR transcript. Deletion of the Kringle domain in the MuSK construct did not attenuate the observed induction of AChR transcription and aggregation. Taken together, our findings provide the first demonstration that increased level of MuSK expression in vivo significantly elevate the aggregation and transcription of AChR at the NMJ in developing Xenopus embryos. 

*Theme:* Development and regeneration

*Topic:* Formation and specificity of synapses

*Keywords:* Receptor tyrosine kinase; Synapse formation; Agrin; Neuregulin; Neuromuscular junction; MuSK; AChR

## 1. Introduction

Reciprocal signaling between motor neurons and muscle fibers is critical for the formation of the neuromuscular junction (NMJ; [23]). One of the hallmarks for postsynaptic specializations during the formation of NMJ is the clustering of acetylcholine receptors (AChRs) on muscle fibers. The mechanisms controlling the aggregation of AChRs are at both transcriptional and post-translational levels. While neuregulin is known to induce transcription of specific genes at subsynaptic nuclei on muscle, agrin is well documented to exert the post-translational control by inducing the clustering of pre-existing AChRs that are initially distributed throughout the muscle membrane [4].

The activation of a muscle-specific receptor tyrosine kinase, MuSK, represents a critical step in the signaling pathway of agrin. The expression of MuSK is precisely colocalized with AChRs at the synapse of adult skeletal muscle [7,25] and MuSK has been shown to mediate agrin-induced tyrosine phosphorylation and aggregation of AChRs in cultured myotubes [12]. Furthermore, MuSK knockout mice die shortly after birth and do not exhibit the aggregation of AChRs at the NMJ [6], a phenotype similar to that observed in agrin knockout mice [10].

The Xenopus system is used in the present study to elucidate the functional roles of MuSK in the formation of NMJ during embryonic development. Sequence analysis of Xenopus MuSK identified in our laboratory reveals similarity with all known forms of MuSK, including Torpedo, human, mouse, rat and chicken [7,9,16,17,25]. Interestingly, the cloning of MuSK from Torpedo, Xenopus and chicken demonstrates shared characteristic features that are different from the known mammalian counterparts. For example, just like chicken and Torpedo MuSK, Xenopus MuSK contains a Kringle domain between the Ig-like IV

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domain and the transmembrane domain. Moreover, the expression of Xenopus MuSK is not restricted to muscle but can also be detected in adult spleen and developing central nervous system, including brain, eye and spinal cord. Similarly, chicken MuSK can be detected in the cerebellum during early embryonic development.

The well-characterized rapid development of NMJ and the external embryonic development in Xenopus provide a useful in vivo system to study the effects of ectopically expressed synaptic proteins during the formation of NMJ. In this study, the functional consequences of overexpressing wild type MuSK and its Kringle-domain deleted (K-del) mutant were examined at the innervated regions in the injected embryos. We report here that an induction of the AChR transcription and aggregation was observed at the NMJ in Xenopus embryos injected with wild type or K-del MuSK mutant, suggesting that MuSK enhanced the formation of NMJ during Xenopus embryonic development.

## 2. Materials and methods

### *2.1. Embryos*

*Xenopus laevis* were purchased from Carolina Biological Supply (Burlington, NC, USA) and were induced to spawn by injection with human chorionic gonadotropin (Sigma, MO, USA). Embryos were obtained by artificial fertilization and were staged according to Nieuwkoop and Faber [21].

### *2.2. DNA constructs*

The cDNAs encoding full length Xenopus MuSK, with or without myc-tag, were subcloned into the expression vector pCMV-Script as previously described [7]. The cDNA construct encoding green fluorescent protein (GFP) was purchased from Quantum (Canada). The K-del MuSK mutant was constructed by PCR with the Kringle domain deleted. Partial cDNA fragments encoding Xenopus AChRα, rapsyn and EF1 were obtained using exact oligoprimers based on published sequences [2,3].

### *2.3. Transfection and tyrosine phosphorylation assay*

The cDNA constructs for wild type MuSK and its K-del mutant were transfected into COS-7 cells using lipofectamine plus (Gibco; [8]). The cells were starved and the lysate was collected for tyrosine phosphorylation assay as previously described [7]. Briefly, the lysate was immunoprecipitated with polyclonal MuSK antibody, followed by immunoblotting with phosphotyrosine antibody (4G10, Upstate Biotechnology). The protein expression of MuSK and its mutant were confirmed by Western blot analysis.

### *2.4. RNA synthesis and injection*

Capped mRNA was synthesized by in vitro transcription of linearized plasmids using the mMessage mMachine transcription kit (Ambion, TX, USA). Xenopus MuSK and GFP cDNA constructs were linearized with XhoI and ApaI, respectively. The quantity and size of synthesized mRNAs were confirmed by running on a formaldehyde gel and 4 ng (in 2 nl) of mRNA was injected into the side of the animal poles of one-cell stage embryos which were previously dejellied using 2% cysteine chloride. RNAs were injected using a pressure-injection system (Medical Systems, PLI-188), together with a micromanipulator (Zeiss MMJ) and a bottom illumination stereomicroscope (Zeiss Stemi SV6). Injection pressure was maintained at 15 p.s.i. and the duration of pressure pulse ranged from 150 to 250 ms to allow a constant volume to be delivered for each injection. The injected embryos were transferred to 0.1×MMR solution, allowed to develop at room temperature, and staged for subsequent analysis.

### *2.5. Immunohistochemical analysis*

For whole mount immunohistochemical analysis, Xenopus embryos (stage 27–40) were deskinned and fixed in MEMFA for 2 h at 4°C. The embryos were washed and blocked with 10% FBS–PBS for 1 h at room temperature. For staining of AChR, the embryos were first incubated with biotin-conjugated α-bungarotoxin ($10^{-7}$ M; Molecular Probe, OR, USA) in 10% FBS–PBS at 37°C for 1 h. The embryos were then washed and incubated with FITC-conjugated streptavidin (1:1000; Cappel, Belgium). The results were confirmed by staining embryos with rhodamine-conjugated α-bungarotoxin (1:1000). For staining of MuSK, the embryos were first permeabilized with Triton X-100 (0.4%) in 10% FBS–PBS, then incubated with a specific MuSK antibody (1:1000) followed by FITC-conjugated secondary antibody (1:1000; Cappel, Belgium) as previously described [7]. The staining of MuSK was performed using two different MuSK antibodies raised against different epitopes and was confirmed by competition experiment using the appropriate peptides. To localize the ectopic MuSK expression, the embryos were injected with myc-tagged MuSK at one-cell stage. Stage 35 embryos were double stained with myc antibody (9E10, 1:200; Calbiochem) followed by FITC-conjugated secondary antibody and MuSK antibody followed by rhodamine-conjugated secondary antibody (1:1000; Cappel).

### *2.6. Collection and analysis of images*

AChR aggregates between myotomes posterior to the head regions were imaged by confocal microscopy. Projected fluorescent images from serial sections of embryos were analyzed using MRC-600 confocal microscope under

two magnifications (10× and 20×; Bio-Rad, CA, USA). Nine Z series for stage 30 and eleven Z series for stage 35 were scanned (at 5-μm intervals), with the brightest plane selected for image analysis. Microscopic settings were maintained unchanged throughout the analysis. The area of AChR aggregates was measured using Metamorph IMAGE ANALYSIS software (Universal Imaging). Ten embryos from each stage were subjected to immunohistochemical analysis for AChR and MuSK. More than six individual experiments were performed for each analysis, and the results of three representative experiments are depicted in Table 1.

### *2.7. Northern blot analysis*

Total RNAs were extracted from embryos (stages 18–41) by lithium chloride precipitation and Northern blot analysis was performed as described previously [7,15]. Probes used include partial cDNA fragments encoding Xenopus MuSK (amino acids nos. 1–731), AChRα (amino acids no. 1–457) and rapsyn (amino acids nos. 1–359). Equal loading of RNA was confirmed using a control probe EF1α.

## 3. Results

### *3.1. Expression profile of MuSK and AChR in Xenopus embryos during development*

Two transcripts of MuSK (~7 kb and ~3 kb) were detected in developing Xenopus embryos (stages 18–41; Fig. 1A). The larger transcript could also be detected in adult Xenopus muscle [7]. Similar to AChRα, the level of transcripts encoding MuSK increased during development and plateaued at ~stage 33, while that of AChRα (~2.3 kb) peaked at a later stage (~stage 41; Fig. 1A). To examine the localization of MuSK protein during the formation of NMJ, whole-mount staining for MuSK and AChR was performed on developing embryos (stages 27–40). AChR aggregates were barely detected between the myotomes at stage 27; AChR expression then increased and became confined to the NMJ at stages 35 and 40 (Fig. 1B). On the other hand, expression of MuSK protein was detected in muscle nuclei in body segments of the embryo at stage 27 and punctated staining was already detectable at the junctions between myotomes. MuSK expression increased at stage 35 and the staining became localized to the NMJ between myotomes (Fig. 1B). Comparison of the protein expression of AChR and MuSK revealed that the region of MuSK staining was more extensive than AChR aggregates at the NMJs. This finding is consistent with our previous observation that MuSK clusters are consistently larger in area than AChR clusters in vitro [7]. Occasionally, MuSK staining was also observed in motor nerves that innervate the NMJs in embryos at stage 40 (Fig. 1C).

The developmental regulation of MuSK transcripts was examined in isolated tail and limb muscles of Xenopus. The larger MuSK transcript was detected in tail muscle at stage 57, increased at stage 58 and decreased at stage 61 (Fig. 1D). While two MuSK transcripts first appeared in limb muscle at stage 56, their expression peaked at stage 59 and was downregulated during later stages of development. The developmental mRNA expression profiles of AChRα and rapsyn in muscle were similar to that observed for MuSK (Fig. 1E). Immunohistochemical analysis of limb muscle in stage 59 embryos revealed small AChR clusters at muscle periphery while MuSK protein was diffusely expressed in muscles with some of the staining colocalized with AChR (Fig. 1F). The extent of colocalization of MuSK and AChR staining increased throughout the later stages of development, and MuSK became confined to the NMJ regions in young Xenopus (1 year old; Fig. 1F).

### *3.2. Overexpression of MuSK in developing embryos*

To demonstrate that the injected mRNA was expressed in Xenopus embryos, GFP was used as control. The synthetic RNA for each construct (GFP or MuSK) was injected into one-cell stage embryos as described in Methods. The embryos with GFP fluorescence were found to be distributed in most cells of the embryos from stages 20 to 35, showing that the injected mRNA was expressed along the whole body segments of embryos analyzed (Fig. 2A). Similar studies were performed with the myc-tagged full length MuSK mRNA to identify the localization of injected MuSK. Whole mount immunohistochemical staining with myc antibody demonstrated that ectopic MuSK expression was detected throughout the embryo and was highly localized at muscle nuclei and NMJs (Fig. 2B). Co-staining experiments demonstrated that ectopically overexpressed MuSK was generally colocalized with the endogenous MuSK (Fig. 2B).

### *3.3. Tyrosine phosphorylation of MuSK and K-del mutant*

Unlike their mammalian counterparts, the forms of MuSK identified in Xenopus, chicken and Torpedo all contain a unique Kringle domain. While the potential function of the Kringle domain is so far unknown, it has been suggested to interact in the protease-mediated signaling pathway [17]. As a first step to examine the functional consequences of overexpressing wild type MuSK and its K-del mutant, we have investigated the ability of these constructs to autophosphorylate when overexpressed in COS-7 cells. Just like the wild type MuSK, the K-del mutant retained the ability to be phosphorylated (Fig. 2C).

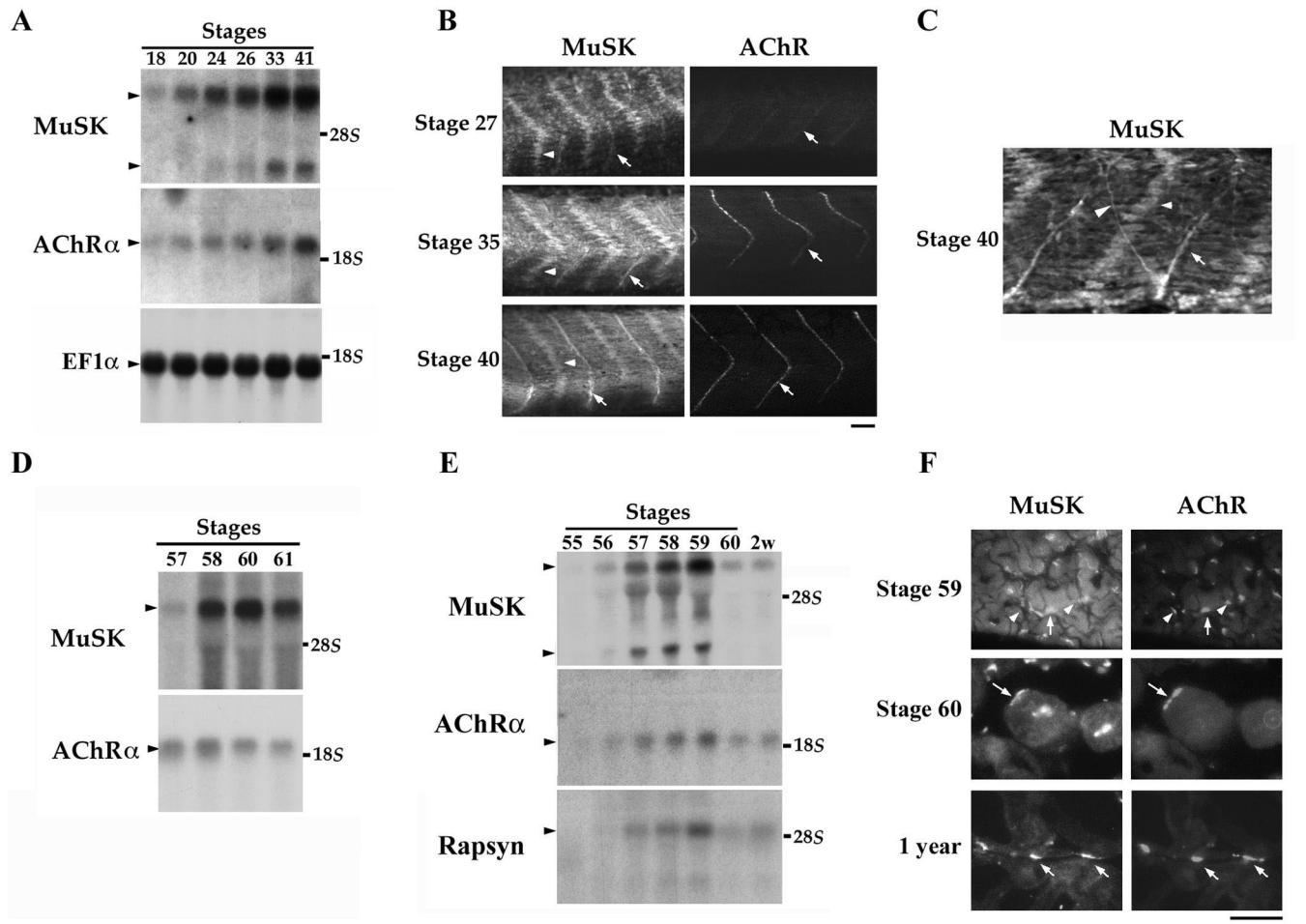


Fig. 1. (A) Northern blot analysis of MuSK and AChRα expression in developing Xenopus embryos. Positions of transcripts encoding different genes are indicated by arrowheads on the left, and ribosomal bands are depicted on the right. (B) Localization of MuSK and AChR protein at NMJ in developing Xenopus embryos by immunohistochemical analysis. Whole mount embryos from stages 27–40 were collected and stained with MuSK antibody (left panel; arrowhead depicts MuSK staining in muscle nuclei while arrow indicates MuSK staining localized to the NMJ). AChR aggregation at the NMJ was detected as described in Methods (right panel; arrow depicts AChR aggregates). Scale bar=25 μm. (C) Expression of MuSK protein in the motor nerve innervating the NMJ in stage 40 embryos (large arrowhead indicates MuSK staining on motor nerve while small arrowhead depicts MuSK staining in muscle nuclei and arrow indicates MuSK staining localized to the NMJ). (D) Northern blot analysis of MuSK and AChRα in tail muscle during embryonic development. (E) Northern blot analysis of MuSK, AChRα and rapsyn in developing limb muscles. (F) Localization of MuSK and AChR protein at the NMJ in developing limb muscles by immunohistochemical analysis. Arrows indicate the colocalization of MuSK and AChR while the arrowheads show the noncongruent MuSK and AChR protein localization.

### *3.4. Overexpression of MuSK induced AChRα transcription in embryos during the formation of NMJ*

The development of embryos injected with MuSK mRNA appeared to be normal compared to uninjected embryos, with no significant difference in survival or morphological defects. To evaluate the expression of injected Xenopus MuSK mRNA, Northern blot analysis using RNAs prepared from embryos of stages 27 and 30 was performed. As described above, the ~7 kb MuSK transcript was detected in Xenopus embryos. An extra transcript of ~3 kb, corresponding to the size of synthetic MuSK RNA, was detected in injected embryos (Fig. 3A). The level of injected MuSK transcript remained relatively unchanged from stage 27 to 30 and persisted in embryos until at least stage 35. Thus, the injected RNA could be retained and persisted in the embryos throughout the developmental stages examined in this study.

To examine the functional consequence of overexpressing MuSK on synaptic function, the mRNA expression of several postsynaptic proteins was analyzed. The mRNA expression for AChRα was found to be induced in MuSK injected embryos at both stages 27 and 30 (Fig. 3A). The induction of AChRα mRNA expression in embryos overexpressing MuSK was found to be consistent in four individual experiments (increase of ~1.7-fold at stage 27; Fig. 3C). On the other hand, there was no significant increase of the rapsyn transcripts in MuSK overexpressing embryos. Equal loading of RNA was confirmed by probing with EF1α cDNA fragment (Fig. 3A).

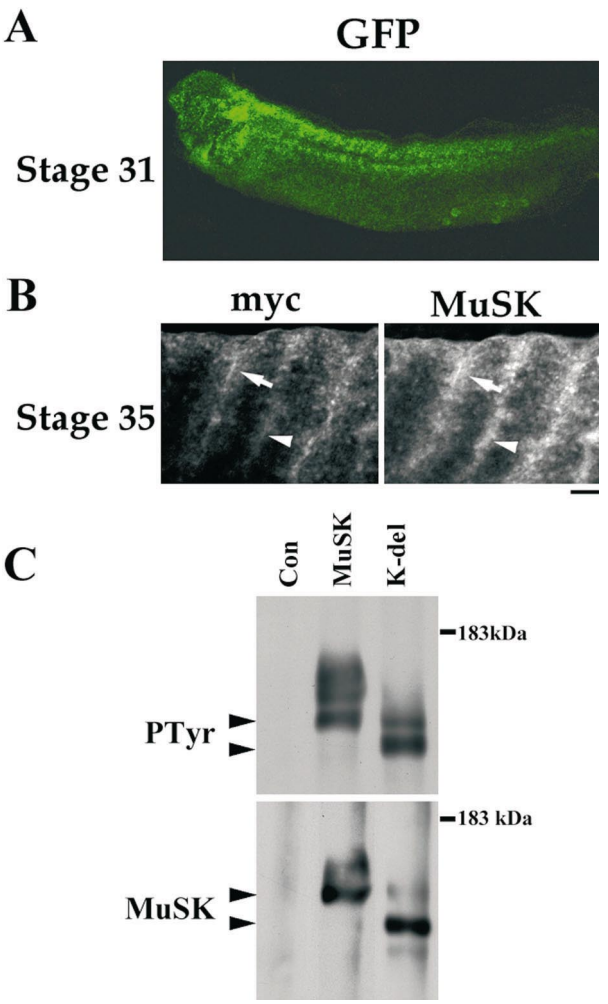


Fig. 2. (A) GFP fluorescence was detected in most cells of the stage 31 embryo which was injected with GFP mRNA. (B) The ectopically expressed MuSK was mainly localized to muscle nuclei (arrowhead) and the NMJ (arrow). Whole-mount double staining on stage 35 embryo injected with myc-tagged MuSK was performed using myc antibody (left panel) and MuSK antibody (right panel). Scale bar, 25 μm. (C) Phosphorylation assay of different MuSK constructs in COS-7 fibroblasts. Autophosphorylation of MuSK was induced in COS-7 cells by overexpressing wild type MuSK or K-del mutant. Western blot analysis of MuSK showed the relative equal loading of MuSK proteins (lower panel).

To investigate whether the Kringle domain identified in Xenopus MuSK was required for the observed effect on the induction of AChR transcript, the K-del MuSK mutant was ectopically expressed in embryos using the same approach. A transcript of ~2.5 kb, corresponding to the size of synthetic K-del MuSK RNA, was detected in the K-del mutant injected embryos collected at stages 27 and 30 (Fig. 3B). Similar increase in AChR transcript, comparable to that of the wild type MuSK, was observed in embryos injected with K-del MuSK construct (increase of ~1.8-fold compared to that of control at stage 27; Fig. 3B and C).

### *3.5. Overexpression of Xenopus MuSK and K-del mutant increased AChR aggregation at the synaptic regions in developing embryos*

To investigate the effect of overexpressed MuSK on the formation of NMJ, AChR aggregation in MuSK injected embryos was compared with that in buffer injected controls. GFP injected embryos were also used as controls. While GFP overexpression did not affect AChR aggregation, overexpression of full length MuSK significantly increased the AChR aggregation in developing embryos at stages 30 and 35 (Fig. 4A) and 32 (data not shown). The increase of AChR aggregation observed in MuSK-overexpressing embryos was mainly localized to the NMJs between myotomes. In addition to a concomitant increase in AChR aggregation in the extrajunctional regions, a significant increase in the area of AChR aggregation was induced at the NMJ of MuSK overexpressing embryos (Fig. 4B). The results from three representative experiments are summarized in Table 1. An ~2-fold increase in the area of AChR aggregates was detected at the NMJ of MuSK injected embryos when compared to buffer-injected control. Similar results were obtained with the K-del MuSK mutant (Fig. 4C and Table 1).

## 4. Discussion

In the present study, we have examined the consequences of overexpressing MuSK or its K-del mutant in Xenopus embryos to address the functional role of this receptor tyrosine kinase in enhancing the formation of nerve–muscle synapses during embryonic development. Our findings provide the first demonstration that increased level of MuSK expression in vivo significantly elevate the aggregation of AChR at the NMJ in developing Xenopus embryos.

The level of AChRα transcripts in Xenopus embryos has previously been characterized to reach the maximum level at stage 41, a stage at which the tadpoles swim freely [2]. Our findings in this study demonstrate that while MuSK and AChRα exhibit similar expression profile in embryos during development, the maximum level of MuSK mRNA expression detected at stage 33 appears to precede that of AChRα. Furthermore, MuSK and AChR expression can already be detected at the intersomitic boundaries during the early critical stages of NMJ formation, e.g. stage 27. Such pattern of cellular localization for MuSK and AChR is consistent with that observed in chicken and rat [3,5]. In the present study, we have demonstrated that prominent expression of MuSK transcripts detected in tail muscle and limb muscle during development declined to a low level upon maturation of the NMJ. Taken together, the expression profile of MuSK observed in Xenopus muscle is similar to that reported for mammalian muscle.

In this study, we found that overexpression of MuSK in

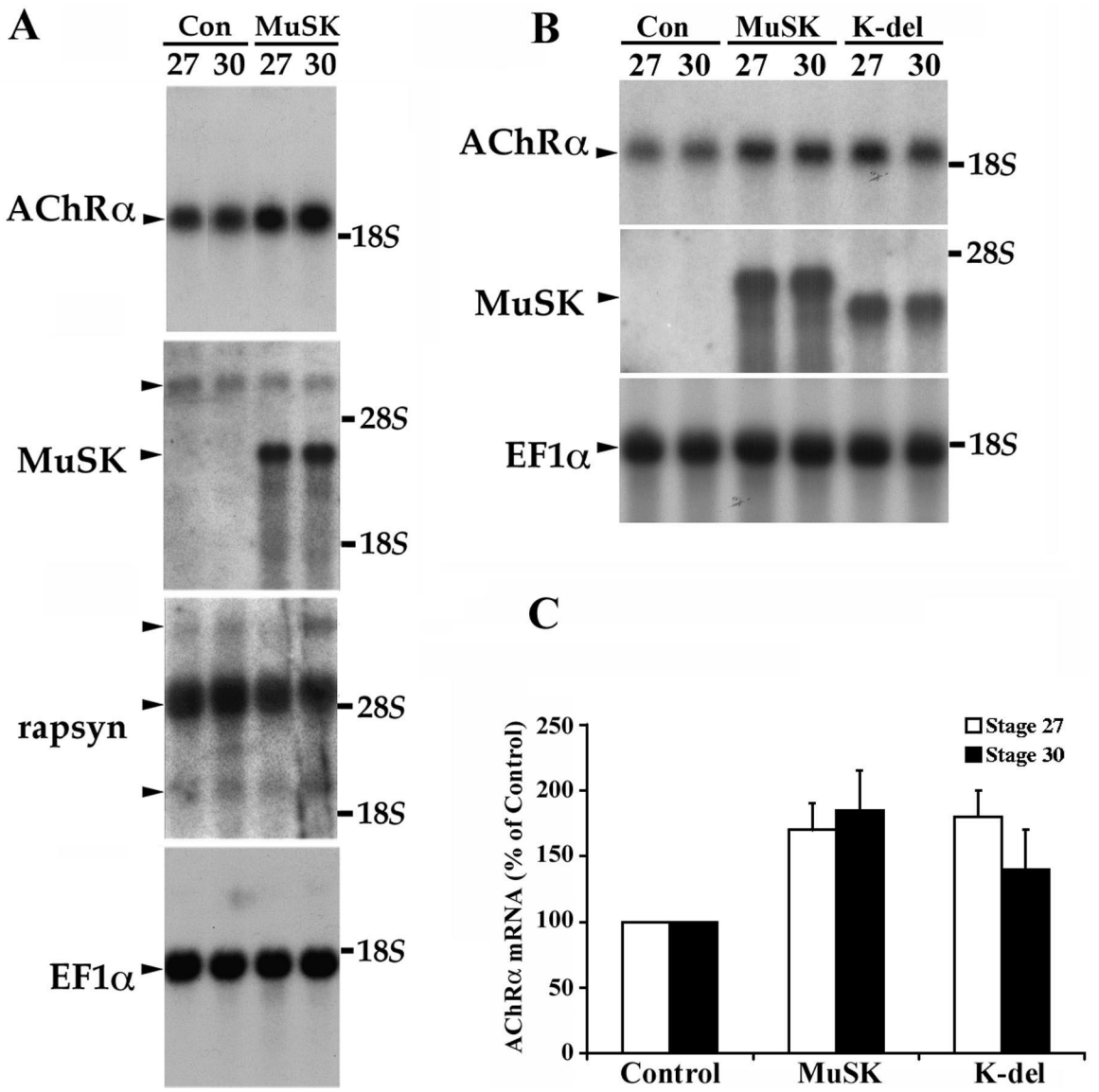


Fig. 3. Northern blot analysis for AChRα transcript in control, wild type MuSK and K-del mutant injected embryos at stage 27 and 30. (A) A consistent increase of ~1.7-fold in the level of AChRα transcript was detected in wild type MuSK overexpressing embryos (MuSK) when compared to buffer-injected controls (Con); the mRNA expression of MuSK, AChRα, rapsyn and EF1α was depicted. (B) Similar increase in the induction of AChRα in the embryos injected with K-del MuSK mutant. The persistent expression of the injected RNA and the equal loading of RNA was confirmed by probing with MuSK and EF1α, respectively. (C) Quantitation of the relative AChRα transcript in embryos injected with buffer, wild type MuSK and K-del MuSK mutant at stage 27 or stage 30.

Xenopus embryos results in a ~2-fold increase in AChR aggregation. Since constitutively active MuSK is capable of activating the downstream signaling pathways [19], it is possible that overexpression of MuSK results in dimerization of the receptors which leads to the enhancement of synaptic development in vivo. However, whether the activation of overexpressed MuSK in Xenopus embryos is dependent on the presence of agrin remains to be determined. In addition to increasing the aggregation of AChRs, overexpression of MuSK in Xenopus embryos also induces the transcription of AChRα in muscle fibers. Our finding is consistent with the report that agrin can regulate subsynaptic transcription in muscle, such as that of AChR [11,18]. It is likely that activated MuSK signaling might stimulate AChR transcription, e.g. by recruiting muscle-derived NRG that activates the ErbB receptor signaling pathway [20]. Indeed, mutant mice lacking MuSK expression exhibits defects in NMJ formation, including the reduction in synapse-specific AChR gene transcription [6].

A unique Kringle domain is present in the forms of MuSK identified in Torpedo, Xenopus and chicken but not in the mammalian orthologs [7,9,16,17,25]. Kringle domain has been identified in many of the extracellular serine

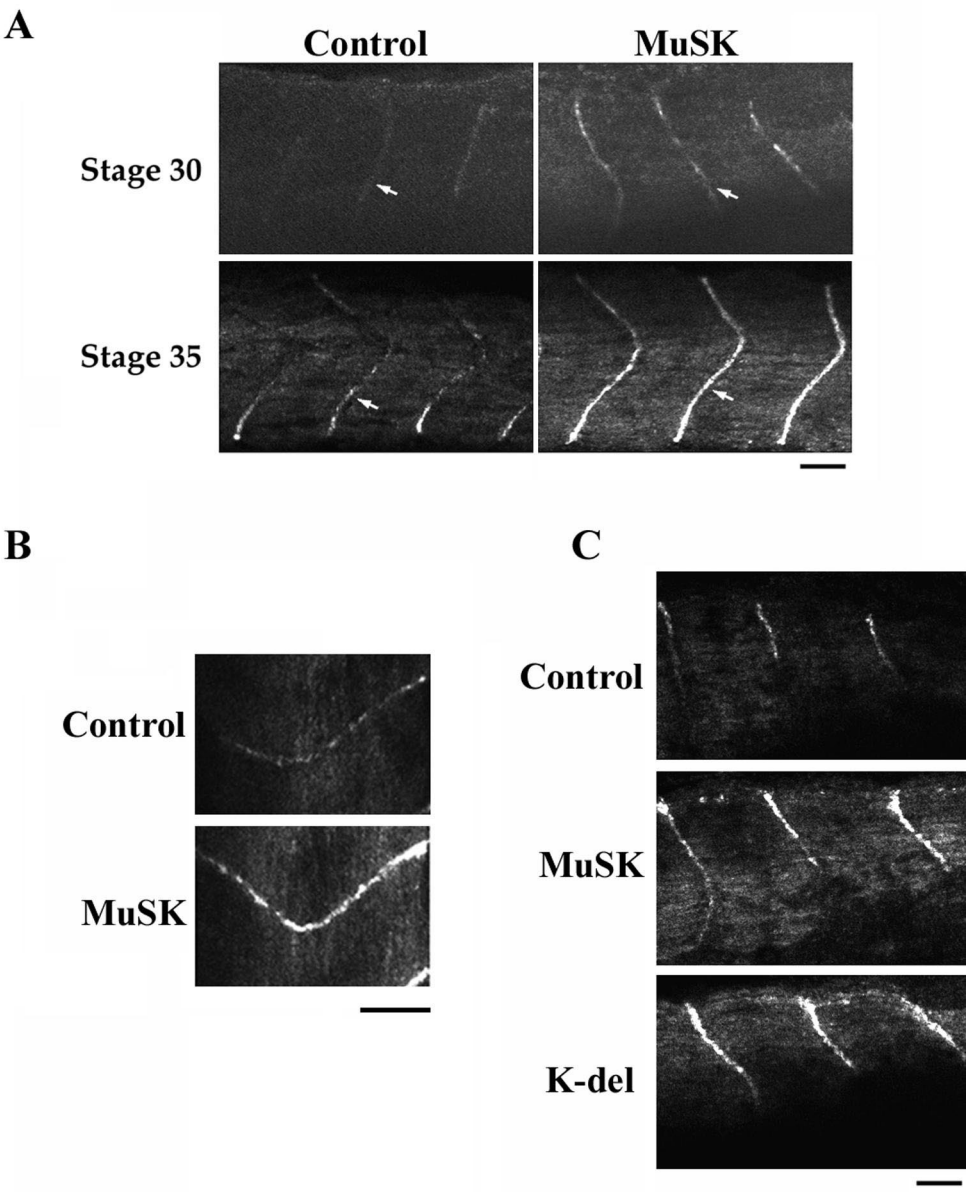


Fig. 4. Overexpression of MuSK or K-del mutant increased the aggregation of AChRs at the developing NMJ. (A) Embryos were injected with buffer (left panel) or MuSK RNA (right panel). After the embryos developed to stage 30 or 35, they were collected and fixed. AChR aggregation was detected as described in Methods. Arrow depicts the AChR aggregates at the innervated regions between myotomes. (B) Higher magnification showing larger area of AChR aggregation in MuSK overexpressing embryos when compared to control. (C) Overexpression of K-del mutant showed similar increase in the aggregation of AChRs at the developing NMJs. Embryos were injected with buffer, wild type MuSK or K-del mutant. Stage 35 embryos were collected for AChR staining. Scale bar=25 μm.

proteases and has been suggested to be involved in the regulation of protease activity. Interestingly, while the presence of this domain in a receptor has only been reported in MuSK and Dnrk in Drosophila [22], it is present in all forms of MuSK identified in Xenopus thus far [Ip et al., unpublished observation]. We report here that Xenopus MuSK lacking the Kringle domain retains the ability to be phosphorylated and aggregate AChR, suggesting that this domain is not required for the AChR clustering activity at the Xenopus NMJ. We are in the process of investigating the involvement of the Kringle domain in mediating other functions of MuSK in Xenopus embryos.

The strategy of overexpressing various synaptic proteins in Xenopus embryos has proven useful in elucidating their functional importance at the neuromuscular synapses in vivo [1,24]. Our findings in this study, together with the recent reports on the ability of agrin to increase AChR aggregation in synaptic regions of embryonic muscle [13,14], have provided further insights into our understanding of the functions of agrin and MuSK in the formation of NMJ during embryonic development.

Table 1
Overexpression of Xenopus MuSK in embryos enhanced the AChR aggregation at the myotomal muscle

| Stage fixed | Buffer injected | | MuSK injected | | Area ratio | K-del mutant injected | | Area ratio |
|---|---|---|---|---|---|---|---|---|
| | *n* | Area (%±S.D.) | *n* | Area (%±S.D.) | | *n* | Area (%±S.D.) | |
| Stage 30 | 8 | 0.9±0.3 | 6 | 1.9±0.5*** | 2.1 | – | ND | – |
| Stage 35 | 9 | 1.9±0.3 | 8 | 2.9±0.3*** | 1.5 | 6 | 2.8±0.4** | 1.5 |
| Stage 30 | 4 | 0.5±0.2 | 6 | 1.4±0.5*** | 2.8 | – | ND | – |
| Stage 35 | 4 | 2.4±0.4 | 4 | 5.2±1.6** | 2.2 | 8 | 4.7±0.8*** | 2.0 |
| Stage 32 | 8 | 0.8±0.3 | 8 | 1.2±0.3** | 1.5 | – | ND | – |
| Stage 35 | 7 | 1.1±0.2 | 9 | 2.7±0.6*** | 2.5 | 7 | 2.6±0.3*** | 2.4 |

Xenopus MuSK was overexpressed in embryos by injecting MuSK mRNA into one-cell stage embryos. The results of three representative experiments are presented in this table. The embryos were fixed at stages 30 and 35; *n*, the number of injected embryos. Area of AChR aggregates expressed as a percentage of total area (%±S.D.). The area of AChR aggregates in MuSK or K-del mutant injected groups was significantly greater than that in the buffer injected controls (one-tailed Student's *t* test; ***, $P<0.005$; **, $P<0.05$). Area ratio refers to the ratio of AChR aggregate area in MuSK/K-del mutant injected groups compared to that of the buffer injected controls.

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