Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Agrin-Deficient Myotube Retains Its Acetylcholine Receptor Aggregation Ability when Challenged with Agrin

San Pun, Yu P. Ng, Jie F. Yang, Nancy Y. Ip, and Karl W. K. Tsim

Department of Biology and Biotechnology Research Institute, Hong Kong University of Science and Technology, Hong Kong, China

Abstract: Agrin is a synapse-organizing molecule that mediates the nerve-induced aggregation of acetylcholine receptors (AChRs) and other postsynaptic components at the developing and regenerating vertebrate neuromuscular junctions. At the neuromuscular junction, three different cell types can express agrin, i.e., neuron, muscle, and Schwann cell. Several lines of evidence suggested that neuron-derived agrin is the AChR-aggregating factor, but the possible roles of muscle-derived agrin in the formation of AChR aggregate are not known. By using the recombinant DNA method, a clonal stable C2C12 cell line transfected with antisense agrin cDNA was created. RNA dot blot and western blot analysis indicated that the expression of agrin in the transfected cell was abolished by DNA transfection. When the agrin-deficient C2C12 cells were induced to form myotubes and subsequently cocultured with agrin cDNA transfected fibroblasts, AChR aggregates were formed in the cocultures. In addition, acetylcholinesterase (AChE) aggregates in agrin-deficient myotubes were also induced by exogenous agrin and the AChE aggregates were colocalized with the AChR aggregates. The agrin-deficient myotubes could also respond to neuron-induced AChR aggregation after coculturing with neuroblastoma cells. Thus, the agrin-deficient myotubes retain their ability to exhibit the agrin- or neuron-induced AChR aggregation. This result suggests that the formation of postsynaptic specializations during development and regeneration is mediated by neuron-derived agrin but not the agrin from muscle. **Key Words:** Antisense—Nerve–muscle coculture—Neuromuscular junction—Neuroblastoma—Synaptogenesis.
J. Neurochem. **69,** 2555–2563 (1997).

During the development of neuromuscular junctions, there will be formation of postsynaptic specializations directed by the motor neuron approaching the muscle fiber. These postsynaptic specializations include aggregation of acetylcholine receptors (AChRs), acetylcholinesterase (AChE; EC 3.1.1.7), and other synaptic components at the synaptic sites (McMahan, 1990; Hall and Sanes, 1993). Several lines of evidence indicate that both the axon terminal- and the basal lamina-induced formation of postsynaptic apparatus on developing and regenerating muscle fibers is mediated by agrin (Nitkin et al., 1987; Reist et al., 1987; Magill-Solc and McMahan, 1988; Hall and Sanes, 1993; Bowe and Fallon, 1995; Gautam et al., 1996). According to the agrin hypothesis (Nitkin et al., 1987; McMahan, 1990), agrin is synthesized in cell bodies of motor neurons in spinal cord and transported along their axons to muscle where it is externalized by the axon terminals to bind to the basal lamina in the synaptic cleft and also to agrin receptor(s) on the muscle fibers, triggering the muscle fibers to form aggregates of AChRs, AChE, and other molecules that comprise the postsynaptic apparatus.

Several lines of evidence support the agrin hypothesis. Agrin purified from the extracts of synapse-rich electric organ of the marine ray (*Torpedo californica*) has been best characterized and shown to induce aggregation of AChRs, AChE, and other synaptic molecules on cultured myotubes (Wallace, 1986). Molecules antigenically similar to agrin that cause AChR aggregation on cultured myotubes have also been extracted from muscle, spinal cord, brain, and other regions (Godfrey et al., 1988*a*; Magill-Solc and McMahan, 1988; Godfrey, 1991). Monoclonal antibodies raised against *Torpedo* agrin have been shown to stain molecules in the synaptic cleft of vertebrate neuromuscular junctions (Reist et al., 1987) and the motor neurons in ventral horn of chick spinal cord (Magill-Solc and McMahan, 1988; Fallon and Gelfman, 1989). By using species-specific anti-agrin antibodies in heterologous nerve–muscle cocultures, results have shown that neuron-derived agrin is the synapse-organizing molecule (Cohen and Godfrey, 1992; Reist et al., 1992). Moreover, Gautam et al. (1996) recently demonstrated that postsynaptic AChR aggregates were markedly reduced

Received April 11, 1997; revised manuscript received July 15, 1997; accepted July 16, 1997.

Address correspondence and reprint requests to Dr. K. W. K. Tsim at Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China.

Abbreviations used: AChE, acetylcholinesterase; AChR, acetylcholine receptor; FITC, fluorescein-5-isothiocyanate; HEK 293, human embryonic kidney fibroblast cell line; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSC, saline–sodium citrate.

in number, size, and density in muscles of agrin knockout mutant mice. Thus, the agrin knockout study also supports the hypothesis.

Although these findings support the hypothesis that neuron-derived agrin is the key synapse-organizing molecule, other lines of evidence oppose this hypothesis. For example, agrin molecules are expressed not only in motor neurons but also in a variety of other cell types, such as Schwann cell and muscle cell at the neuromuscular junction (Godfrey et al., 1988*b*; Magill-Solc and McMahan, 1988; Godfrey, 1991; So et al., 1996). Agrin at the synaptic site, however, could be derived from neuron, muscle, or Schwann cell. It is of interest that the expression of muscle agrin was colocalized with AChR aggregates at the aneural limbs of chick embryo (Fallon and Gelfman, 1989), and they appeared in the limb bud mesenchyme of the chick embryo before muscle differentiation or the ingrowth of motor axons (Godfrey et al., 1988*b*).

Agrin cDNAs have been isolated in rat (Rupp et al., 1991), chick (Tsim et al., 1992), and ray (Smith et al., 1992). Different variants of chick and rat agrin mRNAs due to alternative splicing have been identified by PCR analysis. The presence of an amino acid insertion at the Y/A site can be 0 or 4, and at the Z/B site the number of amino acid insertions can be 0, 8, 11, and 19 (Ferns et al., 1992, 1993; Ruegg et al., 1992; Tsim et al., 1992). These agrin isoforms have previously been shown to differ in their AChR-aggregating activity (Campanelli et al., 1991; Ferns et al., 1992, 1993; Ruegg et al., 1992; Gesemann et al., 1995). The insertion at the Z/B site is particularly important for the soluble AChR-aggregating activity of agrin in cultured myotubes, although all forms of agrin have nearly the same AChR-aggregating activity in the cell-attached agrin assay (Ferns et al., 1992, 1993). By using the cloned agrin cDNA as a probe, agrin transcript was detected in chick muscle and its size of transcript was similar to agrin mRNA derived from brain or spinal cord, albeit the expression was about fivefold lower (Tsim et al., 1992). The primary form of muscle-derived agrin is a distinct isoform with no amino insertion at Y and Z sites, namely, $agrin_{0,0}$; however, a minute amount of $agrin_{4,0}$ is also expressed in muscle (Ruegg et al., 1992). When $agrin_{0,0}$ or $agrin_{4,0}$ cDNA was transfected into fibroblasts and cocultured with myotubes, the transfected cells showed equal potency in AChR-aggregating activity compared with other isoforms of agrin (Ferns et al., 1992, 1993). Thus, the molecular genetic analysis clearly indicated that the form of agrin expressed in muscle could be active in the formation of AChR aggregates, but how it was involved in neuron-induced AChR aggregation is still not clear.

Although muscle-derived agrin may not initiate AChR aggregation, it could be synergistic with or complementary to neuron-derived agrin during the formation of neuromuscular junctions, as proposed by Fallon and his colleagues (Lieth et al., 1992). Moreover, muscle agrin may also be involved in stabilizing the AChR aggregates formed on the muscle cell surface. Thus, the primary objective of this study was to create an agrin-deficient muscle cell line by antisense cDNA transfection and to test the responsiveness of this myotube to form AChR aggregates when challenged with exogenous agrin or cocultured with neurons.

MATERIALS AND METHODS

Materials

Eggs of New Hampshire chicks were purchased from a local farm, and chicks were hatched and kept in the University Animal Care Facility. Cell culture medium, fetal calf serum, and other cell culture reagents were from GibcoBRL (Grand Island, NY, U.S.A.), conjugated secondary antibodies from Cappel (Durham, NC, U.S.A.), anti-AChE monoclonal antibody from Transduction Laboratories (Lexington, KY, U.S.A.), anti-rat agrin antibody AGR-540 from Stress-Gen Biotechnologies (Victoria, British Columbia, Canada), anti-α-tubulin antibody from Sigma (St. Louis, MO, U.S.A.), nitrocellulose papers from MSI (Westborough, MA, U.S.A.), tetramethylrhodamine-conjugated α-bungarotoxin from Molecular Probes (Eugene, OR, U.S.A.), chemiluminescence ECL kit for western blot analysis from Amersham (U.K.), and the protein assay kit was from Bio-Rad (Hercules, CA, U.S.A.). Reagents not specified here were from standard sources.

Cell cultures

The C2C12 cells were originally isolated from the thigh muscle of a 2-month-old C3H mouse (Yaffe and Saxel, 1977). C2C12 cells were maintained as undifferentiated myotubes in Dulbecco's modified Eagle medium supplemented with 20% (vol/vol) heat-inactivated horse serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cultures were incubated at 37°C in a 5% CO_2-humidified incubator and medium was replaced every 3–4 days. To induce myotube fusion, cells were grown in fusion medium that contained only 2% (vol/vol) heat-inactivated horse serum (Ferns et al., 1993). Myotubes were allowed to fuse for 2–3 days before coculture. A human embryonic kidney fibroblast cell line (HEK 293) and neuroblastoma × glioma NG108-15 hybrid cell were cultured in 100-mm culture dishes as previously described (Christian et al., 1978; Pun and Tsim, 1995).

Immunohistochemical staining

Myotube cultures were fixed with ice-cold 2% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS; 80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4, 100 m*M* NaCl, pH 7.6), rinsed with PBS, stained with 10^{-7} *M* tetramethylrhodamine-conjugated α-bungarotoxin for 1 h at room temperature, washed with PBS, fixed in ethanol, and mounted in glycerol. The AChR aggregates were counted as described by Wallace (1986) under a 63× objective on a Zeiss Axiophot equipped with phase and fluorescence optics. The mean number of AChR aggregates per field was determined by counting 20 different fields in each culture. For double labeling, the cells were labeled with rhodamine-conjugated α-bungarotoxin (Tsim et al., 1997) and anti-AChE monoclonal antibody (1:1,000) or anti-rat agrin antibody AGR-540 (1:1,000) for 1 h, followed by fluorescein-5-isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig secondary antibodies.

Western blot analysis

C2C12 cell and muscle were lysed in 0.5% (wt/vol) sodium dodecyl sulfate (SDS) in 50 m*M* phosphate buffer (pH 8.0) and the protein concentrations were determined (Bradford, 1976). Samples were boiled in 0.15 m*M* Tris-base, 6% (wt/vol) SDS, 10% (vol/vol) glycerol, 1% dithiothreitol, and 8 *M* urea and then electrophoresed on a polyacrylamide gel. Electrophoresed proteins were transferred onto a nitrocellulose membrane and stained with ponceau S to confirm that an equal amount of protein was loaded for each sample (Sambrook et al., 1989; Tsim et al., 1997). Membranes were blocked with 5% (wt/vol) dry milk in 20 m*M* Tris-base, pH 7.6, 137 m*M* NaCl, 0.1% (vol/vol) Tween 20, for 1 h at 37°C, followed by incubation with anti-rat agrin antibody AGR-540 (1:1,000). Membranes were also stained with anti-AChE antibody at 1:1,000 and anti-α-tubulin antibody at 1:5,000. For the blocking experiment, the anti-agrin antibody was treated with extract from agrin-expressing fibroblasts or with control fibroblast extract, before and during the antibody recognition period. Immunoreactivity was detected by ECL western blot system using peroxidase-conjugated goat anti-mouse or anti-rabbit Ig antibodies, following instructions from the supplier.

RNA dot blot analysis

For RNA dot blot assay, RNAs isolated from C2C12 cells (Chomczynski and Sacchi, 1987) were dot blotted onto a nylon membrane (MSI) using Bio-Dot apparatus (Bio-Rad) as described by Sambrook et al. (1989). An ~1-kb cDNA probe of rat agrin ($agrin_{185-1,058}$; Rupp et al., 1991) was used and probes were labeled with [α-^{32}P]dCTP (Amersham) by using a commercially available labeling kit (Pharmacia, Uppsala, Sweden). Hybridization was performed at 42°C overnight in 50% (vol/vol) formamide, 6× saline–sodium citrate (SSC), 5× Denhardt's solution, 0.5% (wt/vol) SDS, and 50 μg/ml denatured salmon sperm DNA. After hybridization, the filters were washed with decreasing concentrations of SSC to a final concentration of 0.1× SSC with 0.1% (wt/vol) SDS at 55°C, then wrapped in Saran wrap and exposed to X-Omat film (Kodak, Rochester, NY, U.S.A.) with intensifying screen (Okamoto, Japan) at −70°C.

cDNA cloning and stable cell transfection

The pcDNA 3 plasmid (Invitrogen, La Jolla, CA, U.S.A.), containing a G418-resistant gene and under control of a cytomegaloviral promoter, was used as the mammalian expression vector throughout this study. Partial cDNA encoding rat $agrin_{185-1,058}$ was prepared by digestion of full-length rat agrin cDNA with *Xho*I restriction enzymes (Rupp et al., 1991; see Fig. 1). The cDNA inserts were silica gel-purified (Geneclean II, Bio 101, La Jolla, CA, U.S.A.) and ligated at the corresponding restriction enzyme site of pcDNA 3 for subcloning. The identity of cDNA constructs was confirmed by DNA sequencing. C2C12 cells were transfected by calcium phosphate precipitation (Sambrook et al., 1989). For stable transfection, C2C12 cells transfected with sense S-$agrin_{185-1,058}$ cDNA or antisense AS-$agrin_{185-1,058}$ cDNA were grown in culture medium supplemented with 400 μg/ml G418 (Pun and Tsim, 1995). After 3 weeks of antibiotic selection, stable G418-resistant cells were sorted into 96-well plates by a flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). The cells were assayed for antisense and sense mRNA expression by RNA dot blot analysis. In the transient transfection of agrin cDNA, cDNA encoding full-length $agrin_{4,8}$ or $agrin_{4,0}$ was transfected into HEK 293 cells by the calcium phosphate method. The stable agrin-expressing HEK 293 cell line was selected by the same procedure used for C2C12 cells. The agrin-expressing fibroblast was confirmed by antibody staining, as described by Tsim et al. (1992), by using anti-rat agrin antibody (AGR-540) at 1:1,000 dilution. The agrin cDNA transfected HEK 293 cells were cocultured with the C2C12 myotubes for 2 days before the assay for AChR aggregation.

RESULTS

The cDNA encoding rat $agrin_{185-1,058}$ was constructed and subcloned into pcDNA 3 in both sense and antisense orientation, namely, S-$agrin_{185-1,058}$ and AS-$agrin_{185-1,058}$, respectively (Fig. 1). The extreme 5′ end of agrin cDNA was chosen in the antisense transfection analysis, because it was proved to be the best region in blocking agrin expression in other cell types (S. Pun and K. W. K. Tsim, manuscript submitted). Both cDNAs were transfected into C2C12 cells by the calcium phosphate method, and the efficiency of transfection was ~10% as determined by a control β-galactosidase cDNA. Stable clonal cell lines were obtained by single cell sorting; two clones for AS-$agrin_{185-1,058}$ (CA-1 and CA-2) and one clone for S-$agrin_{185-1,058}$ cDNA (CS-1) stably transfected cell lines were obtained. They all showed positive signals in our dot blot analysis (Fig. 2A). The wild-type C2C12 cells served as control and showed background staining; the level of endogenous muscle agrin mRNA in C2C12 cells was too low to be detected (Fig. 2A). To determine if antisense cDNA transfection blocked the agrin protein expression, we collected the cell lysates from CA-1 and CA-2 cells (AS-$agrin_{185-1,058}$ cDNA transfected), sense control CS-1 (S-$agrin_{185-1,058}$ cDNA transfected), and wild-type C2C12 cells for anti-agrin immunoblotting analysis. Expression of agrin was completely abolished in AS-$agrin_{185-1,058}$ cDNA transfected CA-1 and CA-2 cells (Fig. 2B). Expression of agrin in S-$agrin_{185-1,058}$ cDNA transfected control CS-1 cells and wild-type C2C12 cells was unaffected by the transfection; both of them expressed an agrin-immunoreactive protein at ~200 kDa (Fig. 2B). Control proteins, AChE at ~68 kDa and α-tubulin at ~55 kDa, were relatively unchanged in these transfected cells (Fig. 2B). This indicated that the specific blockage of agrin biosynthesis in agrin-deficient C2C12 cells was caused by the antisense agrin RNA that was derived from the incorporated expression vector.

The detected ~200-kDa protein for agrin in C2C12 myotubes is different from that reported in muscle mass (Gautam et al., 1996; Kröger and Mann, 1996). To determine the specificity of the ~200-kDa agrin band, we performed two idependent experiments: (1) to run a low-percentage acrylamide gel, and (2) to block the recognized band by treating the anti-agrin antibody with an excess amount of recombinant rat agrin. Figure 3A shows that the rat muscle expresses an agrin protein at >300 kDa, and a smaller size (<200 kDa), representing degraded products, is also

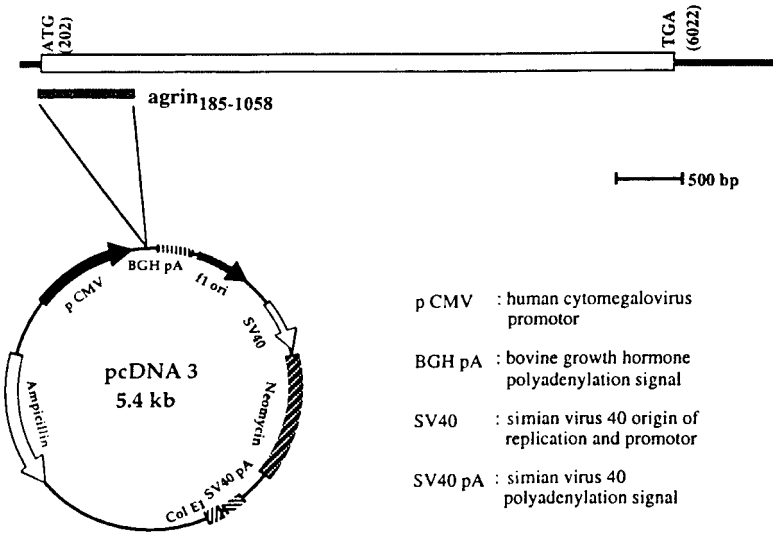

FIG. 1. Construction of agrin antisense cDNA in a mammalian expression vector. A map shows the functional domain of agrin and the location of antisense agrin cDNA construct. The open box is the coding region; the boldfaced line is the noncoding region; the hatched line is the inserted DNA fragment; and the nucleotide numbers are in parentheses. The numbering is according to rat agrin cDNA as described by Rupp et al. (1991). The DNA fragment was subcloned into pcDNA 3 mammalian expression vector. AS-agrin$_{185-1,058}$ represents the antisense construct from 185 to 1,058 bp. The same DNA fragment was inserted in the sense orientation denoted as S-agrin$_{185-1,058}$. The integrity of the construct was confirmed by DNA sequencing. Bar, 500 bp.

revealed, as reported previously (Kröger and Mann, 1996). No high molecular weight protein is detected in either CS-1 or CA-1 cells. The control recombinant agrin was detected at ~200 kDa, ~120 kDa, and ~96 kDa, indicating the degraded products from the transfected fibroblasts. Agrin bands of C2C12 myotubes and transfected fibroblasts, recognized by anti-agrin antibody, were specifically blocked by treating the antibody with an excess amount of recombinant agrin (Fig. 3B). These lines of evidence indicate strongly

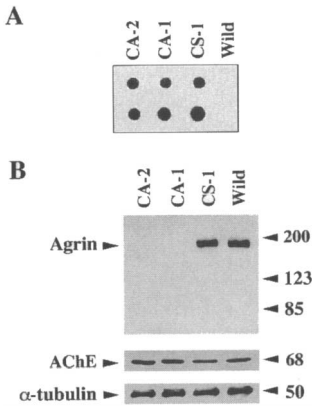

FIG. 2. Antisense agrin cDNA transfection blocks agrin expression in C2C12 myotubes. **A:** RNA dot blot analysis of agrin stably transfected C2C12 cells. RNAs (5 μg) isolated from C2C12 cells were dot blotted onto nylon membrane using Bio-Dot apparatus. A ~1-kb cDNA probe of rat agrin (agrin$_{185-1,058}$; Rupp et al., 1991) was labeled with [α-^{32}P]dCTP. CA-1 and CA-2, antisense agrin cDNA transfected C2C12 cells; CS-1, sense agrin cDNA transfected C2C12 cells; wild, wild-type C2C12 cells. Only the transfected cells showed positive hybridization. **B:** Proteins (20 μg) from AS-agrin$_{185-1,058}$ cDNA stably transfected myotubes (CA-1 and CA-2, antisense), S-agrin$_{185-1,058}$ cDNA transfected myotubes (CS-1, sense control), and wild-type C2C12 myotubes (Wild) were loaded onto a 7.5% SDS-polyacrylamide gel for western blot analysis by using anti-rat agrin antibody AGR-540. The expression of agrin-like protein (~200 kDa) was abolished in CA-1 and CA-2 myotubes, whereas the agrin expression in CS-1 control and wild-type C2C12 myotubes was unaffected. Both AChE at ~68 kDa and α-tubulin at ~55 kDa served as control proteins and were not affected by the DNA transfection. Molecular markers are shown in kilodaltons.

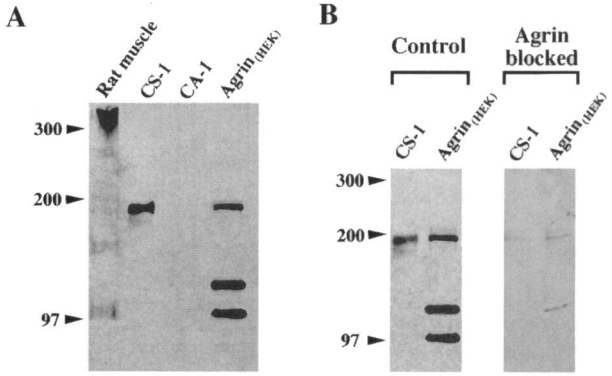

FIG. 3. The ~200-kDa band in C2C12 myotubes is blocked specifically by treating the anti-agrin antibody with an excess amount of recombinant agrin. **A:** Proteins (20 μg) from the extracts of embryonic rat muscle, CS-1 (sense transfected control), CA-1 (antisense transfected), and agrin$_{(HEK)}$ (HEK 293 cells transfected with full-length rat agrin$_{4,8}$ cDNA) were loaded onto a 6.0% SDS-polyacrylamide gel for western blot analysis by using anti-rat agrin antibody AGR-540. Rat muscle agrin was expressed as a broad band at >300 kDa on the top of the gel, and degraded products were also revealed. The agrin band at ~200 kDa was detected in the CS-1 but not in the CA-1 cell line. The recombinant agrin served as control and migrated at ~200 kDa, ~120 kDa, and ~96 kDa. Molecular markers are shown in kilodaltons. **B:** Proteins (20 μg) from the extracts of CS-1 myotubes and agrin$_{(HEK)}$ were loaded onto a 6.0% SDS-polyacrylamide gel for western blot analysis by using anti-rat agrin antibody AGR-540. The antibody was treated with agrin extract from agrin$_{4,8}$ cDNA transfected HEK 293 cells before and during the antibody incubation. The protein bands, recognized by anti-agrin antibody, were blocked specifically by the recombinant agrin. Molecular markers are shown in kilodaltons.

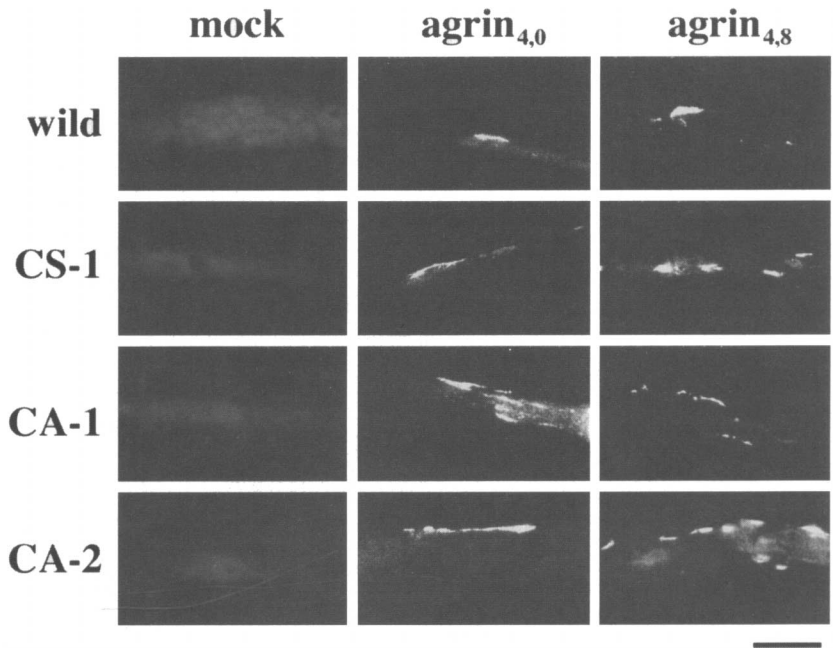

FIG. 4. Agrin induces AChR aggregation in agrin-deficient myotubes. C2C12 myotubes were cocultured with rat $agrin_{4,0}$ or $agrin_{4,8}$ cDNA transfected HEK 293 cells, or mock transfected HEK 293 cells. Myotubes were wild-type C2C12 (wild), S-$agrin_{185-1,058}$ cDNA stably transfected (CS-1), and AS-$agrin_{185-1,058}$ cDNA transfected control (CA-1 and CA-2). Cultures were stained with rhodamine-conjugated α-bungarotoxin and viewed by fluorescence optics. Horizontally, panels are C2C12 myotube (wild), CS-1 myotube (sense transfected C2C12 myotubes), and CA-1 and CA-2 myotubes (antisense transfected C2C12 myotubes). Vertically, panels are pcDNA 3 vector alone (mock) and HEK 293 cells transfected with rat $agrin_{4,0}$ cDNA ($agrin_{4,0}$) and rat $agrin_{4,8}$ cDNA ($agrin_{4,8}$). Bar, 20 μm.

that the ~200-kDa protein band recognized in C2C12 myotubes, indeed, is agrin derived from muscle. The smaller size of agrin in C2C12 cells could be a distinct characteristic of cultured myotubes, or a degraded product from higher molecular weight protein.

All the stable transfectants could be induced to form myotubes on reduction of the serum concentration and they were tested for their responsiveness to exogenous agrin in causing the formation of AChR aggregates. When the agrin-deficient myotubes were cocultured with $agrin_{4,8}$ (agrin isoform with four- and eight-amino acid insertion at Y and Z sites, respectively) cDNA transfected fibroblasts, the formation of AChR aggregates on the surface of myotubes was unaffected compared with wild-type C2C12 or sense agrin cDNA transfected C2C12 myotubes (Fig. 4). Not only could the active $agrin_{4,8}$-expressing fibroblast induce the AChR aggregation in the agrin-deficient myotubes, the so-called inactive $agrin_{4,0}$ also showed similar AChR-aggregating activity in the coculture analysis (Fig. 4). The AChR aggregates, induced by agrin cDNA transfected fibroblasts, were normally at least 5 μm in length, detected at the edge and the bottom of muscle fibers (Fig. 4). The characteristics of these aggregates were very similar to those of previous reports (Ferns et al., 1992, 1993). In addition, the number of agrin-induced AChR aggregates on both agrin-expressing and agrin-deficient myotubes was quantified; there was no significant difference ($p \geq 0.54$, t test) between all types of myotubes being analyzed, i.e., wild type, CS-1, CA-1, and CA-2 (Fig. 5). Moreover, the sensitivity of the agrin-deficient myotubes to form agrin-induced AChR aggregates was similar to that of the control myotubes. When a submaximal amount (2 U) of recombinant agrin (CBA-1, as described by Tsim et al., 1992) was applied to the transfected myotubes, the number of AChR aggregates in agrin-deficient myotubes was 105 ± 15% (mean ± SEM, n = 4) of the control agrin-expressing myotubes. Besides, the agrin-induced AChR aggregates on the surface of agrin-de-

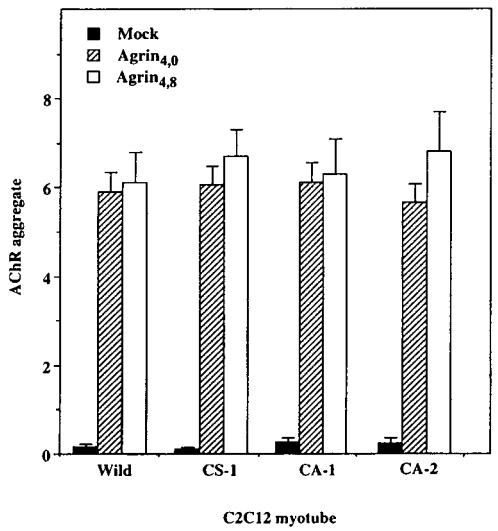

FIG. 5. Quantification of AChR aggregates in agrin-deficient myotubes. Cocultures were performed as in Fig. 4. The AChR aggregates were stained with rhodamine-conjugated α-bungarotoxin and counted under a 63× objective on a Zeiss Axiophot equipped with phase and fluorescence optics. Myotubes are wild-type C2C12 (Wild), AS-$agrin_{185-1,058}$ cDNA transfected (CA-1 and CA-2), and S-$agrin_{185-1,058}$ cDNA stably transfected control (CS-1). Data are number of AChR aggregates per field and are mean ± SEM values (n = 4).

ficient myotubes were found predominantly in the vicinity of agrin-expressing fibroblasts (Fig. 6). Thus, these results indicate that the formation of AChR aggregate on the myotube surface was mediated by exogenously applied agrin but not the intrinsic muscle agrin.

The formation of AChE aggregates was also tested in agrin-deficient myotubes. The myotubes were cocultured with agrin transfected fibroblasts and then double stained with rhodamine-conjugated α-bungarotoxin and anti-AChE monoclonal antibody. The agrin-deficient myotubes retained their full responsiveness to agrin-induced AChE aggregation, and the AChE aggregates were also colocalized with the AChR aggregates (Fig. 7). Moreover, the transfected myotubes were cocultured with neurons to test the neuron-induced AChR aggregation in the agrin-deficient myotubes. Neuroblastoma × glioma hybrid cell line NG108-15 was chosen and they were shown to induce AChR aggregates when cocultured with myotubes (Busis et al., 1984; Pun et al., 1995). When the myotubes were cocultured with NG108-15 cells, both antisense agrin transfected myotubes (CA-1 and CA-2) and sense agrin transfected myotubes (CS-1), and wild-type C2C12 myotubes, exhibited the aggregation of AChRs (Fig. 8A). These myotubes showed no significant difference ($p \geq 0.64$, t test) in their responsiveness to the AChR-aggregating activity induced by NG108-15 cells (Fig. 8B).

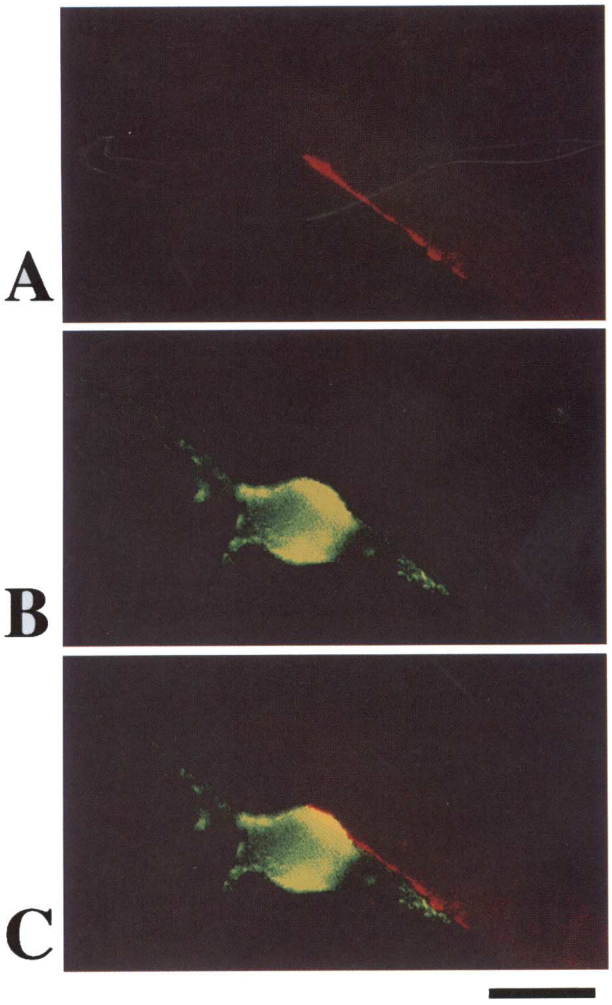

FIG. 6. AChR aggregate formed in mutant C2C12 myotube is in the vicinity of agrin-expressing fibroblast. AS-agrin$_{185-1,058}$ cDNA transfected C2C12 cells (CA-2) were induced to fuse and cocultured with agrin$_{4,8}$ cDNA transfected HEK 293 cells overnight. The culture was fixed and double stained with rhodamine-conjugated α-bungarotoxin and anti-agrin antibody followed by FITC-conjugated goat anti-mouse Ig antibody. **A:** View with rhodamine optics for AChR aggregates. **B:** View with fluorescein optics for agrin expression. **C:** A superimposed view of A and B. The transfected fibroblast shows agrin immunoreactivity (green) in the vicinity of the induced AChR aggregates (red). Bar, 20 μm.

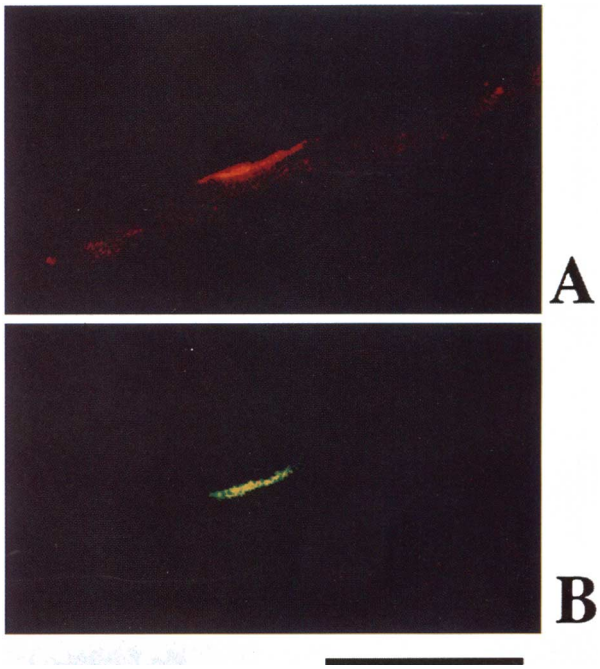

FIG. 7. AChE aggregate is colocalized with AChR aggregate. AS-agrin$_{185-1,058}$ cDNA transfected C2C12 cells (CA-2) were induced to fuse and were cocultured with agrin$_{4,8}$ cDNA transfected HEK 293 cells overnight. The culture was fixed and double stained with rhodamine-conjugated α-bungarotoxin (**A**) and anti-AChE antibody (**B**) followed by FITC-conjugated goat anti-mouse Ig antibody. All the rhodamine and fluorescein staining is colocalized. Bar, 20 μm.

DISCUSSION

Several lines of evidence indicate that muscle-derived agrin may play a role in formation and/or maintenance of the postsynaptic apparatus in the developing neuromuscular junctions (Godfrey et al., 1988*b*; Fallon and Gelfman, 1989; Lieth et al., 1992). The following two models had been proposed by Fallon and his colleagues (Lieth et al., 1992) for the functions of muscle-derived agrin in organizing the agrin- or neuron-induced AChR aggregates: (1) Muscle agrin may

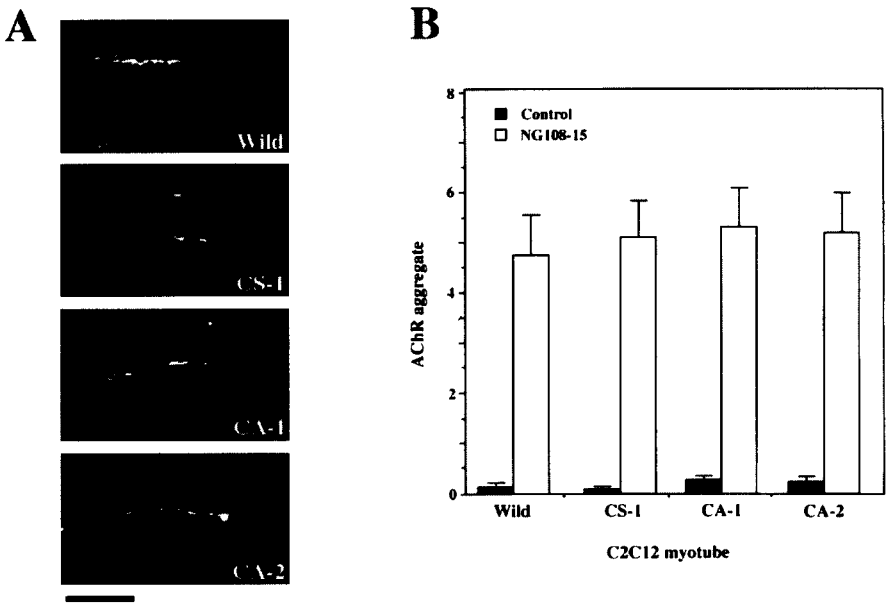

FIG. 8. Coculture of NG108-15 cells with agrin-deficient myotubes. NG108-15 cells were cocultured with agrin-deficient myotubes for 2 days and the AChR aggregates were determined. Myotubes are C2C12 (Wild), sense transfected (CS-1), and antisense transfected (CA-1 and CA-2). **A:** Cultures were stained with rhodamine-conjugated α-bungarotoxin and viewed under fluorescence optics. Bar, 20 μm. **B:** The AChR aggregates were counted under a 63× objective on a Zeiss Axiophot equipped with phase and fluorescence optics. Control is the myotube cultured alone. The mean number of AChR aggregates per field was determined by counting 20 different fields in each culture. Data are mean ± SEM values (n = 4).

exist on the muscle surface as an inactive precursor and external stimuli would liberate the active forms that would induce the AChR aggregation on the surface of muscle fibers; and (2) muscle agrin may stabilize and/or promote the maturation of AChR aggregates induced by external stimuli. The results in the present study demonstrated that agrin-deficient myotubes retain their full AChR aggregation ability when exogenous agrin or neurons are challenged. Therefore, the first proposed model that muscle agrin could be activated during the formation of AChR aggregates appears to be invalidated. Although we do not have direct evidence to address the second model, the magnitude and the responsiveness of AChR aggregation were similar between agrin-expressing and agrin-deficient myotubes when they were challenged with agrin or neurons. Thus, the second model may not be feasible. Our laboratory is currently determining the rate of disappearance of the agrin-induced AChR aggregates in our agrin-deficient myotubes, to determine the possible role of muscle agrin in stabilizing the formed AChR aggregates.

Previous studies from our laboratory showed that the antisense agrin cDNA transfection blocked agrin expression in neuroblastoma cell line NG108-15, and the transfection completely abolished the NG108-15 cell-induced AChR aggregation when the agrin-deficient neurons were cocultured with myotubes (S. Pun and K. W. K. Tsim, manuscript submitted). Taken together with our previous studies, our findings from using antisense cDNA transfection further support the "agrin hypothesis" that agrin produced by neurons induces the AChR aggregation on the surface of myotubes. Another approach, by using anti-agrin antibody in neuron–muscle cocultures, has also drawn similar conclusions. By using species-specific anti-agrin antibodies, Reist et al. (1992) showed that neuron-derived agrin induces AChR aggregation on myotubes, whereas muscle-derived agrin does not affect AChR aggregation in the heterologous neuron–muscle coculture. Moreover, Cohen and Godfrey (1992), in their heterologous neuron–muscle cocultures, found that axons of embryonic frog spinal cord neurons in culture supply agrin to synapses that were formed with muscle cells. Although the interpretation of results was dependent on the specificity of the antibody used, their conclusions were in line with those of our studies using the molecular genetic approach. Regardless of whether the antibody or the genetic approach is adopted, the conclusion is that neuron-derived agrin, but not muscle-derived agrin, is the molecule that initiates AChR aggregation during the formation of neuromuscular junctions.

Our antisense study is supplementary to the analysis in agrin knockout mutant mice (Gautam et al., 1996). These investigators cannot exclude the possibility that in agrin-deficiency mutant mice the defect in the formation of neuromuscular junctions could be due to the lack of agrin expression from muscle; indeed, the mutant mice have a reduction of agrin in both motor neuron and muscle fiber. However, by using the antisense approach, the agrin expression from either neuron or muscle can be blocked selectively, and this could provide a clear demonstration that neuron-derived agrin induces the synapse formation in vitro. In contrast to our results, a small number of AChR aggregate was detected in the nerve–muscle contacts of agrin-deficiency mutant mice (Gautam et al., 1996). The formation of some postsynaptic specializations in the mutant mice could be a result of incomplete blockage of agrin, in particular, the $agrin_{0,0}$ and $agrin_{4,0}$ from the motor neurons.

Although muscle-derived agrin may not be involved in inducing the AChR aggregation, the possible role of muscle-derived agrin could not be excluded in the formation of neuromuscular junctions. First, muscle expresses agrin both in vivo and in vitro, and their expression profile is related to the critical period of neuromuscular junction formation (Godfrey et al., 1988*b*; Tsim et al., 1992). Second, muscle agrin is associated with basal lamina and it is colocalized with neuron- or agrin-induced AChR aggregate. Third, muscle agrin is present before the appearance of AChR aggregates on the muscle cell surface during the early stage of chick development. Moreover, the expression of muscle agrin is colocalized with AChR aggregates formed in the aneural muscle cell in vivo (Fallon and Gelfman, 1989). Last, muscle expresses an isoform of agrin having a zero amino acid insertion at both Y and Z sites. However, $agrin_{0,0}$ cDNA when transfected into fibroblasts and cocultured with myotubes could induce the aggregation of AChRs. These lines of evidence suggest that muscle-derived agrin may be involved in the formation of neuromuscular junctions, and thus, alternate functions of muscle agrin should be considered seriously.

By using coculture of agrin cDNA transfected Chinese hamster ovary cells with ciliary ganglion neurons, Campagna et al. (1995) proposed that muscle agrin can induce the presynaptic differentiation of neurons. Their conclusions were based on the following three observations: (1) motor neurons adhere to agrin; (2) agrin is inhibitory to the growth of extending motor neurites; and (3) agrin induces the aggregation of synaptic vesicle protein synaptotagmin in motor neurons. These observations fully support the possible role that muscle-derived agrin played during the formation of neuromuscular junctions. The result from agrin knockout mutant mice also supports the presynaptic functions of muscle agrin (Gautam et al., 1996). In agrin-deficient mutant mice, the presynaptic nerve was defective in presynaptic specializations that include the perturbation of axonal branching and they were vesicle poor and loosely associated with Schwann cell processes. However, the defect of agrin expression in the mutant mice is not restricted only to muscle. Thus, our agrin-deficient myotubes could provide a better model to clarify the possible functions of muscle-derived agrin in directing presynaptic specializations during the formation of neuromuscular junctions. Indeed, recent results from our laboratory have demonstrated that the agrin-deficient neurons exhibit a different pattern of neurite outgrowth and also the expression of some neuronal proteins.

Acknowledgment: We are grateful to Lisa Yung, Tina Dong, and H. Y. Choi from our laboratory and to William Chau from the University Animal Care Facility for their expert technical assistance. We thank Dr. Richard Scheller of Stanford University for the rat agrin cDNAs. The research was supported by grants from Research Grants Council of Hong Kong and Biotechnology Research Institute of Hong Kong University of Science and Technology.

REFERENCES

Bowe M. A. and Fallon J. R. (1995) The role of agrin in synapse formation. *Annu. Rev. Neurosci.* **18,** 443–462.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* **72,** 248–254.

Busis N. A., Daniels M. P., Bauer H. C., Pudimat P. A., Sonderegger P., Schaffner A. E., and Nirenberg M. (1984) Three cholinergic neuroblastoma hybrid cell lines that form few synapses on myotubes are deficient in acetylcholine receptor aggregation molecules and large dense core vesicles. *Brain Res.* **324,** 201–210.

Campagna J. A., Ruegg M. A., and Bixby J. L. (1995) Agrin is a differentiation-inducing "stop signal" for motoneurons in vitro. *Neuron* **15,** 1365–1374.

Campanelli J. T., Hoch W., Rupp F., Kreiner T., and Scheller R. H. (1991) Agrin mediates cell contact-induced acetylcholine receptor clustering. *Cell* **67,** 909–916.

Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-choroform extraction. *Anal. Biochem.* **162,** 156–159.

Christian C. N., Daniels M. P., Sugiyama H., Vogel Z., Jacques L., and Nelson P. G. (1978) A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. *Proc. Natl. Acad. Sci. USA* **75,** 4011–4015.

Cohen M. W. and Godfrey E. W. (1992) Early appearance of and neuronal contribution to agrin-like molecules at embryonic frog nerve-muscle synapses formed in culture. *J. Neurosci.* **12,** 2982–2992.

Fallon J. R. and Gelfman C. E. (1989) Agrin related molecules are concentrated at acetylcholine receptor cluster in normal and aneural developing muscle. *J. Cell Biol.* **108,** 1527–1535.

Ferns M., Hock W., Campanelli J. T., Rupp F., Hall Z. W., and Scheller R. H. (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. *Neuron* **8,** 1079–1086.

Ferns M., Campanelli J. T., Hoch W., Scheller R. H., and Hall Z. W. (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* **11,** 491–502.

Gautam M., Noakes P. G., Moscoso L., Rupp F., Scheller R. H., Merlie J. P., and Sanes J. R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85,** 525–535.

Gesemann M., Denzer A. J., and Ruegg M. A. (1995) Acetycholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J. Cell Biol.* **128,** 625–636.

Godfrey E. W. (1991) Comparison of agrin-like proteins from the extracellular matrix of chicken kidney and muscle with neural agrin, a synapse organizing protein. *Exp. Cell Res.* **195,** 99–109.

Godfrey E. W., Dietz M. E., Morstad A. L., Wallskog P. A., and Yorde D. E. (1988*a*) Acetylcholine receptor-aggregating proteins are associated with the extracellular matrix of many tissues in *Torpedo*. *J. Cell Biol.* **106,** 1263–1272.

Godfrey E. W., Siebenlist R. E., Wallskog P. A., Walters L. M., Bolender D. L., and Yorde D. E. (1988*b*) Basal lamina components are concentrated in premuscle masses and at early acetylcholine receptor clusters in chick embryo hindlimb muscles. *Dev. Biol.* **130,** 471–486.

Hall Z. W. and Sanes J. R. (1993) Synaptic structure and development: the neuromuscular junction. *Cell* **72**/*Neuron* **10** (Suppl.), 99–121.

Kröger S. and Mann S. (1996) Biochemical and functional characterization of basal lamina-bound agrin in the chick central nervous system. *Eur. J. Neurosci.* **8,** 500–509.

Lieth E., Cardasis C. A., and Fallon J. R. (1992) Muscle-derived agrin in cultured myotubes: expression in the basal lamina and

at induced acetylcholine receptor clusters. *Dev. Biol.* **149,** 41–54.

Magill-Solc C. and McMahan U. J. (1988) Motor neurons contain agrin-like molecules. *J. Cell Biol.* **107,** 1825–1833.

McMahan U. J. (1990) The agrin hypothesis. *Cold Spring Harb. Symp. Quant. Biol.* **55,** 407–418.

Nitkin R. M., Smith M. A., Magill C., Fallon J. R., Yao M. M., Wallace B. G., and McMahan U. J. (1987) Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. *J. Cell Biol.* **105,** 2471–2478.

Pun S. and Tsim K. W. K. (1995) Truncated form of pro-acetylcholine receptor-inducing activity (ARIA) induces AChR α-subunit but not AChE transcripts in cultured chick myotubes. *Neurosci. Lett.* **198,** 107–110.

Pun S., So K. L., and Tsim K. W. K. (1995) Agrin from NG108-15 induces acetylcholine receptor aggregation on primary rat myotube cultures. *Soc. Neurosci. Abstr.* **21,** 1776.

Reist N. E., Magill C., and McMahan U. J. (1987) Agrin-like molecules at synaptic sites in normal, denervated, and damaged skeletal muscles. *J. Cell Biol.* **105,** 2457–2469.

Reist N. E., Werle M. J., and McMahan U. J. (1992) Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron* **8,** 865–868.

Ruegg M. A., Tsim K. W. K., Horton S. E., Kröger S., Escher G., Gensch E. M., and McMahan U. J. (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron* **8,** 691–699.

Rupp F., Payan D. G., Magill-Solc C., Cowan D. M., and Scheller R. H. (1991) Structure and expression of a rat agrin. *Neuron* **6,** 811–823.

Sambrook J., Fritsch E. F., and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd edit. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Smith M. A., Magill-Solc C., Rupp F., Yao Y.-M. M., Schilling J., Snow P., and McMahan U. J. (1992) Isolation and characterization of a cDNA that encodes an agrin homolog in the marine ray. *Mol. Cell. Neurosci.* **3,** 406–417.

So K. L., Pun S., Wan D. C. C., and Tsim K. W. K. (1996) Cerebellar granule cells express a specific isoform of agrin that lacks the acetylcholine receptor aggregating activity. *FEBS Lett.* **379,** 63–68.

Tsim K. W. K., Ruegg M. A., Escher G., Kröger S., and McMahan U. J. (1992) cDNA that encodes active agrin. *Neuron* **8,** 677–689.

Tsim K. W. K., Choi R. C. Y., Dong T. T. X., and Wan D. C. C. (1997) A globular, not asymmetric, form of AChE is expressed in chick motor neurons: down-regulation towards maturity and after denervation. *J. Neurochem.* **68,** 479–487.

Wallace B. G. (1986) Aggregating factor from *Torpedo* electric organ induces patches containing acetylcholine receptors, acetylcholinesterase and butyrylcholinesterase on cultured myotubes. *J. Cell Biol.* **102,** 783–794.

Yaffe A. and Saxel O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270,** 725–727.