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IDENTIFICATION OF AGRIN IN ELECTRIC ORGAN EXTRACTS AND LOCALIZATION OF AGRIN-LIKE MOLECULES IN MUSCLE AND CENTRAL NERVOUS SYSTEM

BY MARTIN A. SMITH, YUNG-MAE M. YAO, NOREEN E. REIST, CATHERINE MAGILL, BRUCE G. WALLACE AND U. J. McMAHAN

Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, USA

SUMMARY

The portion of the muscle fibre's basal lamina that occupies the synaptic cleft at the neuromuscular junction contains molecules that cause the aggregation of acetylcholine receptors and acetylcholinesterase on regenerating muscle fibres. Agrin, which is extracted from basal lamina-containing fractions of the *Torpedo* electric organ and causes the formation of acetylcholine receptor and acetylcholinesterase aggregates on cultured myotubes, may be similar, if not identical, to the acetylcholine receptor- and acetylcholinesterase-aggregating molecules at the neuromuscular junction. Here we summarize experiments which led to the identification of agrin and established that the basal lamina at the neuromuscular junction contains molecules antigenically similar to agrin. We also discuss results which raise the possibility that agrin-like molecules at the neuromuscular junction are produced by motor neurones.

INTRODUCTION

The basal lamina sheaths of muscle fibres in skeletal muscles survive trauma that causes the muscle fibres and the axons that innervate them to degenerate. New myofibres regenerate within the sheaths, axons grow to the original synaptic sites on them, and neuromuscular function is re-established. At the regenerated neuromuscular junctions, as at the original ones, the myofibre surface is characterized by high concentrations of acetylcholine receptors (AChRs) and acetylcholinesterase (AChE), both of which are crucial for synaptic transmission. Our studies on regenerating neuromuscular junctions in the frog have revealed that the synaptic portion of the myofibre basal lamina sheath contains molecules that direct the formation of AChR and AChE aggregates on regenerating myofibres (Burden, Sargent & McMahan, 1979; McMahan & Slater, 1984; Anglister & McMahan, 1985). Among the questions raised by this observation are the following. How are the synaptic organizing molecules in the basal lamina regulated? What is their mechanism of action? Do these molecules play a role in the long-term maintenance of the

Key words: agrin, neuromuscular junction, acetylcholine receptors, acetylcholinesterase.

neuromuscular junction as well as in restoring neuromuscular function after trauma? Are these the same molecules that mediate the formation of AChR and AChE aggregates at developing neuromuscular junctions in the embryo?

As a step towards answering these questions we have undertaken a series of studies aimed at identifying the synaptic organizing molecules at the neuromuscular junction and making specific markers for them. We selected as a source of such molecules the electric organ of *Torpedo californica*, which has a concentration of cholinergic synapses far greater than that of skeletal muscle. In previous reports (Godfrey *et al.* 1984; Wallace *et al.* 1985; Wallace, 1986; Magill *et al.* 1986) we documented that basal lamina-containing fractions of the electric organ are enriched for molecules, called agrin, that cause the formation of patches on cultured myotubes at which AChRs and AChE are aggregated. We demonstrated further that similar molecules can be extracted from muscle (Godfrey *et al.* 1984), but in much smaller amounts, and that monoclonal antibodies against agrin recognize molecules concentrated in the synaptic cleft at the neuromuscular junction (Fallon *et al.* 1985). These findings led to the agrin hypothesis: agrin is similar to the AChR- and AChE-aggregating molecules in the synaptic basal lamina (Fallon *et al.* 1985; Wallace *et al.* 1985). Here we present a brief account of new studies that have led to the identification and purification of agrin and have revealed that agrin-like molecules in the synaptic cleft at the neuromuscular junction are components of the basal lamina, which is consistent with the agrin hypothesis. We also present evidence that the cell bodies of motor neurones contain agrin-like molecules, which raises the possibility that the AChR- and AChE-aggregating molecules in the synaptic basal lamina are produced by the presynaptic cellular component of the neuromuscular junction.

IDENTIFICATION AND PURIFICATION

In a previous report (Nitkin *et al.* 1983) we showed that agrin activity can be purified several thousand-fold by routine gel filtration and ion exchange chromatography. To identify and further purify agrin we have used immunoaffinity techniques.

We made monoclonal antibodies by immunizing mice with a partially purified preparation of agrin and screened the resulting hybridomas for those secreting antibodies that immunoprecipitated agrin activity (Fallon *et al.* 1985). At least seven of our anti-agrin antibodies recognize different epitopes (e.g. Magill *et al.* 1987). We found that at least four of the seven antibodies immunoprecipitated polypeptides of relative molecular masses (M_r) 70, 95, 135 and 150K (M_r 1000 = K) from our electric organ extracts (Fig. 1). Each of the monoclonal antibodies that immunoprecipitated native agrin was tested by immunoblot analysis for its ability to bind to denatured agrin. Specific labelling was detected for one of the monoclonal antibodies; it again bound to polypeptides at 70, 95, 135 and 150K (data not shown; but see Fig. 2).

To learn which of the polypeptides recognized by the antibodies had agrin activity we combined gel filtration column chromatography with immunoblot analysis. As shown in Fig. 2, the 150K and 95K polypeptides comigrate with the AChR/AChE-

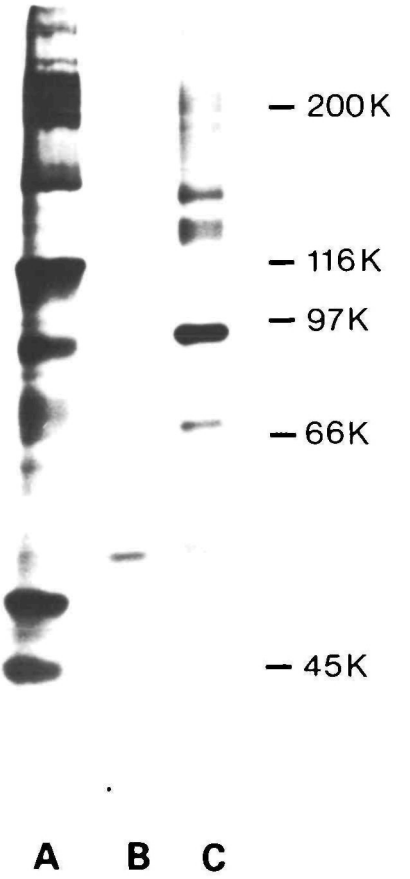


Fig. 1. Anti-agrin antibodies bind to polypeptides of 70, 95, 135 and 150K. Samples (3000 units) of a partially purified preparation of agrin (Cibacron pool; R. M. Nitkin, M. A. Smith, J. R. Fallon, Y. M. Yao, B. G. Wallace & U. J. McMahan, in preparation) were incubated with Sepharose beads to which normal mouse serum or an anti-agrin monoclonal antibody had been covalently bound. The beads were washed extensively and bound polypeptides were eluted, precipitated with trichloroacetic acid, separated by SDS polyacrylamide gel electrophoresis and stained with silver (R. M. Nitkin *et al.* in preparation). (A) 30 units Cibacron pool; (B,C) peptides eluted from (B) normal mouse serum or (C) anti-agrin monoclonal antibody (11D2) coupled beads. Numbers on the right indicate the positions of standards of known relative molecular mass. Similar results were obtained with monoclonal antibodies that recognized three other epitopes.

aggregating activity. Thus our antibodies recognize in electric organ extracts two forms of agrin plus two polypeptides which do not have AChR/AChE-aggregating activity.

We have now purified microgram amounts of agrin and the other related polypeptides by employing immunoaffinity techniques. Isolation of each of the polypeptides by preparative polyacrylamide gel electrophoresis has permitted analysis of their N-terminal amino acid sequence. Our aim is to develop probes which

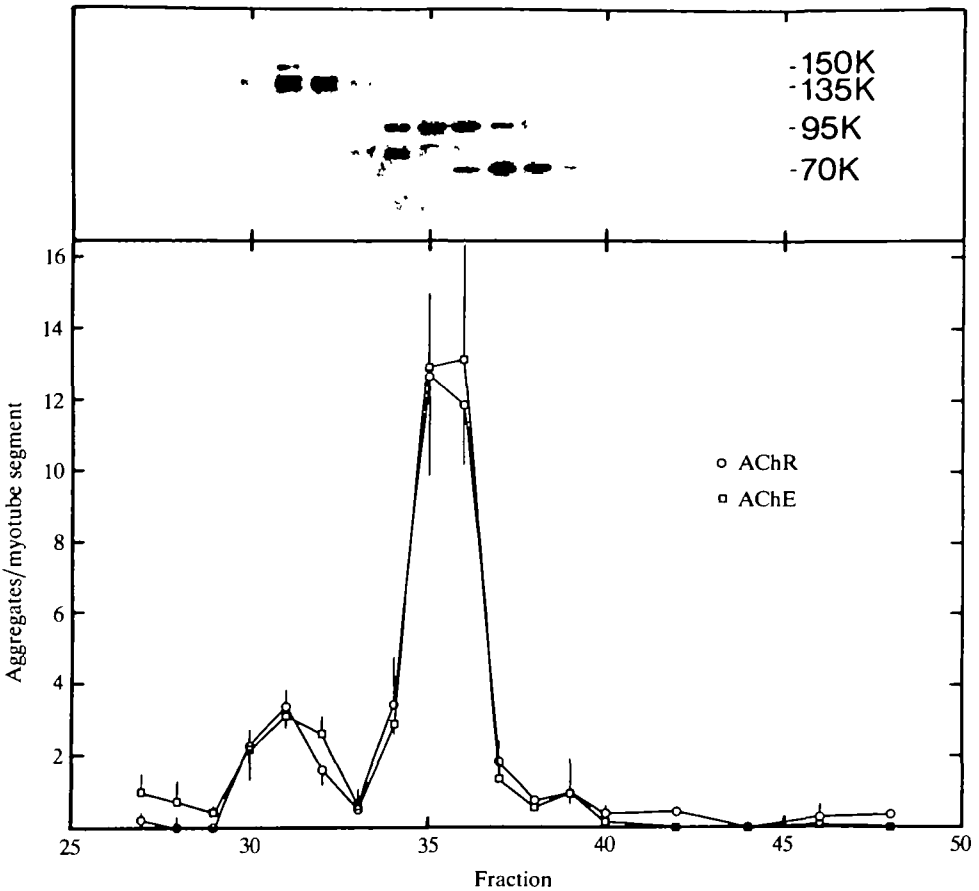


Fig. 2. Acetylcholine receptor (AChR, ○)- and acetylcholinesterase (AChE, □)-aggregating activities comigrate with 150 and 95K polypeptides recognized by anti-agrin antibodies. 0·75 ml of a partially purified preparation of agrin (Cibacron pool, R. M. Nitkin, M. A. Smith, J. R. Fallon, Y. M. Yao, B. G. Wallace & U. J. McMahan, in preparation) was applied to a 120×1 cm column of Sephacryl S-200 equilibrated and eluted with buffer containing 0·5 mol l^{-1} NaCl, 0·2 mol l^{-1} sodium bicarbonate, 5% glycerol, 0·02% azide, pH 9·0. Lower panel: fractions (1·5 ml) were collected and assayed for AChE- and AChR-aggregating activity (Wallace, 1986). Data is presented a mean ± S.E.M. ($N = 3$). Upper panel: samples (0·75 ml) of each fraction were precipitated with trichloroacetic acid and analysed by SDS polyacrylamide gel electrophoresis and immunoblotting with an anti-agrin antibody (see R. M. Nitkin *et al.* in preparation). AChE- and AChR-aggregating activities comigrated with each other and with the 150 and 95K agrin polypeptides, the 135 and 70K agrin-like polypeptides did not appear to possess any AChR/AChE-aggregating activity.

will enable us to use molecular genetic techniques to examine such questions as how the agrin polypeptides are related to each other, where and when they are synthesized, and if there are related peptides in other tissues or in muscles of other animal species.

Three polypeptides that cause AChR aggregation on cultured myotubes have been identified in neural tissues by other workers. Any or all of these molecules may play a

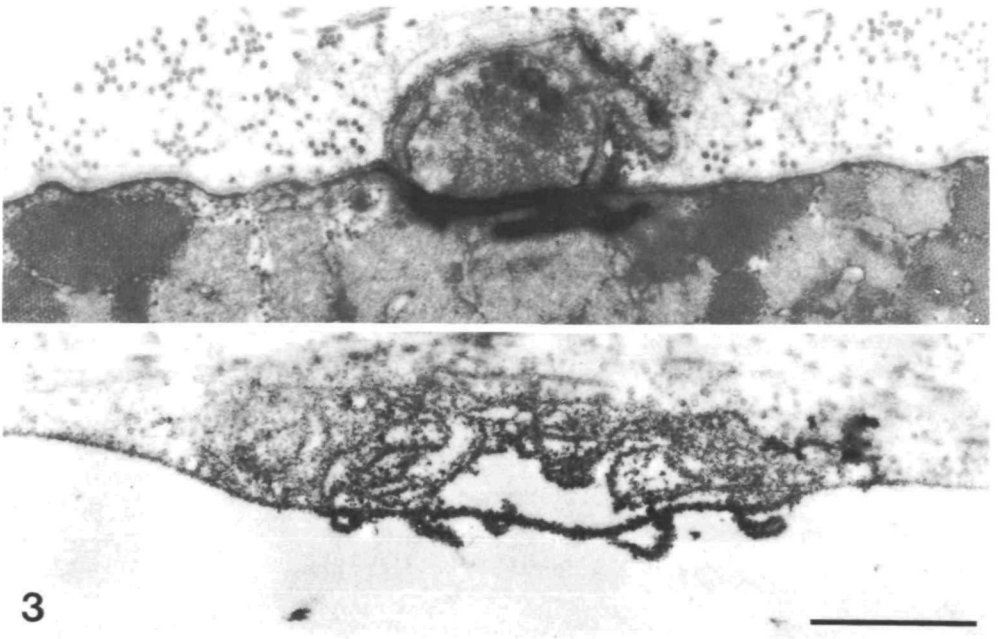


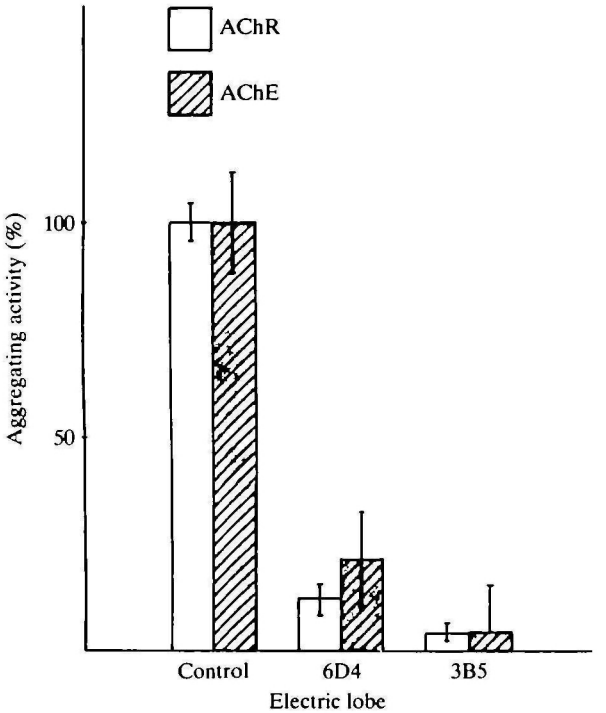
Fig. 3. Monoclonal antibodies against agrin recognize molecules concentrated in and stably bound to the synaptic basal lamina at neuromuscular junctions. Top: normal neuromuscular junction in cutaneous pectoris muscle of frog (*Rana pipiens*). Bottom: site of a neuromuscular junction 3 weeks after damaging the cutaneous pectoris muscle by crushing it. Myofibre, axon terminal and Schwann cell degenerated and were phagocytized in response to the trauma but the basal lamina of the myofibre and Schwann cell persisted. Antibody binding in the damaged muscle is localized to the synaptic portion of the myofibre basal lamina and to the Schwann cell basal lamina, presenting a staining pattern identical to that at the normal neuromuscular junction. [The muscles were stained with a mixture of anti-agrin antibodies 3B5 and 5B1. Details of our staining procedures are in Fallon *et al.* (1985).] Scale bar, 1 μm.

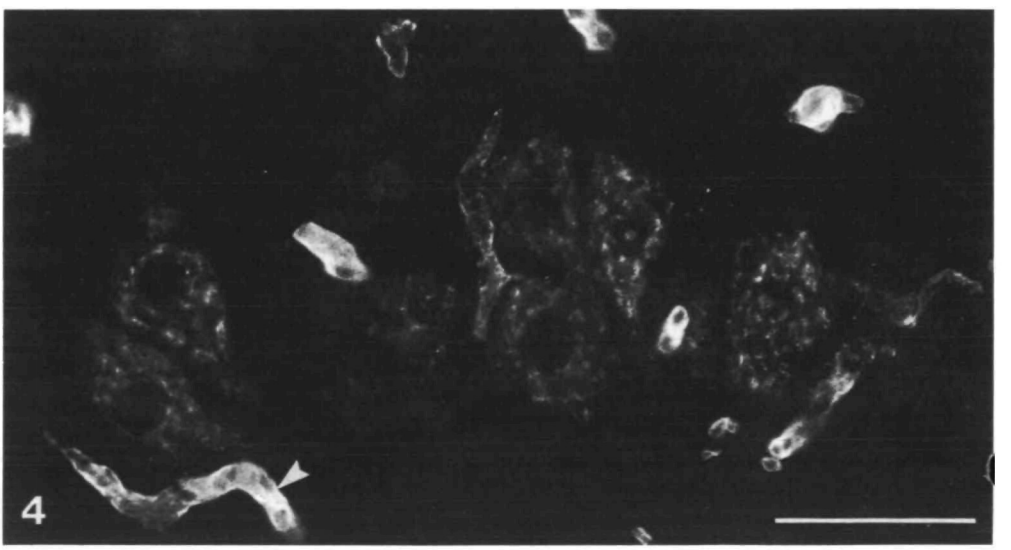
role in the formation of AChR aggregates at the neuromuscular junction. They are a 42K polypeptide extracted from chick brain (Usdin & Fischbach, 1986), an 84K polypeptide, called sciatin, which is extracted from sciatic nerves and is virtually identical to transferrin (e.g. Oh & Markelonis, 1982), and calcitonin gene-related peptide (CGRP) which is 23K and is present in motor neurones (e.g. New & Mudge, 1986). The relative molecular mass of each clearly differs from that of the two forms of agrin. Moreover, agrin has little, if any, effect on the rate of receptor insertion into the myotube plasma membrane while the others have a profound effect in this regard. Thus, agrin is distinct from any of these molecules.

AGRIN-LIKE MOLECULES IN THE SYNAPTIC BASAL LAMINA

To determine whether agrin-like molecules in the synaptic cleft at the neuromuscular junction are components of the basal lamina we first damaged the frog's cutaneous pectoris muscle by crushing it (Anglister & McMahan, 1985; McMahan & Slater, 1984). This resulted in degeneration and phagocytosis of all cellular

components of the neuromuscular junctions – myofibres, axon terminals and Schwann cells – while leaving intact much of the myofibres' basal lamina sheaths. Regeneration of myofibres and axons was prevented (McMahan & Slater, 1984). Three weeks after damage, the 'muscle' was removed from the frog and the empty basal lamina sheaths were incubated with anti-agrin antibodies. As shown in Fig. 3, the antibodies stained the synaptic portion of the myofibre basal lamina intensely.





Thus, molecules antigenically similar to agrin are concentrated in the synaptic basal lamina and these molecules remain adherent to the basal lamina for several days after muscle degeneration, as do the basal lamina's AChR- and AChE-aggregating molecules (McMahan & Slater, 1984).

The synaptic basal lamina is not the only structure in muscle stained by anti-agrin antibodies. Fig. 3 illustrates that the antibodies also stain Schwann cell basal lamina. In addition, although the extrajunctional surface of twitch muscle fibres did not stain, the antibodies stained the extrajunctional surface of slow skeletal muscle fibres, but at a much lower level than at the neuromuscular junction. In certain species the antibodies stained the surface of smooth muscle fibres in the walls of blood vessels. The surface of smooth muscle fibres, like that of skeletal muscle fibres, is coated with basal lamina. Thus, agrin may be antigenically related to one or more basal lamina molecules that have a broad distribution in muscle.

AGRIN-LIKE MOLECULES IN THE CENTRAL NERVOUS SYSTEM

One likely source of the AChR- and AChE-aggregating molecules in the synaptic basal lamina is the axon terminal. If the axon terminal were the source and if agrin were related to the AChR- and AChE-aggregating molecules in the synaptic basal lamina, one might expect to detect agrin in extracts of CNS regions rich in the cell bodies of motor neurones. As illustrated in Fig. 4, we indeed found that extracts of the electric lobe of the *Torpedo* brain, the region of the brain occupied by the cell bodies of the motor neurones that innervate the electric organ, contained molecules that caused the formation of AChR and AChE aggregates on cultured myotubes. The active molecules were immunoprecipitated with anti-agrin antibodies, indicating that they are similar, if not identical, to agrin. We have now detected functionally and antigenically similar molecules in extracts of spinal cord, another region rich in the cell bodies of motor neurones, from *Torpedo*, frog and chick.

We stained the electric lobe and spinal cords with the anti-agrin antibodies to determine the distribution of agrin-like molecules. In all cases the cell bodies of motor neurones stained (Fig. 4). The stain was distributed in patches in the cytoplasm, indicating that it is associated with organelles. No other neurones in the electric lobe and spinal cord (lumbosacral region) stained; thus, among neurone cell bodies in these regions, agrin-like molecules are selectively localized to those of

Fig. 4. Agrin-like molecules in the central nervous system. Top: an extract from the electric lobe of *Torpedo* brain causes the formation of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) aggregates on cultured chick myotubes, as does agrin. Each of two anti-agrin monoclonal antibodies 6D4 and 3B5 immunoprecipitates the activity. The extract was made by a method similar to that used for making electric organ extracts. Tissue was homogenized in Puck's saline and centrifuged at 15 900 ***g*** for 20 min. The pellet was then extracted overnight in 0·2 mol l^{-1} bicarbonate buffer, at pH 9·0, containing 5% glycerol. The extract was centrifuged at 150 000 ***g*** for 3 h before use. Bottom: a frozen section of the chick spinal cord stained with a mixture of anti-agrin antibodies 5B1 and 11D2. Stain is concentrated within the cell bodies of motor neurones and appears to be associated with cytoplasmic organelles. Stain is also associated with capillaries (arrowhead). Scale bar, 50 μm. Our method for immunoprecipitation is described in Fallon *et al.* (1985).

motor neurones. Stain was also associated with capillaries and pia mater, which have a basal lamina. We are currently performing experiments to determine whether the agrin-like molecules in the motor neurones have agrin activity and whether these molecules are released at axon terminals to become incorporated into the synaptic basal lamina.

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