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SYNTHESIS AND TRANSPORT OF AGRIN-LIKE MOLECULES IN MOTOR NEURONS

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Summary

Several lines of evidence indicate that agrin, or a protein very similar to it, directs the formation and maintenance of the postsynaptic apparatus at the neuromuscular junction. We discuss the results of studies involving immunohistochemical, biochemical and *in situ* hybridization techniques that support the hypothesis that agrin or agrin-like molecules active at the junction are produced by motor neurons.

Introduction

Studies conducted in this laboratory have led to the discovery of agrin, a protein believed to play a central role in the formation of the postsynaptic apparatus of neuromuscular synapses on skeletal muscle fibers (Nitkin *et al.* 1987). The postsynaptic apparatus at the neuromuscular synapse, as at other synapses throughout the nervous system, is made up of several structural specializations, each composed of aggregates of molecules or organelles. Some of the specializations are directly involved in synaptic transmission. For example, the plasma membrane has a dense aggregate of the receptors for the neurotransmitter acetylcholine, and the surface of the muscle fiber has an aggregate of acetylcholinesterase (AChE), the enzyme that terminates the action of the transmitter on the muscle fiber. It has long been known that the motor axon terminal at the neuromuscular synapse induces the formation and maintenance of the postsynaptic apparatus (e.g. Anderson and Cohen, 1977; Frank and Fischbach, 1979; Bevan and Steinbach, 1977). It is also known that the portion of the basal lamina of the myofiber that occupies the synaptic cleft has components that induce the formation of the postsynaptic apparatus on regenerating muscle fibers (Burden *et al.* 1979; McMahan and Slater, 1984; Anglister and McMahan, 1985). Results from our studies suggest that agrin, or a molecule very similar to it, mediates the induction by the axon terminal and synaptic basal lamina of many, if not all, of the structural specializations in the postsynaptic apparatus. According to the agrin

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hypothesis (Nitkin *et al.* 1987; Magill-Solc and McMahan, 1988), agrin is synthesized in the cell bodies of motor neurons, transported along their axons to muscle and released by the axon terminals into the synaptic cleft, where it binds to synaptic basal lamina and interacts with receptors on the muscle fibers to trigger the formation of the postsynaptic apparatus in the embryo and to maintain it in the adult.

Several lines of evidence support the agrin hypothesis. (1) Agrin has been extracted and purified from basal-lamina-containing fractions of the electric organ of the marine ray *Torpedo californica* (Godfrey *et al.* 1984; Nitkin *et al.* 1987), a tissue richly innervated by motor axons. (2) When agrin is applied to cultured chick myotubes it induces them to form patches on their surface that contain aggregates of acetylcholine receptors, acetylcholinesterase and other components of the postsynaptic apparatus (Wallace *et al.* 1985; Wallace, 1989; McMahan and Wallace, 1989). (3) Antibodies against agrin stain the cell bodies of motor neurons in the brain and spinal cord in the embryo and adult of several species of animals (Magill-Solc and McMahan, 1988). (4) Extracts of motor neurons contain molecules that induce cultured chick myotubes to form patches containing components of the postsynaptic apparatus similar to those induced by agrin, and the active molecules are immunoprecipitated by anti-agrin antibodies (Magill-Solc and McMahan, 1988). (5) Anti-agrin antibodies stain the basal lamina in the synaptic cleft at the neuromuscular synapse (Reist *et al.* 1987). Thus, agrin is known to induce the formation of postsynaptic apparatus *in vitro*, and motor neuron cell bodies, in both embryos and adults, as well as synaptic basal lamina, contain agrin or a molecule very similar to it.

Here we review evidence that the cell bodies of motor neurons contain agrin and present initial results from new studies which confirm that agrin is synthesized in motor neurons and is transported in an anterograde direction along their axons.

Cell bodies of motor neurons contain agrin

Our library of anti-agrin monoclonal antibodies contains at least five antibodies that are directed against different epitopes on agrin (Reist *et al.* 1987). All of these stain the synaptic basal lamina at neuromuscular synapses in *T. californica*. Some also stain the synaptic basal lamina in frog and/or chicken (Fig. 1; see also Reist *et al.* 1987). The staining of skeletal muscle is not restricted to synaptic basal lamina. For example, in all three species patches of stain are associated with the Schwann cell basal lamina (Fig. 1), while in *T. californica* and chicken the basal lamina of the extrajunctional region of slow muscle fibers and the basal lamina of smooth muscle in blood vessels stain. Thus, our antibodies reveal that although agrin and/or antigenically related molecules are concentrated in the synaptic basal lamina such molecules have a broad distribution in muscle.

The anti-agrin antibodies that stain the synaptic basal lamina in a particular species also stain the motor neurons in that species, which would be expected if the agrin-like molecules in the synaptic cleft were identical, or closely related, to those

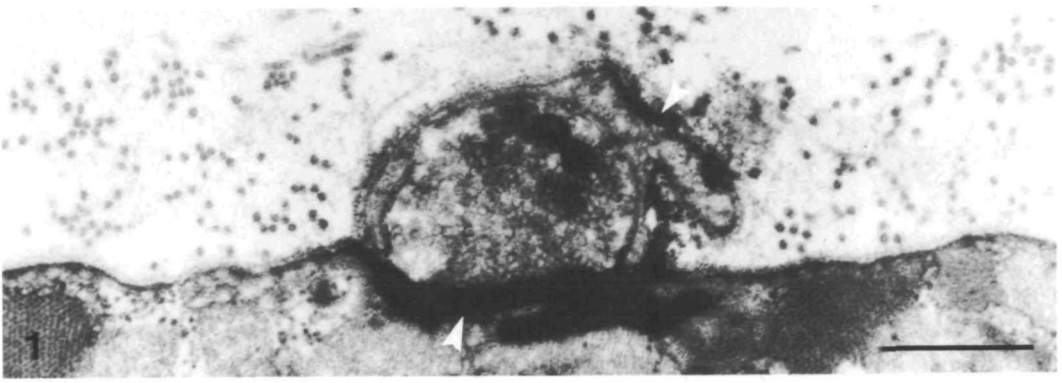

Fig. 1. Anti-agrin antibodies bind to molecules that are concentrated in the basal lamina of the synaptic cleft and of the Schwann cell (arrowheads) at the neuromuscular junction. Frog muscle stained with monoclonal antibody (mAb) 5B1. Scale bar, 1 μm.

in the motor neurons (Magill-Solc and McMahan, 1988). Fig. 2A is a light micrograph of anti-agrin-stained motor neurons of the electric lobe of the brain of *T. californica*, the portion of the brain that innervates the electric organ. The staining of frog spinal motor neurons, which innervate skeletal muscle, is shown in Fig. 2B. In both cases the stain is concentrated in patches in the cytoplasm, suggesting that agrin-like molecules are associated with organelles. Indeed, electron microscopy has revealed that the stain is concentrated in the Golgi apparatus, which processes proteins for secretion (Magill-Solc and McMahan, 1988).

Capillaries in the central nervous system (CNS) are also stained by anti-agrin antibodies (Figs 2 and 3); the stain is concentrated in the basal lamina that lies between the capillary endothelium and astrocyte foot-processes (Magill-Solc and McMahan, 1988). Thus, in the CNS, molecules antigenically related to agrin have a broad distribution. However, motor neurons are distinct among neurons in that they are the only ones in which anti-agrin staining has been detected and, therefore, either they are the only neurons that contain agrin or they have a relatively high concentration of such molecules (Magill-Solc and McMahan, 1988).

Staining of motor neurons of the chick spinal cord was observed in adults, neonates and embryos (Figs 2 and 3) (Magill-Solc and McMahan, 1988). Although faint, it could be detected in the lumbosacral region of the spinal cord of chick as early as embryonic day 5–6, the time at which motor neurons in this region of the spinal cord form neuromuscular junctions.

Motor neurons contain mRNA that encodes agrin-like molecules

The observation that the Golgi apparatus of motor neurons is stained by anti-agrin monoclonal antibodies strongly suggests that the motor neurons synthesize agrin-like molecules. We have now tested this hypothesis by searching for mRNA that encodes agrin in motor neurons. Our approach was as follows.

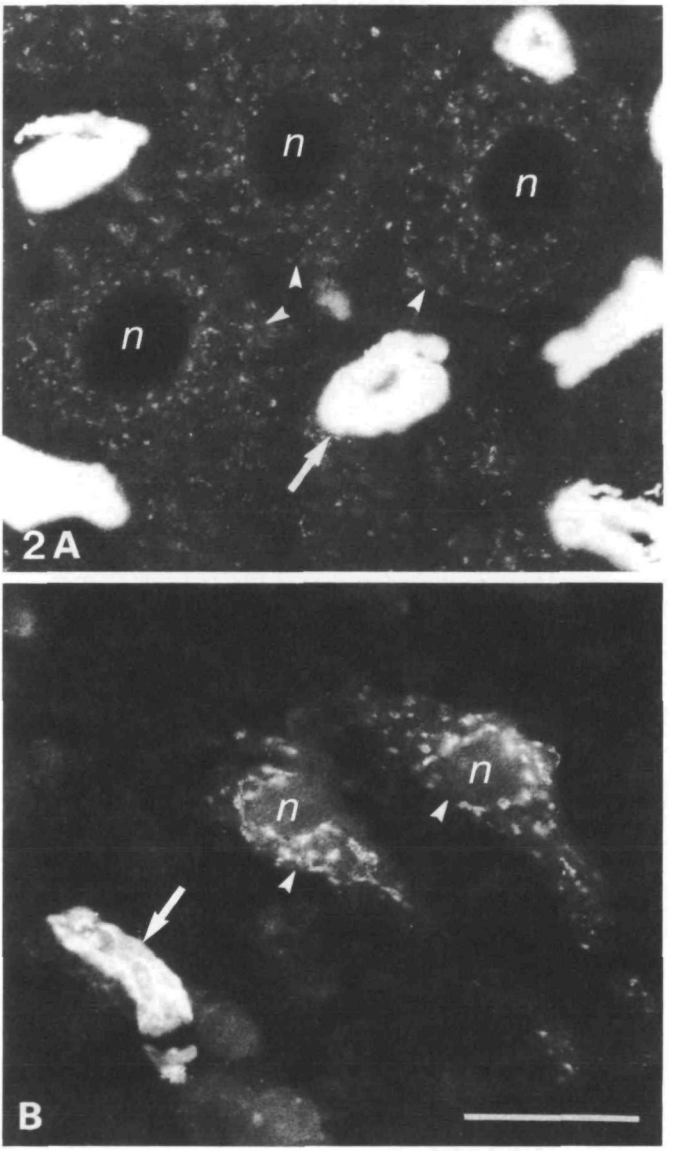

Fig. 2. Anti-agrin antibodies stain the cell bodies of motor neurons and the capillaries in the CNS of neonatal *Torpedo californica* and adult frog. (A) Electric lobe of newborn (1 week) *T. californica* stained with mAb 6D4. (B) Ventral horn in the spinal cord of an adult frog stained with mAbs 3B5 and 5B1. Neuron, arrowhead; neuronal nucleus, *n*; capillary, arrow. Scale bar, 50 μm.

We first immunoprecipitated the polypeptides in the electric organ basal lamina extracts that were recognized by anti-agrin monoclonal antibodies (Nitkin *et al.* 1987). There were four such polypeptides. Two (M_r 150×10^3 and 95×10^3) were found to have acetylcholine receptor (AChR) and acetylcholine esterase (AChE)

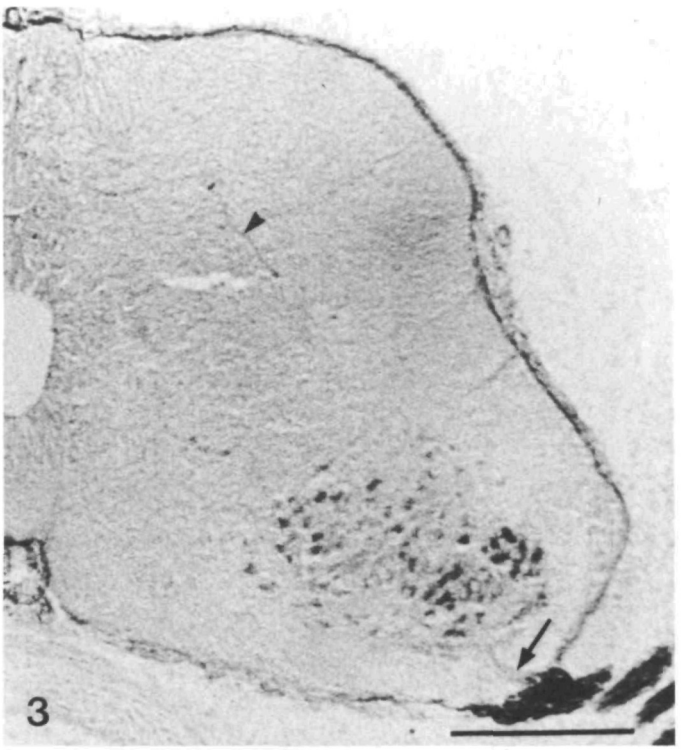

Fig. 3. Selective staining of motor neurons and non-neural structures in embryonic chick by an anti-agrin mAb. The right half of a cross-section of the lumbosacral region of a spinal cord from a day 10 chick embryo incubated with mAb 5B1. Motor neurons and the pial surface of the spinal cord are intensely stained. Capillaries (arrowhead) at this stage of development are lightly stained; compare with intense staining of capillaries at a later stage of development in Fig. 2. Glial cells and other neurons are not stained. The intensely stained structures outside the spinal cord are ventral roots; much of the stain is probably in the Schwann cell basal lamina, which is known to stain intensely in the adult (Reist *et al.* 1987). The lightly stained region (arrow) of the spinal cord extending from the motor column to the ventral root was observed at higher magnification to be composed of narrow cell processes having a nearly uniform diameter, probably motor axons. Scale bar, 200 μm.

aggregating activity and thus were agrin; two (M_r 135×10^3 and 70×10^3) were inactive. Antiserum against the four polypeptides was then used to screen a cDNA library constructed in lambda gt-11 from poly(A+) RNA that had been extracted from the electric lobe of *Discopyge ommata*, a marine ray related to *T. californica* (M. Smith, F. Rupp, C. Magill-Solc, R. Scheller and U. J. McMahan, unpublished observations). One clone initially isolated was determined by epitope selection of the antiserum to encode a fusion protein containing antigenic determinants shared with all four *T. californica* agrin (M_r 150×10^3 and 95×10^3) and agrin-like (M_r 135×10^3 and 70×10^3) proteins (M. Smith, F. Rupp, C. Magill-Solc, R. Scheller and U. J. McMahan, unpublished observations). This recombinant cDNA clone,

called agrin-6, has been characterized by sequencing analysis (M. Smith, F. Rupp, C. Magill-Solc, R. Scheller and U. J. McMahan, unpublished observations). It contains an insert of 4.5 kb and shows no strong homologies to previously characterized matrix-associated proteins, such as laminin, fibronectin, collagen or entactin. This cDNA encodes an amino acid sequence that is very similar to the *N*-terminal sequence of the M_r 95×10^3 and 70×10^3 agrin proteins. Eleven out of fourteen amino acids are identical; one exchange is due to the conservative mutation from Val to Leu. This homology is many orders of magnitude beyond random expectation and the minor differences in sequence are expected to be due to the fact that different species of marine rays were used for the cloning and protein determination. Using the electric ray agrin-6 clone as a probe, cDNA clones homologous to *T. californica* agrin were isolated and characterized from chick brain and rat spinal cord libraries (chick: K. Tsim, M. Ruegg, S. Kroger, Q. Escher, C. Magill and U. J. McMahan, unpublished observations; rat: F. Rupp, C. Magill, D. Cowan, R. Scheller and U. J. McMahan, unpublished observations). Both have been sequenced and show strong homology with the *T. californica* agrin-6 clone. Northern blot analysis of RNA from various tissues of ray, chick and rat indicates that the corresponding message is concentrated in brain and spinal cord of all three species.

To determine whether motor neurons contain mRNA that encodes agrin-like proteins, we did *in situ* hybridizations with probes against the agrin clone on sections of spinal cord and brain of *D. ommata*. We used the agrin-6 clone to generate both anti-sense and sense ^{35}S-labelled RNA probes. The anti-sense probes are complementary to the mRNA and will hybridize to it, thereby revealing the localization of the agrin mRNA. The sense probe is identical to the agrin mRNA and will not hybridize to the mRNA, but serves as a control for non-specific RNA binding. When *in situ* hybridizations were done on the medulla of *D. ommata*, labelling was found to be concentrated in the electromotor neurons of the electric lobe and in motor neurons of the brainstem (Fig. 4A). *In situ* hybridizations on sections of the spinal cord also revealed labelling concentrated in motor neurons (Fig. 4B). Labelling was also found in the ventral horns of the spinal cord of day 17 rat embryos. Thus, motor neurons in embryos and adults contain an mRNA that codes for agrin-like molecules, as would be expected if they synthesized agrin for development, maintenance and regeneration of the neuromuscular synapse.

Anterograde transport of agrin-like molecules in motor axons

A ligature placed on a nerve causes proteins produced in the cell bodies of the axons forming the nerve to accumulate predominantly in the axons on the cell body side of the ligature (e.g. Ranish and Ochs, 1972; Booj *et al.* 1986). Thus, if agrin is produced in the cell bodies of motor neurons and is transported along their axons to neuromuscular junctions, one might expect to observe an accumulation of anti-agrin-stained molecules predominantly on the cell body side of such a

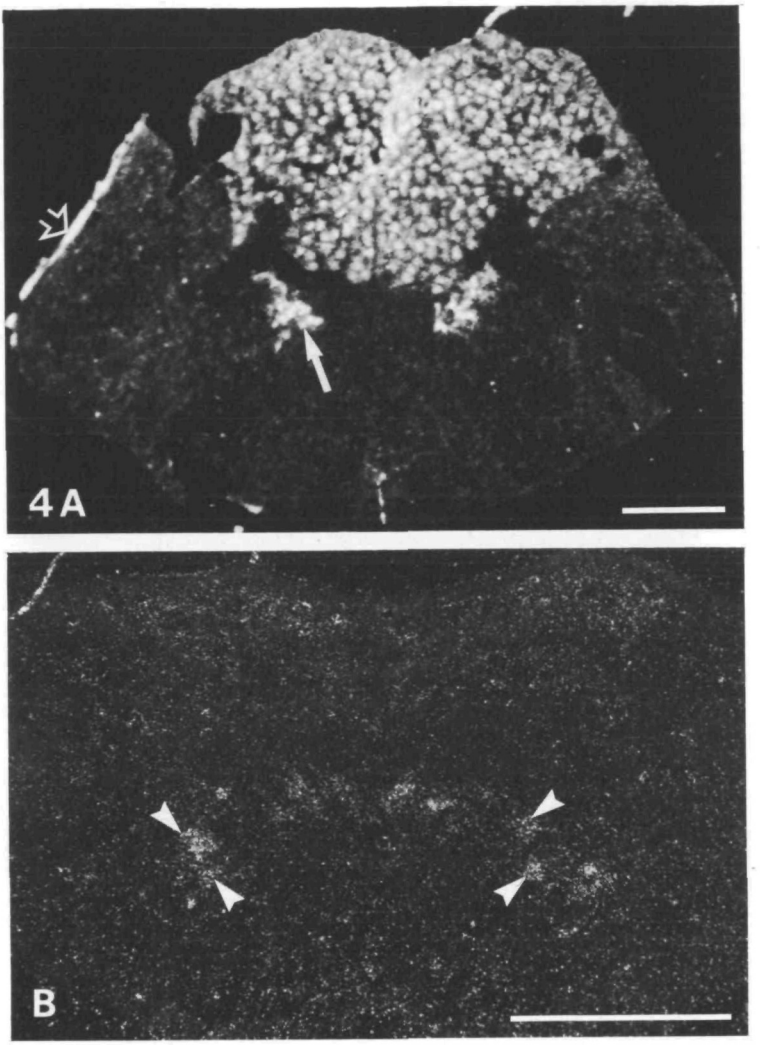

Fig. 4. Localization of agrin mRNA in motor neurons of *Discopyge ommata* by *in situ* hybridization. Darkfield autoradiography. (A) Cross-section from medulla. Grains are concentrated over the electromotor neurons of the electric lobe and the motor nucleus (arrow) of nerves VII and IX. There is no labelling of the pial surface, although a piece of the meninges which is still adherent to the surface of the brain scatters light, thereby appearing to be labelled (open arrow). (B) Cross-section from spinal cord. Grains are concentrated over the large neurons in the ventral horn which are motor neurons (arrowheads). The association of grains with motor neurons was confirmed in adjacent sections by staining with Cresyl Violet (not shown). Scale bars, 500 μm.

ligature. Accordingly, we placed a ligature on the sciatic nerve of the frog. Axons in normal frog sciatic nerves do not stain with anti-agrin antibodies. However, within 3 h after ligating the nerves many of the axons on the cell body side of the ligature were stained, while there were relatively few stained axons on the

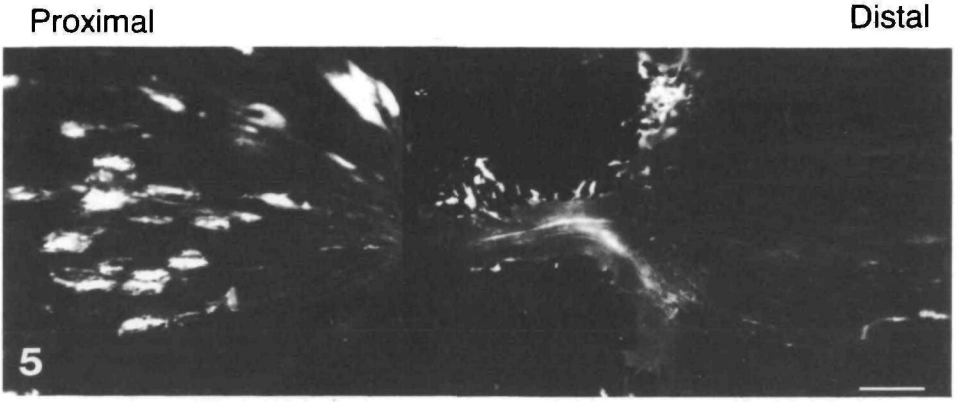

Fig. 5. Anti-agrin antibodies recognize molecules that accumulate in axons on the proximal (cell body) side of a ligature applied to the sciatic nerve of frog. A longitudinal section through a segment of nerve taken 2 days after ligation. Monoclonal antibody staining (mAbs 3B5 and 5B1) is highly concentrated in axons on the proximal side of the ligature, while there is relatively little staining on the distal side. Scale bar, 100 mm.

terminal side (Fig. 5). Moreover, when extracts of the cell body side of the nerve were applied to chick myotube cultures they were found to contain three- to fourfold more AChR aggregating activity than similar extracts from the terminal side of the ligature and 10-fold more activity than normal nerves. Most of the activity in the ligated nerves (about 70%) was immunoprecipitated by anti-agrin monoclonal antibodies, as expected. In experiments where we severed the ventral roots supplying motor axons to the sciatic nerve 3 days prior to making the ligation, we detected no axonal staining nor did we detect AChR aggregating activity in extracts of such nerves, indicating that the agrin-like molecules we found in ligated nerves with undamaged ventral roots were derived from the cell bodies of motor neurons rather than from the cell bodies of other components (sensory and autonomic) of the nerve.

Discussion

Our observation that the extrajunctional basal lamina of certain muscle fibers in adult chickens and *T. californica* stains with anti-agrin antibodies (Reist *et al.* 1987) and the finding by others (Godfrey *et al.* 1988; Fallon and Gelfman, 1989) that the muscle fiber basal lamina in aneural and normal embryonic chick limbs stains similarly indicates that muscle fibers make molecules antigenically similar to agrin. Thus, it is conceivable that agrin-like molecules produced by muscle are taken up by axon terminals at the neuromuscular junction and are transported retrogradely to the motor neuron cell bodies. The results of experiments we present here and elsewhere, however, indicate that most, if not all, of the agrin-like molecules we detect in motor neurons are produced by the motor neurons themselves: (1) motor

neurons contain mRNA for synthesizing agrin-like molecules; (2) agrin-like molecules are concentrated in the Golgi apparatus of the motor neuron, which processes proteins for secretion; and (3) agrin-like molecules accumulate along ligated axons in a manner indicating that their transport is primarily in an anterograde direction. These findings are fully consistent with the hypothesis that agrin, or a molecule very similar to it, mediates the motor-neuron- and basal-lamina-induced aggregation of AChRs and other postsynaptic components at the neuromuscular junction on skeletal muscle fibers.

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