Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3938–3941, May 1990
Neurobiology

Asymmetric distribution of dystrophin in developing and adult *Torpedo marmorata* electrocyte: Evidence for its association with the acetylcholine receptor-rich membrane

(morphogenesis/synapse/cytoskeleton)

B. J. JASMIN*, A. CARTAUD*, M. A. LUDOSKY*, J. P. CHANGEUX†, AND J. CARTAUD*‡

*Biologie Cellulaire des Membranes, Institut Jacques Monod, Centre National de la Recherche Scientifique, Université Paris 7, 2 Place Jussieu, 75251, Paris Cédex 05, France; and †Neurobiologie Moléculaire, Département des Biotechnologies, Institut Pasteur, 25 Rue du Docteur Roux, 75724 Paris Cédex 15, France

Contributed by J. P. Changeux, February 26, 1990

ABSTRACT **Dystrophin has been shown to occur in *Torpedo* electrocyte [Chang, H. W., Bock, E. & Bonilla, E. (1989) *J. Biol. Chem.* 264, 20831–20834], a highly polarized syncytium that is embryologically derived from skeletal muscle and displays functionally distinct plasma membrane domains on its innervated and noninnervated faces. In the present study, we investigated the subcellular distribution of dystrophin in the adult electrocyte from *Torpedo marmorata* and the evolution of its distribution during embryogenesis. Immunofluorescence experiments performed on adult electrocytes with a polyclonal antibody directed against chicken dystrophin revealed that dystrophin immunoreactivity codistributed exclusively with the acetylcholine receptor along the innervated membrane. At the ultrastructural level, dystrophin immunoreactivity appears confined to the face of the subsynaptic membrane exposed to the cytoplasm. In developing electrocytes (45-mm embryo), dystrophin is already detectable at the acetylcholine receptor-rich ventral pole of the cells before the entry of the electromotor axons. Furthermore, we show that dystrophin represents a major component of purified membrane fractions rich in acetylcholine receptor. A putative role of dystrophin in the organization and stabilization of the subsynaptic membrane domain of the electrocyte is discussed.**

The Duchenne/Becker muscular dystrophy gene has recently been identified by reversed genetics and localized on human chromosome X (reviewed in refs. 1–3). Its product, dystrophin, has a predicted molecular mass of 427 kDa and is absent from (or deficient in) diseased muscle. Inspection of its amino acid sequence reveals that the dystrophin molecule consists of four domains, three of which share various degrees of sequence homology with the cytoskeletal proteins spectrin and α-actinin. In normal muscle cells, dystrophin is localized at the sarcolemma (4–8). However, its precise function remains to be elucidated.

The electrocyte of *Torpedo marmorata* is a syncytium embryologically derived from skeletal muscle stem cells (9) and, consistent with this lineage, dystrophin has been observed in this tissue (10). The electrocyte is a highly polarized cell with two functionally distinct and structurally different plasma membrane domains (see references in refs. 9 and 11), along its innervated and noninnervated faces. It may thus represent a unique model with which to investigate the function of dystrophin. In the present work, we address the question of dystrophin subcellular distribution in the adult electrocyte in relation to the polarity of the cell. Furthermore, in the course of our ongoing studies on synaptogenesis, we examine the developmental stage at which dystrophin becomes associated with the membrane. Finally, we compare properties of dystrophin from the electric organ with those of dystrophin found in *Torpedo* skeletal muscles.

MATERIALS AND METHODS

Anti-Dystrophin Antibody. The polyclonal antibody used in this study was raised in rabbits from a fusion protein which included a 556-amino acid fragment (residues 1173–1728) of the spectrin-like domain of chicken dystrophin. The antibody recognizes dystrophin in several species from human to fish.

Immunocytochemistry. Freshly dissected *T. marmorata* columns of electrocytes were fixed in 3% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, at 4°C. Subsequently, the tissue was impregnated with 25% (wt/vol) sucrose and rapidly frozen. Electric organ from 40- to 80-mm (body length) embryos was treated similarly. Caudal muscles of adult *Torpedo* were also freshly dissected, but immediately frozen.

Indirect immunofluorescence experiments were performed on cryostat sections (4 μm). Dystrophin was detected by using the polyclonal antibody (dilution 1:100) revealed with tetramethylrhodamine-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The acetylcholine receptor (AChR)-rich membrane was identified by labeling with fluorescein isothiocyanate-conjugated α-bungarotoxin (1 μg/ml; Sigma). Photomicrographs were obtained with a Leitz photomicroscope equipped with epifluorescence illumination (filters for rhodamine and fluorescein), using Plan ×63 (n.a., 1.40) or ×100 (n.a., 1.32) immersion optics. T-Max films were used and set at 800 ASA, and were developed accordingly.

Immunogold labeling (see ref. 11 for details) was performed by incubating cryostat sections with the anti-dystrophin antibody followed by incubation with protein A that was conjugated to colloidal (10-nm) gold. Nonspecific binding was prevented by preincubation of the sections with 4% bovine serum albumin and 1% fish gelatin in phosphate-buffered saline. After washing, sections were fixed again with 2% glutaraldehyde and 0.1% tannic acid in 0.1 M cacodylate buffer (pH 7.4), postfixed with 1% osmium tetroxide, dehydrated in a series of ethanol solutions, and embedded in epoxy resin. Thin sections were observed in a Philips EM 410 electron microscope without further staining, and micrographs were obtained on Kodak electron microscopy 4489 film.

Preparation of Membrane Fraction, SDS/PAGE, and Immunoblotting. AChR-enriched membranes were prepared from fresh electric tissue according to Mitra *et al.* (12) with the addition of the antiprotease agents leupeptin and pepsta-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: AChR, acetylcholine receptor.

‡To whom reprint requests should be addressed.

tin A (5 μg/ml; Sigma). Microsomal fractions of caudal muscles of *T. marmorata* and mammalian muscles (rat) were prepared as described by Mitchell *et al.* (13) in the presence of benzamidine (0.8 mM), leupeptin (0.5 μg/ml), aprotinin (0.5 μg/ml), pepstatin A (0.25 μg/ml) and phenylmethylsulfonyl fluoride (0.2 mM) as antiproteolytic agents.

Proteins of the membrane fractions were separated by one-dimensional SDS/6% PAGE (14) in a slab cell (Mini-Protean II, Bio-Rad) operating at 200 V for 45 min. In the experiments in which dystrophin from electrocyte and from skeletal muscle membrane fractions were compared on the same gel, proteins were run for a further 25 min after the dye front had reached the bottom of the gel. This allowed for a better separation of the high molecular mass proteins.

Immunoblotting experiments were performed according to Towbin *et al.* (15). Dystrophin was detected by incubation of the nitrocellulose strip with the antibody (1:200 to 1:500). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) was used as a second antibody and the color reaction was developed with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reaction (Kirkegaard & Perry Laboratories).

RESULTS

As an initial approach, we performed immunofluorescence experiments to reveal the presence of dystrophin antigens in transverse cryostat sections of adult electric tissue. In these sections, the two distinct plasma membrane surfaces of the electrocytes, innervated and noninnervated, can be easily identified. The immunoreactivity observed with the anti-dystrophin antibody strictly codistributed with the AChR identified by α-bungarotoxin labeling (Fig. 1 *A* and *A'*). Thus, the dystrophin antibody reacted with the innervated surface of the electrocytes.

At the electron microscopic level, the subcellular localization of dystrophin immunoreactivity was achieved by immunogold labeling. Gold particles were observed all along the innervated membrane of the electrocytes and appeared strictly confined to the cytoplasmic surface of the subsynaptic membrane (Fig. 1 *B* and *C*). No significant labeling was observed at the level of the noninnervated membrane (Fig. 1*D*) or elsewhere in the cytoplasm.

Since this polypeptide appeared as a component of the subsynaptic membrane, it was of interest to study the incorporation of dystrophin in its membrane domain during *Torpedo* electrocyte embryogenesis. We have selected two stages of development: embryos of 40–45 mm and 75–80 mm, which correspond, respectively, to an early stage at which AChRs are already present at the ventral pole of newly formed electrocyte in the absence of innervation (16, 17) and to a stage at which the innervated electrocyte displays a flattened asymmetric structure. Dystrophin immunoreactivity was detected at the AChR-rich ventral pole of the 45-mm-embryo electrocyte (Fig. 2). It was also present at the later developmental stages (data not shown). Although we have not quantified the intensity of the fluorescence in these experiments, we consistently observed that in 45-mm embryos, dystrophin fluorescence was much higher than that of the AChR. In contrast, in the adult electrocyte under the same conditions, the fluorescence associated with the AChR was higher than that of dystrophin.

The antigen(s) recognized by the antibody was identified in immunoblotting experiments performed on AChR-enriched membrane fractions. The anti-dystrophin antibody detected a high molecular mass protein (Fig. 3*A*). In both *Torpedo* AChR-

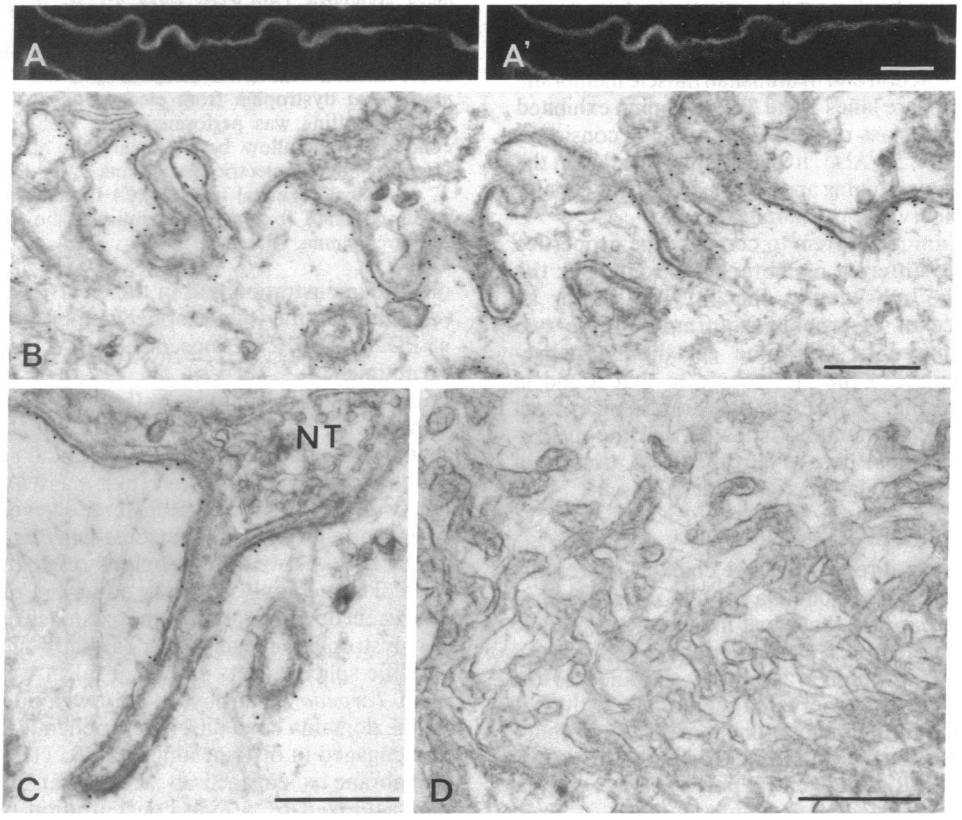

FIG. 1. Subcellular localization of dystrophin in adult electrocyte. (*A* and *A'*) Double fluorescence experiment showing the codistribution of AChRs (*A*) and dystrophin (*A'*) in a cryostat section of adult electric tissue. The same field was observed with fluorescein-labeled α-bungarotoxin to detect AChR and rhodamine indirect immunofluorescence to detect dystrophin. (*B* and *C*) Immunogold localization of dystrophin, showing its association with the cytoplasmic face of the subsynaptic membrane. NT, nerve terminal. (*D*) In the same experiment, no labeling of the noninnervated membrane was observed. (Bar = 25 μm in *A*/*A'*; bar = 0.5 μm in *B–D*.)

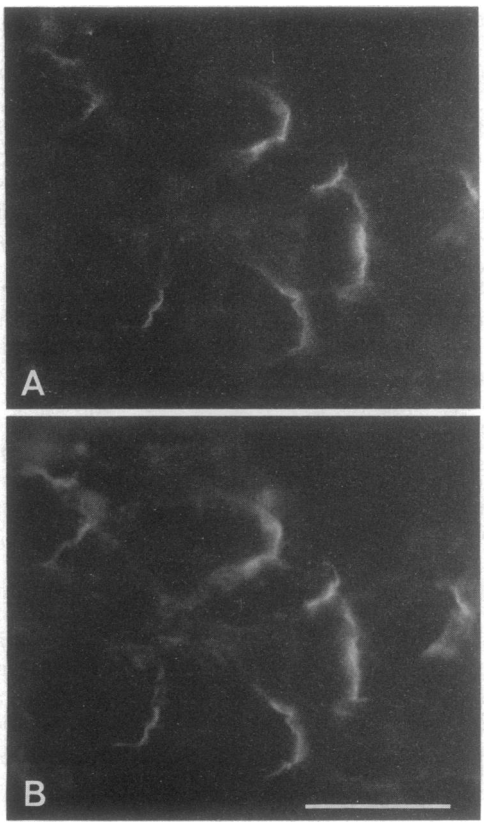

FIG. 2. Double fluorescence experiment showing the codistribution of AChR (*A*) and dystrophin (*B*) in cryostat sections of embryonic (45-mm stage) *T. marmorata* electric tissue. Note the strict correspondence of the two labelings at the ventral pole of developing electrocytes. (Bar = 50 μm.)

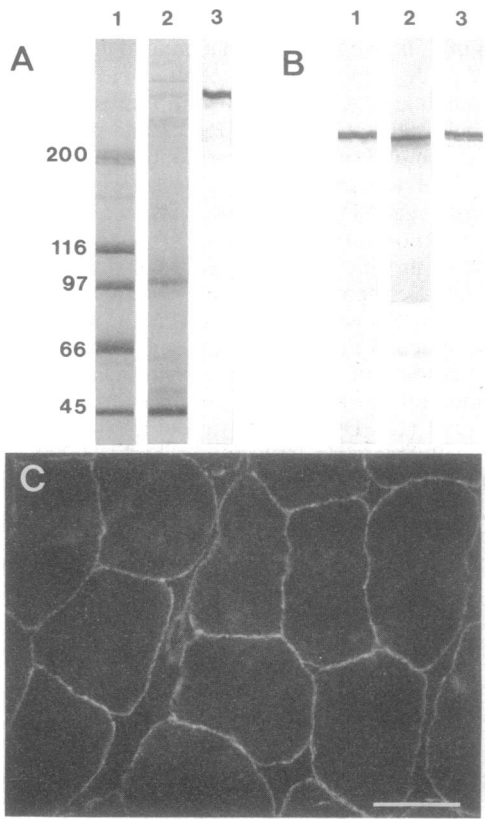

FIG. 3. (*A*) SDS/6% PAGE–immunoblot analysis of dystrophin in AChR-enriched membrane fraction from adult *T. marmorata* electric tissue. Lane 1, Coomassie blue staining of high molecular mass standards (Bio-Rad; sizes at left in kilodaltons); lane 2, Coomassie blue staining of the proteins of the AChR-enriched membrane fraction; lane 3, immunoblot of AChR-enriched membrane fraction with anti-dystrophin antibody. (*B*) Comparison of *T. marmorata* dystrophin from electric tissue and skeletal muscle. Immunoblotting was performed after SDS/6% PAGE run under conditions that allow better separation of high molecular mass proteins. Lane 1, electric tissue; lane 2, rat skeletal muscle; lane 3, *T. marmorata* skeletal muscle. (*C*) Immunofluorescence of transverse section of *Torpedo* caudal muscle, showing intense staining of the sarcolemma. (Bar = 50 μm.)

enriched membrane fraction and mammalian muscle membrane fraction (Fig. 3*B*, compare lanes 1 and 2), dystrophin exhibited an apparent molecular mass of ≈400-kDa, a value consistent with other reports (1, 4, 5). Also, it was readily detected in the gel after Coomassie blue staining and thus appears to be a major component of the innervated membrane.

Torpedo offers a unique system to compare the properties of dystrophin in two different membrane environments: the sarcolemma and the subsynaptic domain. To test for an eventual occurrence of dystrophin isoforms in these two systems, we compared in the same gel the "synapse-associated" dystrophin of the electrocyte to that found in skeletal muscle (see Fig. 3*C* for immunofluorescence) from the same animal. The immunoblot showed that the dystrophin obtained from these two dystrophin-rich tissues of *Torpedo* had close molecular masses (Fig. 3*B*).

DISCUSSION

A polyclonal antibody directed against chicken dystrophin recognizes specifically an ≈400-kDa protein in the electrocyte of *Torpedo marmorata* that most likely corresponds to dystrophin. In adult electrocytes, immunogold experiments demonstrated that dystrophin was strictly confined to the cytoplasmic surface of the innervated membrane of the cell and that it represented a major component of AChR-enriched membrane fractions. Furthermore, dystrophin was found to codistribute with AChR at the ventral pole of electrocytes at an early developmental stage before innervation.

Chang *et al.* (10) have reported, in agreement with our findings, that dystrophin is present in *Torpedo* electric tissue as a major component of AChR-enriched membranes yet, in contrast to our results, they also observed immunofluorescent staining of the noninnervated membrane. Here, we provide a direct demonstration that in the electric organ, dystrophin is restricted to the subsynaptic membrane and that like the 43-kDa protein, it is exposed at the cytoplasmic surface of this membrane.

In attempts to identify dystrophin isoforms, we used one-dimensional SDS/PAGE to compare membrane fractions originating from *Torpedo* electric tissue and skeletal muscle. We found that the apparent molecular masses of the dystrophin in the two preparations were close. However, additional experiments are necessary to examine whether more subtle differences exist between dystrophins originating from these tissues. This certainly deserves attention, especially in light of the recent demonstration of the existence of an autosomal gene for dystrophin (18) and the possibility of alternative, tissue-specific splicing of its precursor mRNA (19).

In *Torpedo* electrocyte, the two opposite plasma membrane domains have different biochemical compositions and are engaged in distinct functions (9, 11). The noninnervated membrane is engaged in active transport of ions and is characterized by a high concentration of Na^+,K^+-ATPase and by the cytoskeletal proteins spectrin and ankyrin. As shown in other systems (20, 21), spectrin and ankyrin are most likely involved in the maintenance of the polarized distribution and possibly, in the topographic assembly of Na^+,K^+-ATPase. Conversely, the subsynaptic membrane is

responsible for the genesis of electrical discharges. It contains high levels of AChR molecules as well as several nonreceptor membrane-associated peripheral proteins. These include the 43-kDa (22), 54-kDa (23), 58-kDa (24), 87-kDa (25), and non-spectrin 270- to 300-kDa (26) proteins as well as dystrophin (present study and ref. 10). With the exception of the 43-kDa and 54-kDa proteins, for which functions in AChR stabilization (see references in refs. 17 and 27) and in anchoring of intermediate filaments (23) have been hypothesized, respectively, the role of the other peripheral proteins is not well documented.

The structural homology of dystrophin and spectrin has led to the proposal that both are engaged in membrane stabilization (1, 2, 5, 7, 28). Since most studies on this issue dealt with skeletal muscles in which these two proteins codistribute at the sarcolemma (29), their respective function could not be easily distinguished. However, an indication that they may have different molecular roles comes from the observation that in Duchenne muscular dystrophy patients, who lack muscular dystrophin, spectrin is distributed normally (30). In *Torpedo* electrocyte, the divergent and exclusive localization of dystrophin and spectrin indicates that the two proteins are involved in distinct molecular interactions. In this regard, the C-terminal domain of dystrophin, which does not share any sequence homology with known proteins (1), may confer its specificity of interactions with membrane glycoproteins (28) and/or with other cytoskeletal components.

In the electrocyte, dystrophin may be linked to the innervated membrane via interactions with a "membrane receptor" sharing functional analogies with ankyrin, the membrane receptor of spectrin (see references in ref. 31). A candidate for this role may be the 43-kDa protein, which, like ankyrin (32), is an acylated amphitropic peripheral membrane protein (33). In this context, studies of the morphogenesis of the excitable membrane in embryos may shed some light on the interactions that dystrophin establishes with other components of the membrane. We focused our attention on the 45-mm stage, at which the assembly of the AChR-rich membrane domain begins (16). As observed by immunofluorescence, dystrophin is already a major component of this membrane. Interestingly, at this stage of development, the 43-kDa protein is mostly cytoplasmic (17). Therefore, the molecular interactions of dystrophin with the developing subsynaptic membrane do not seem to require the presence of the 43-kDa protein.

The occurrence of dystrophin at an early developmental stage of the subsynaptic membrane raises the possibility that it plays an active role in the assembly of this membrane domain. A recent model (34) suggests that the anion transporter, one of the major integral proteins of the erythroid membrane, is gradually recruited onto pre-assembled peripheral cytoskeletal binding sites involving spectrin and ankyrin during membrane morphogenesis. Similarly, in the developing electrocyte, dystrophin may participate to the formation of a submembrane cytoskeletal net that could favor AChR clustering. Furthermore, since laminin (17) as well as other components of the basal lamina (35) are also present at the 45-mm developmental stage, dystrophin may also contribute to a transmembrane signaling system involving components of the extracellular matrix. Accordingly, dystrophin would represent a crucial element involved in the assembly of the subsynaptic membrane and, thus, in synapse formation.

We thank Dr. Jean J. Léger for the generous gift of the anti-dystrophin antibody and Drs. E. L. Benedetti and R. Couteaux for critical reading of the manuscript. The constant support of the Association Française contre les Myopathies, the Collège de France, the Centre National de la Recherche Scientifique, and the Institut National pour la Santé et la Recherche Médicale is gratefully acknowledged. B.J.J. is the recipient of a Postdoctoral Fellowship in Sciences from the North Atlantic Treaty Organization (via the Natural Sciences and Engineering Research Council of Canada).

1. Hoffman, E. P. & Kunkel, L. M. (1989) *Neuron* **2,** 1019–1029.
2. Monaco A. P. (1989) *Trends Biochem. Sci.* **14,** 412–415.
3. Mandel, J. L. (1989) *Nature (London)* **339,** 584–586.
4. Hoffman, E. P., Knudson, C. M., Campbell, K. P. & Kunkel, L. M. (1987) *Nature (London)* **330,** 754–758.
5. Zubrzycka-Gaarn, E. E., Bulman, D. E., Karpati, G., Burghes, A. H. M., Belfall, B., Klamut, H. J., Talbot, J., Hodges, R. S., Ray, P. N. & Worton, R. G. (1988) *Nature (London)* **333,** 466–469.
6. Arahata, K., Ishiura, S., Ishiguro, T., Tsukahara, T., Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E. & Sugita, H. (1988) *Nature (London)* **333,** 861–863.
7. Watkins, S. C., Hoffman, E. P., Slayter, H. S. & Kunkel, L. M. (1988) *Nature (London)* **333,** 863–866.
8. Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salviati, G., DiMauro, S., Kunkel, L. M., Hoffman, E. P. & Rowland, L. P. (1988) *Cell* **54,** 447–452.
9. Changeux, J. P. (1981) *Harvey Lect.* **75,** 85–254.
10. Chang, H. W., Bock, E. & Bonilla, E. (1989) *J. Biol. Chem.* **264,** 20831–20834.
11. Kordeli, E., Cartaud, J., Nghiêm, H. O., Pradel, L. A., Dubreuil, C., Paulin, D. & Changeux, J. P. (1986) *J. Cell Biol.* **102,** 748–761.
12. Mitra, A. K., McCarthy, M. P. & Stroud, R. M. (1989) *J. Cell Biol.* **109,** 755–774.
13. Mitchell, R. D., Palade, P. & Fleischer, S. (1983) *J. Cell Biol.* **96,** 1008–1016.
14. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
15. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
16. Witzemann, V., Richardson, G. & Boustead, C. (1983) *Neuroscience* **8,** 333–349.
17. Kordeli, E., Cartaud, J., Nghiêm, H. O., Devillers-Thiéry, A., & Changeux, J. P. (1989) *J. Cell Biol.* **108,** 127–139.
18. Love, D. R., Hill, D. F., Dickson, G., Spurr, N. K., Byth, B. C., Marsden, R. F., Walsh, F. S., Edwards, Y. H. & Davies, K. E. (1989) *Nature (London)* **339,** 55–58.
19. Walsh, F. S., Pizzey, J. A. & Dickson, G. (1989) *Trends Neurosci.* **12,** 235–238.
20. Morrow, J. S., Cianci, C. D., Ardito, T., Mann, A. S. & Kashgarian, M. (1989) *J. Cell Biol.* **108,** 455–465.
21. Nelson, W. J. & Hammerton, R. W. (1989) *J. Cell Biol.* **108,** 893–902.
22. Sobel, A., Weber, M. & Changeux, J. P. (1977) *Eur. J. Biochem.* **80,** 215–224.
23. Cartaud, A., Courvalin, J. C., Ludosky, M. A. & Cartaud, J. (1989) *J. Cell Biol.* **109,** 1745–1752.
24. Froehner, S. C. (1984) *J. Cell Biol.* **99,** 88–96.
25. Carr, C., Fischbach, G. D. & Cohen, J. B. (1989) *J. Cell Biol.* **109,** 1753–1764.
26. Woodruff, M. L., Theriot, J. & Burden, S. J. (1987) *J. Cell Biol.* **104,** 939–946.
27. Froehner, S. C. (1986) *Trends Neurosci.* **9,** 37–41.
28. Campbell, K. P. & Kahl, S. D. (1989) *Nature (London)* **338,** 259–262.
29. Repasky, E. A., Granger, B. L. & Lazarides, E. (1982) *Cell* **29,** 821–833.
30. Appleyard, S. T., Dunn, M. J., Dubowitz, V., Scott, M. L., Pittman, S. J. & Shotton, D. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 776–780.
31. Bennett, V. (1985) *Annu. Rev. Biochem.* **54,** 273–304.
32. Staufenbiel, M. & Lazarides, E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 318–322.
33. Musill, S., Carr, C., Cohen, J. B. & Merlie, J. P. (1988) *J. Cell Biol.* **107,** 1113–1121.
34. Lazarides, E. & Woods, C. (1989) *Annu. Rev. Cell Biol.* **5,** 427–452.
35. Richardson, G. P., Fiedler, W. & Fox, G. Q. (1987) *Cell Tissue Res.* **247,** 651–665.