SUBSTRUCTURE OF AMPHIBIAN MOTOR END PLATE

Evidence for a Granular Component Projecting from the Outer Surface of the Receptive Membrane

JACK ROSENBLUTH

From the Departments of Physiology and Rehabilitation Medicine, New York University Medical Center, New York 10016

ABSTRACT

End-plate membrane has been examined at amphibian myoneural junctions by means of transmission electron microscopy of thin tissue sections. The postjunctional membrane exhibits morphologically specialized dense, convex patches which are located superficially facing the axon terminal but do not extend into the depths of the junctional folds. In the specialized regions the plasma membrane is ~120 Å thick and trilaminar. The outer dense lamina is thickened by the presence in it of granular elements ~60–120 Å in diameter which are spaced semiregularly at ~100–150-Å intervals and which border the junctional cleft directly. In these regions the concentration of the granules is of the order of $\sim 10^4/\mu m^2$, which is in the same range as the estimated concentration of receptor sites at other vertebrate cholinergic junctions. Filamentous projections can sometimes be seen extending from the granules to the overlying basement membrane, and in oblique views a reticular pattern may appear both in these patches and in the basement membrane. The cytoplasmic surface of the specialized membrane is covered with an amorphous and filamentous dense material whose distribution coincides with that of the granules visible in the outer layer and which may be connected to them across the membrane. In unosmicated specimens stained with permanganate and uranyl acetate the specialized regions exhibit the same morphological features but stand out sharply in contrast to adjacent areas of unspecialized membrane which appear only faintly. Such preparations are particularly useful in assessing the extent of the specialized membrane. It is proposed that the granules visible at the outer surface of the end-plate membrane represent acetylcholine receptors and that in amphibians, as in annelids, the receptors at myoneural junctions are concentrated into patches which occupy less than the total postjunctional membrane surface area.

INTRODUCTION

Annelid myoneural junctions have been shown to exhibit a prominent structural specialization of the postjunctional membrane consisting of arrays of granules seated in the membrane which give rise to projections extending into the junctional cleft (20, 24). It was suggested originally that these regularly arrayed postjunctional elements might represent transmitter receptor sites or acetylcholinesterase. However, the latter now seems an unlikely possibility in view of the fact that a very similar structural specialization has recently been demonstrated at junctions where neither acetylcholinesterase nor any other degradative enzyme would be expected (22).

In previous studies of vertebrate myoneural junctions the postjunctional membrane has been characterized by the presence of the "subneural apparatus" consisting of cleftlike junctional folds (1, 6, 18) which are conspicuous in twitch muscles but are virtually absent in certain slow muscles (10, 17). A "subsarcolemmal cytoplasmic density" has also been noted (12) as well as an apparent "thickening" or increase in density of the postjunctional membrane (3, 9, 17, 27). However, the significance of these specializations is not clear.

In view of the evidence suggesting that the periodically disposed elements at the outer surface of invertebrate postjunctional membranes may represent visible receptors, a closer look has been taken at the postjunctional membrane in a vertebrate in order to define its substructure more clearly. By means of transmission electron microscopy and with the help of special stains it has been possible to resolve new details in the outer surface of the vertebrate postjunctional membrane and to show that it too exhibits a granular substructure, occurring in discrete patches, very similar to that found in the invertebrates. Although the granules are more difficult to visualize in the vertebrate and apparently also are not so regularly arrayed, their approximate concentration is of the same order of magnitude as that of receptor sites at vertebrate cholinergic junctions elsewhere (4). It is therefore proposed that these granules, which are visible at the very surface of the vertebrate postjunctional membrane and are responsible for its characteristic appearance, also represent the transmitter receptors. Patches of membrane specialized in this way are distinctive enough to permit determination of the extent of the receptive surface area at skeletal myoneural junctions and may prove useful in following changes in the distribution of receptors under abnormal conditions. A report of these findings was presented at the 1973 meeting of the American Association of Anatomists and has been published in abstract form (21, 25).

MATERIALS AND METHODS

Specimens of tail muscle were obtained from swimming tadpoles of either *Bufo marinus* or *Rana pipiens* in most cases at stages well past bilateral closure of the operculum, but before tail resorption had begun. Animals were anesthetized in 10% ethyl carbamate and the tissue was fixed without dissection in 1–5% glutaraldehyde in 0.1 M phosphate buffer. After a minimum of 2 h the specimens were rinsed and then postfixed in buffered 1–2% osmium tetroxide and dehydrated and embedded in Araldite. In some instances osmium tetroxide postfixation was omitted. Thick sections were stained with 1% toluidine blue in 0.5% sodium borate for survey purposes. Thin sections were stained with 2% potassium permanganate for 15 min followed by a rinse with 10 drops of 5% citric acid. The sections were then stained with 2% uranyl acetate in 75% ethanol at 60°C for 15 min and rinsed in water. In some cases either the permanganate or uranyl stain was omitted. The preparative procedure for earthworm tissues was given previously in detail (20). Except for survey pictures, electron micrographs were generally taken at an initial magnification of × 40,000 with a Philips EM 300 instrument operating at 60 kV.

RESULTS

Before presenting detailed observations on the vertebrate junctions the structure of annelid junctions will be recapitulated briefly in order to provide a basis for comparison. In preparations of glutaraldehyde-osmium tetroxide-fixed earthworm muscle stained with potassium permanganate and uranyl acetate (Fig. 1) postjunctional membranes display the following components: The cytoplasmic surface of the membrane is marked by a continuous dark line, the "inner dense lamina" (19), which is usually coated on its cytoplasmic surface by an amorphous and filamentous dense material exhibiting no periodic structure. Immediately external to the inner dense lamina is a thin lucent "middle" lamina. The latter separates the inner dense lamina from the beaded "outer dense lamina" which contains an array of granules ~70 Å in diameter. These granules impinge on the middle lucent lamina and protrude from the outer surface of the membrane as well. In addition, they

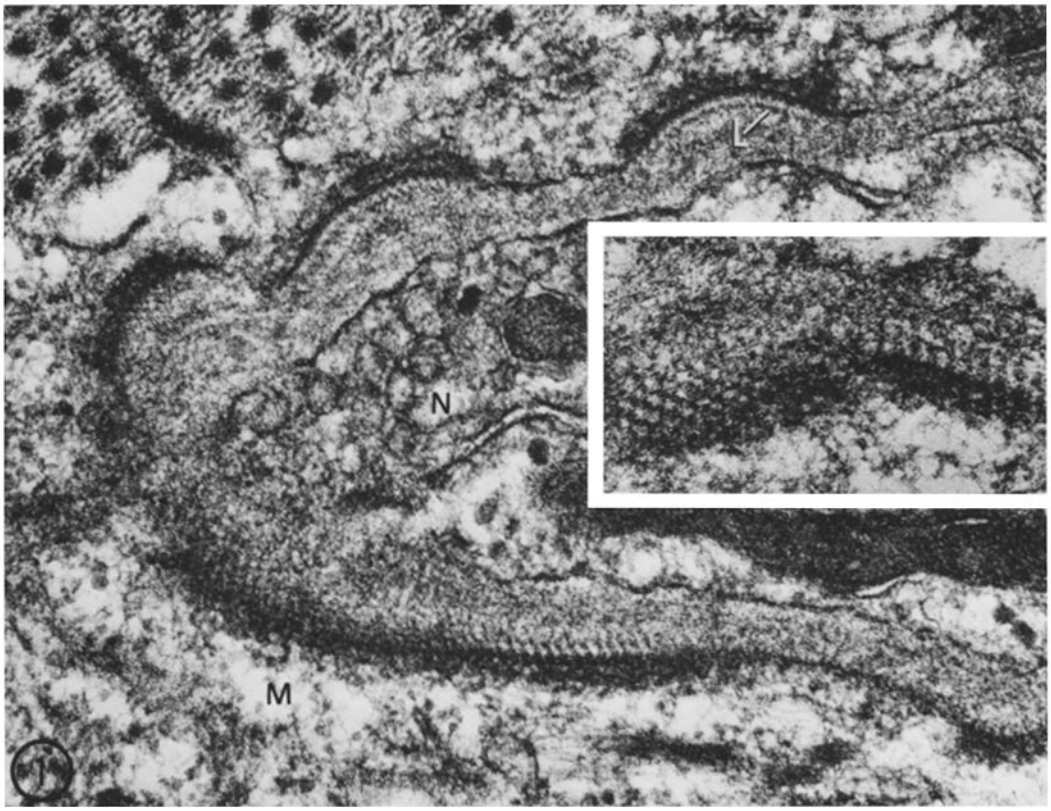
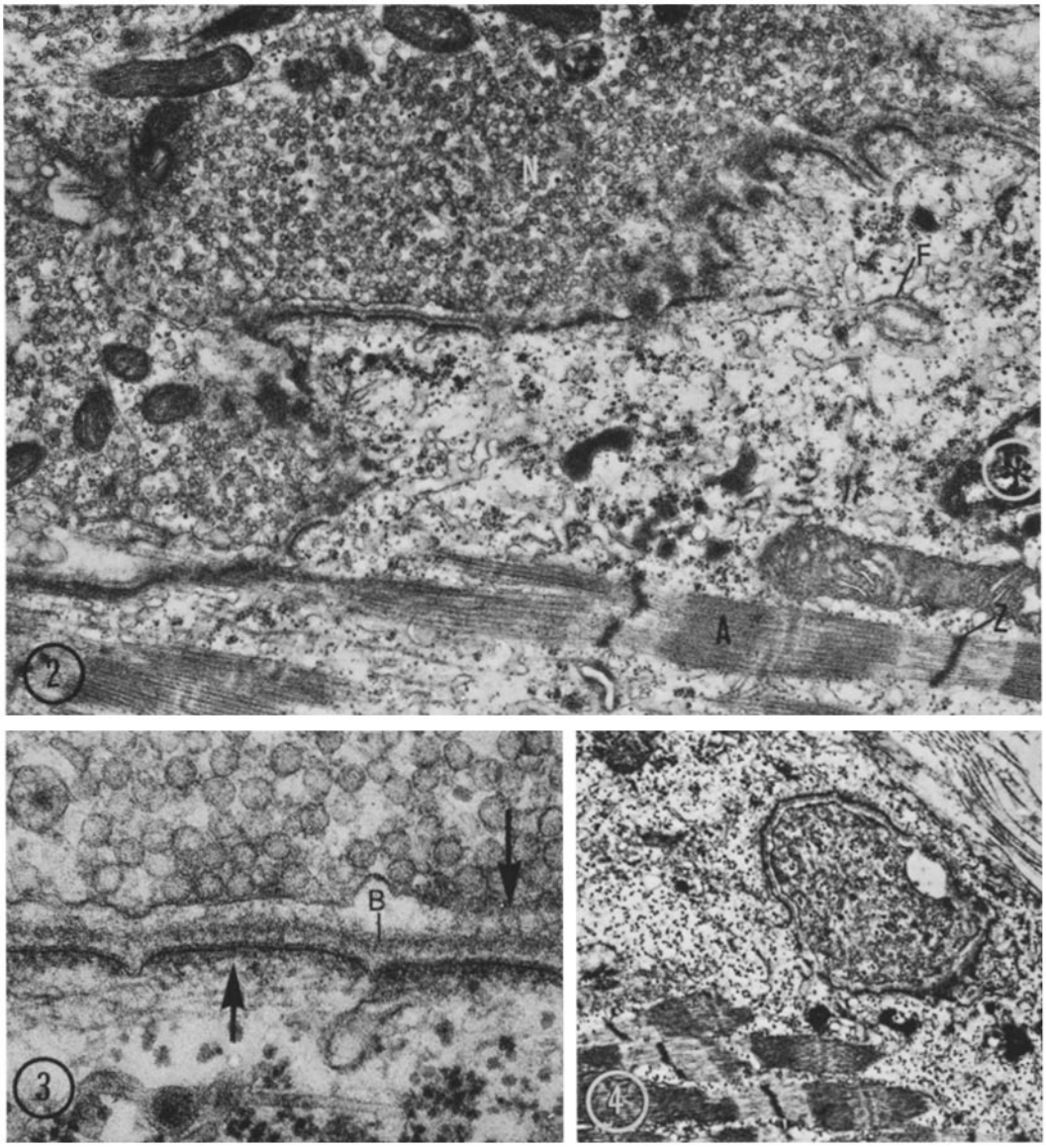


FIGURE 1 Earthworm myoneural junction. A bundle of nerve fibers (*N*) is surrounded by muscle processes (*M*). At least five regions of postjunctional membrane specialization are visible, sectioned at various angles. The specialized membrane is concave and is coated on its cytoplasmic surface by an amorphous material. Dumbbell-shaped projections can be seen arising from the outer surface of the membrane at the bottom of the figure. Towards the left the membrane tilts and the projections form a reticular pattern. At the upper right of the figure the tips of the projections are visible forming a discrete lamina (*L*) parallel to the plasma membrane but their shafts do not show up. *Inset:* Higher magnification of a specialized postjunctional patch. At the right projections are seen in side view and at the left they are cut obliquely forming a distinct reticular pattern. × 100,000. *Inset*, × 130,000.

give rise to projections extending ~200 Å into the junctional cleft. The tips of the projections, which are thicker than the shafts, often show up even when the shafts themselves cannot be seen and form what appears to be another lamina external to the end-plate membrane (*L* in Fig. 1). This extra lamina is separate from the basement membrane, which is much farther away from the postjunctional membrane and does not follow its irregularities so precisely. In oblique views of the membrane the granules and projections appear in hexagonal array with a spacing of ~160 Å and sometimes form reticular patterns (Fig. 1, inset). In specimens that have not been postfixed with osmium tetroxide but have been stained by the permanganate-uranyl acetate method (20), the plasma membranes in general are not well visualized. However, the postjunctional membrane exhibits the same structures just described, i.e., a continuous dense inner lamina, which has a coating applied to its cytoplasmic surface, a lucent middle lamina, a beaded outer dense lamina containing granules, and, extending from these granules, projections whose bulbous tips form still another lamina ~200 Å external to the "unit" membrane.

In amphibian muscle prepared with both glutaraldehyde and osmium tetroxide, myoneural junctions can be identified readily by virtue of the vesicle-containing nerve endings in apposition to muscle processes (Figs. 2, 4). The end-plate mem-



FIGURES 2–15 Details of amphibian myoneural junctions.

FIGURE 2 Longitudinally sectioned myofibrils with distinct A bands (*A*) and Z lines (*Z*) are visible in a muscle fiber whose membrane is juxtaposed to a large, vesicle-containing nerve ending (*N*). A portion of a muscle cell nucleus (*) and junctional fold (*F*) are also visible. Note that the membrane of the junctional fold appears much less dense than the sarcolemma directly apposed to the nerve ending. × 20,000.

FIGURE 3 Detail of Fig. 2 showing the vesicle-containing nerve terminal at the top facing the muscle cell at the bottom. The muscle cell membrane appears doubled and scalloped. Amorphous material coats the cytoplasmic surface of the membrane and a basement membrane (*B*) is interposed between the nerve and muscle cells. Filaments appear to connect the basement membrane with both the axolemma and sarcolemma (arrows). × 70,000.

FIGURE 4 Low power tangential view of myoneural junction at which the axon appears to be embedded in the muscle fiber. The dense scalloped end-plate membrane is distinct from that of both the axon and superficial muscle cell membrane facing connective tissue at the upper right. × 17,000.

brane tends to be convex outwards and scalloped (Figs. 2–6) in contrast to the specialized postjunctional membrane in the earthworm which is invariably either concave or flat (Fig. 1). Even at intermediate magnifications (Fig. 3) it is sometimes apparent that the portion of the amphibian postjunctional membrane facing the axon differs from adjacent membranes in that it is apparently doubled (overall width ~120 Å). In higher magnification views of this membrane (Figs. 6–8) it is possible to resolve a continuous dense inner lamina ~30 Å thick which has an amorphous cytoplasmic coating applied to it, a lucent middle lamina ~20 Å thick, and an outer dense lamina which is ~70 Å thick and discontinuous, but which lacks the obvious regularity visible in annelid membranes. This specialized membrane extends part way into the junctional folds and there both the thickened outer dense lamina and the cytoplasmic coating end abruptly (Fig. 6).

A semiregular spacing of granules is discernible in the outer dense lamina of this membrane. In Fig. 5 inset, for example, the elements repeat at ~100-Å intervals and in Figs. 7 and 8 their spacing is ~140 Å. It was pointed out previously (20) that such apparently discrepant spacings could both be derived from the same array. On the other hand, the fact that it is more difficult to find orderly arrays of granules at amphibian junctions than at those of annelids suggests that the apparent lack of order in the amphibian is probably real. Estimation of the concentration of granules at the amphibian end-plate membrane is therefore more difficult than at annelid membranes. Where spac-

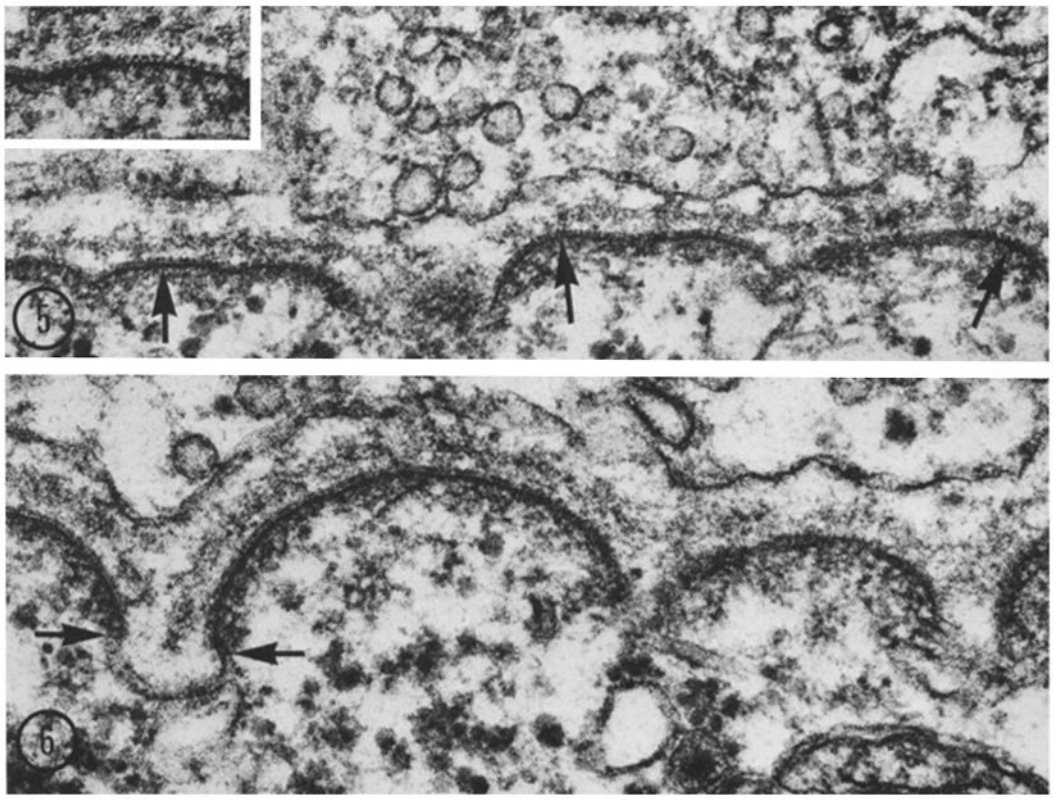


FIGURE 5 The muscle cell membrane here consists of a continuous inner dense lamina and an outer dense lamina which appears to be composed of granules (arrows). These granules are ~70 Å in diameter but do not exhibit a regular spacing in this region. *Inset:* Higher magnification showing one region in which the granules of the outer dense lamina are spaced at ~100 Å intervals. × 90,000. *Inset*, × 130,000.

FIGURE 6 At this junction the specialized membrane covers the convexities of the scallops but does not extend into a junctional fold (arrows). The granularity in the outer dense lamina of the specialized membrane is clear. × 110,000.

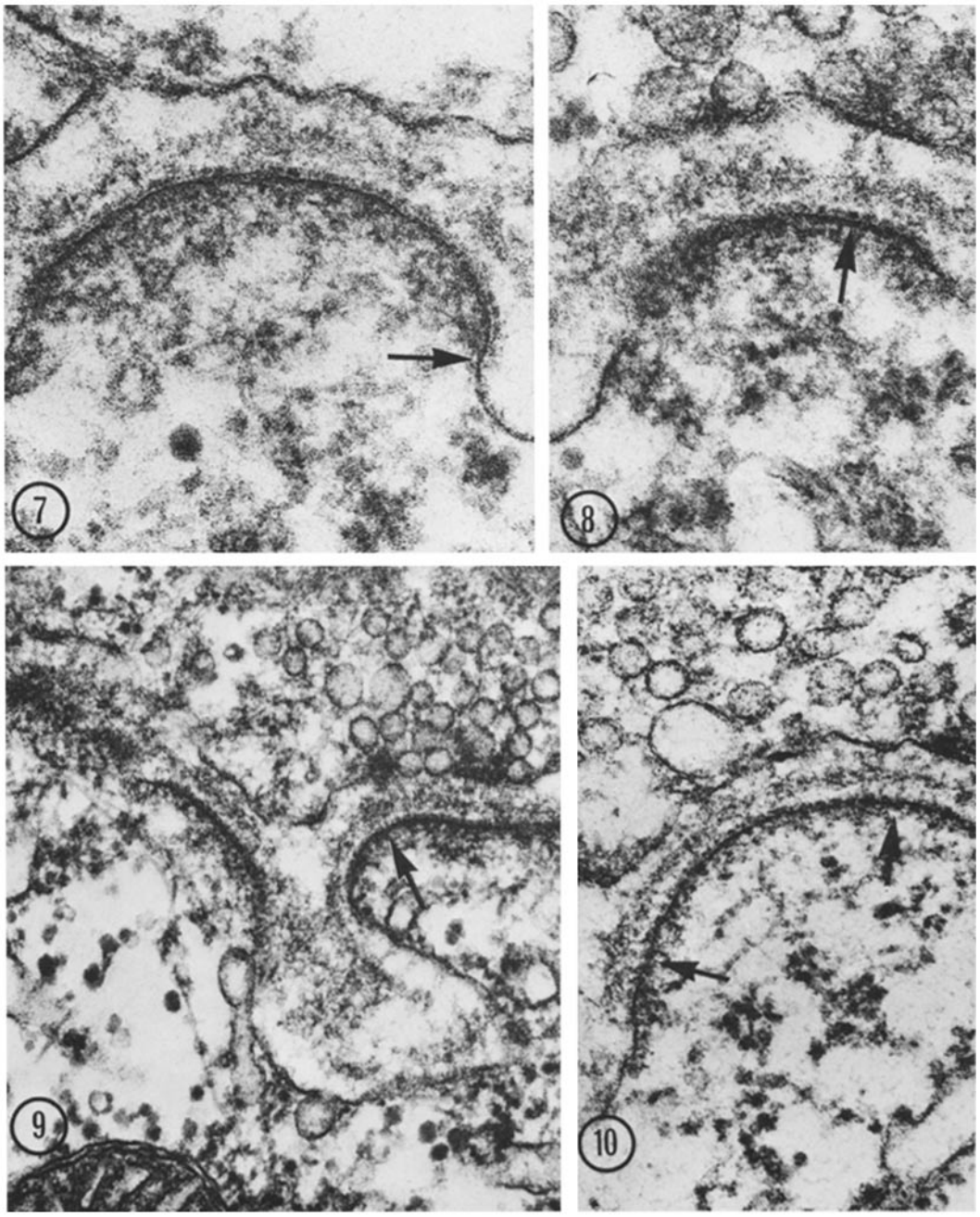


FIGURE 7 Granules located in the outer dense lamina of the postjunctional membrane appear separated from the continuous inner dense lamina by a narrow lucent region ~20 Å in width. Both the coating on the inner surface of the membrane and the granules in the outer dense lamina end abruptly at the arrow near the origin of a junctional fold. × 160,000.

FIGURE 8 Granules (arrow) are clearly visible in the outer dense lamina of the end-plate membrane, in this case facing a dense plaque on the axolemma. × 160,000.

FIGURE 9 End plate showing specialized patches of membrane on either side of the mouth of a junctional fold. At the arrow filamentous projections appear to extend to the overlying basement membrane. ×80,000.

FIGURE 10 Specialized patch of end-plate membrane showing periodic granules. Filamentous projections extend to the overlying basement membrane in several areas (arrows). × 120,000.

ings can be discerned at the amphibian junctions they are in a range compatible with a concentration of the order of $\sim 10^4$ granules/μm^2. Granules in regular hexagonal array at a concentration of $\sim 10^5/\mu m^2$ would be spaced at intervals of ~34 Å (15–48 Å); at a concentration of $\sim 10^3/\mu m^2$ their spacing would be ~340 Å (152–481 Å).

The granules exhibit an apparent variation in width from ~60 Å (Fig. 9) to ~120 Å (Fig. 7) which could reflect a real variation in their size or irregularity in their shape. However, this variation could also arise partly from broadening of the images due to imprecise superimposition of the granules located at different levels within the thickness of the section, especially if their spacing is not uniform. These granules resemble those recently found in negatively stained membrane fragments isolated from the electric organ of *Torpedo* (5, 16).

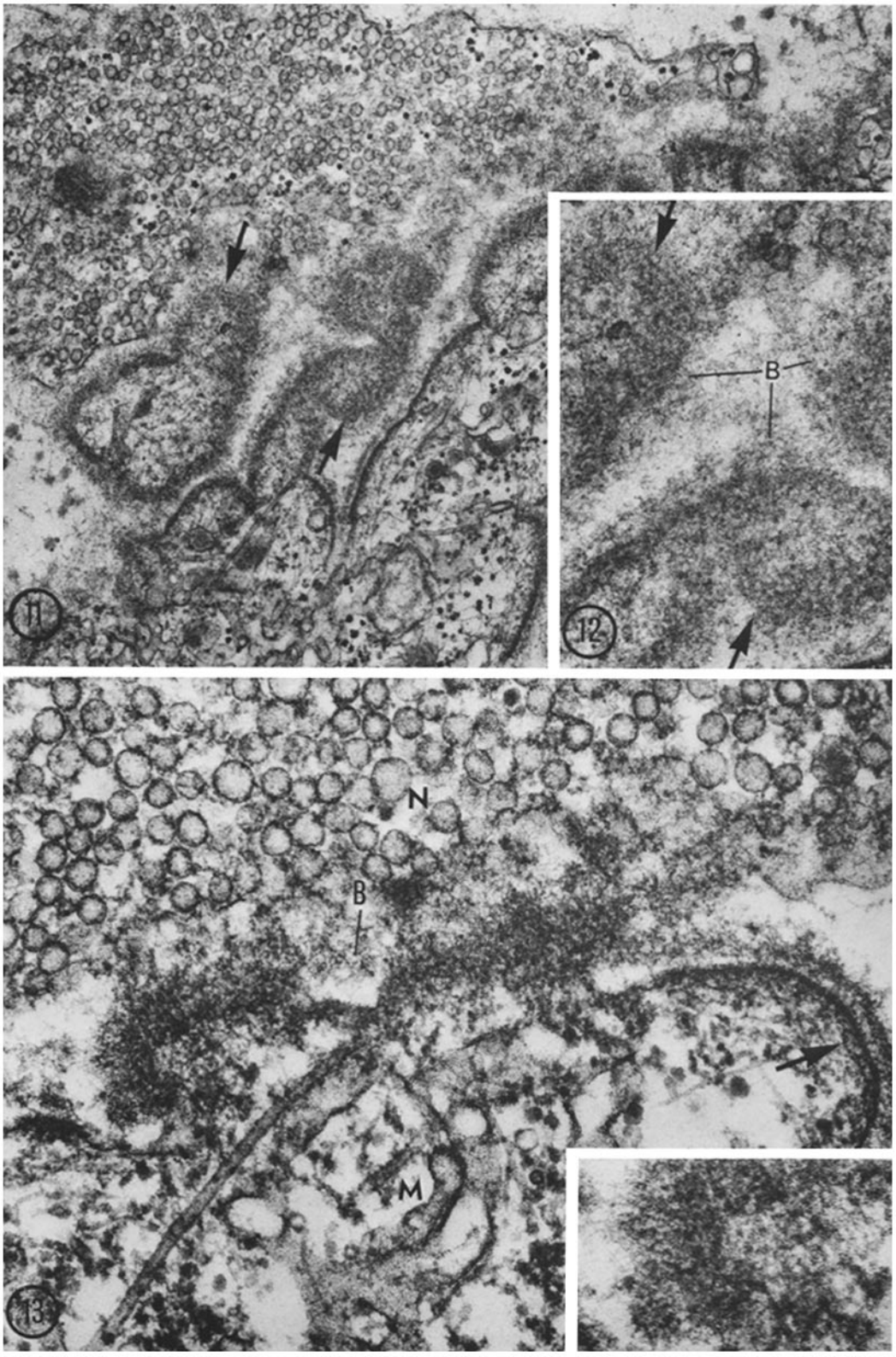
One of the most distinctive characteristics of the postjunctional membrane in annelids is the presence of regular arrays of projections arising from the granules (Fig. 1). The shafts of these projections are, however, not always seen; often only their bulbous tips are visible as an apparently separate lamina. In the amphibian, filaments can sometimes be seen extending from the granules in the postjunctional membrane to the overlying basement membrane (Figs. 9, 10, 13). These appear to be comparable to the more distinct projections that occur at the annelid junctions but may be more difficult to visualize here because they are less orderly in arrangement and therefore do not superimpose congruently within the thickness of the section. Furthermore, the basement membrane, in the case of the amphibian, is much closer to the postjunctional membrane (~200 Å) than it is in annelids and therefore if a lamina were formed at the amphibian junction by the bulbous tips of the projections it would be superimposed on the basement membrane and not visible separately.

Occasionally, the postjunctional membrane is cut obliquely and its surface is visible with its various layers superimposed on each other as well as on other constituents above and below it. In such preparations a distinct reticular pattern sometimes appears (Figs. 11–13) closely resembling that seen in annelid membranes (Fig. 1, inset). The repeating units of this pattern are spaced at ~100–200-Å intervals. However, because of the superimposition the exact location of the structures giving rise to this pattern is ambiguous. It could be formed by the granules themselves viewed at an oblique angle and partially overlapping; it could arise from the projections extending from the granules to the basement membrane, or it could be formed by interconnections between the granules within the plane of the plasma membrane and thus not visible in transverse sections through the junctions. The basement membrane itself sometimes also exhibits lattice-like patterns (Figs. 9, 13) or striations (11) which are most apparent in oblique or tangential sections. This network, whose dimensions are similar to the spacing of the granules in the endplate membrane, may be formed by the tips of the projections extending from the granules (Figs. 9, 10) and thus be equivalent to lamina (*L*) in Fig. 1, which at annelid junctions is separate from the basement membrane.

In amphibian specimens not exposed to osmium tetroxide but stained with permanganate and uranyl acetate both the continuous inner dense lamina and the discontinuous outer dense lamina containing granules show up in contrast to adjacent areas of the muscle cell membrane which are poorly delineated in such preparations (Figs. 14, 15). Thus with respect to staining properties as well the membrane at the vertebrate junction corresponds to that at the annelid junctions. The unosmicated specimens, here as in annelids (20, 24), are particularly useful in defining the extent of the specialized membrane, for adjacent nonspecialized membranes are not trilaminar and appear only faintly. Examination of unosmicated specimens thus offers a means of quantitating the specialized surface area at vertebrate motor endplates by a relatively simple and rapid procedure.

DISCUSSION

This paper reports a morphological specialization of the vertebrate postjunctional membrane consisting of arrays of granules visible in the outer dense lamina of the membrane facing the axon terminal. The specialized membrane is readily identifiable because of its affinity for certain heavy metal stains especially in unosmicated specimens where its trilaminar structure stands out vividly in contrast to adjacent membranes which are only faintly visible in such preparations. This specialization resembles the hexagonally arrayed granules and projections found previously on postjunctional membranes at invertebrate myoneural junctions with respect to location, staining properties, and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size and concentration of granules, and is similar also to a postsynaptic membrane specialization seen in the amphibian central nervous system (23). It appears, therefore, that this structural specialization represents a widespread component of chemically transmitting junctions.

The essential features of this specialization in its various locations are the presence of more or less regularly disposed elements visible in the outer part of the postjunctional membrane directly bordering the junctional cleft. In the case of annelid myoneural junctions (20, 24) these repeating dense elements have the form of granules which give rise to elongated projections extending into the junctional cleft. At amphibian myoneural junctions, only the granules can be seen distinctly and the outer dense lamina of the membrane is thickened by their presence; at insect flight muscle junctions (22), only the projections are apparent arising directly from the membrane without visible separation from it. Although morphologically variable, these regularly disposed elements in all cases have an affinity for permanganate-uranyl acetate stains even in unosmicated specimens where plasma membranes in general are visualized only faintly. The outer dense lamina is quite different in appearance from the continuous inner dense lamina which is usually coated with an amorphous cytoplasmic material (12).

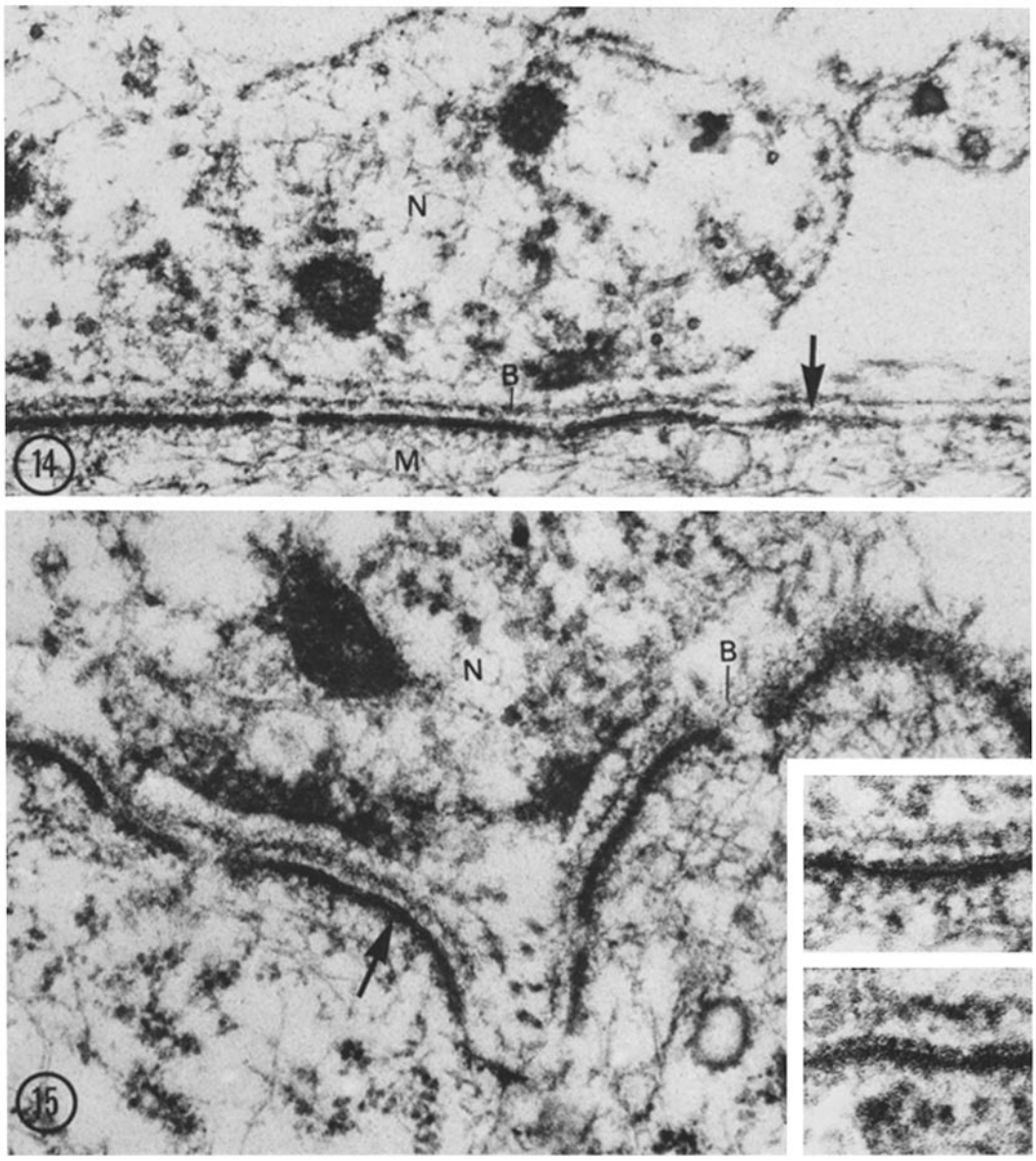
The staining properties and configuration of this specialization suggest that the membrane in this region contains protein or glycoprotein granular units in a semiregular array in a concentration of $\sim 10^4/\mu m^2$. Although these elements are visible only in the outer dense lamina of the membrane, it has been suggested previously (24) that they may in fact extend through and through the membrane in the manner of the erythrocyte membrane protein described by Marchesi et al. (13). The fact that the distribution of the coating on the cytoplasmic surface coincides with that of granules at the outer surface supports the view that the two are continuous across the membrane and that the granules visible in the outer dense lamina of the membrane represent only the outermost portions of collar buttonlike units. This specialization is also characterized by its patchy distribution; i.e., it does not extend over the entire postjunctional membrane. The point is particularly obvious in the leech (24) where the specialized patches occupy only a small percentage of the postjunctional membrane. At the amphibian junctions as well although the specialized postjunctional patches are more extensive, they are conspicuous primarily at the superficial convexities of the end-plate membrane. Acetylcholinesterase, in contrast, is thought to extend into the depths of the junctional folds (7), a distribution which most closely follows that of the basement membrane (cf. reference 2).

Three possible functions can be ascribed to the amphibian end-plate membrane specialization. Superficially it resembles that at myotendinous junctions and other attachment regions suggesting a possible role in mechanical attachment. This similarity is probably fortuitous, however, since in both annelid and insect muscle a clear morphological distinction can be seen between the postjunctional membrane specialization and that at attachment plaques elsewhere along the surface of the same cell type. The possibility also exists that the membrane specialization represents the location of acetylcholinesterase. However, a very similar membrane specialization has been found at junctions where no such enzyme would be expected. The most intriguing possibility, and the one that is proposed here, is that the granules in the special-

FIGURE 11 Oblique view of amphibian myoneural junction. In several places where the postjunctional membrane is cut obliquely it exhibits a reticular pattern barely visible at this magnification (arrows). Filaments and glycogen are prominent in the end-plate sarcoplasm. × 30,000.

FIGURE 12 Detail of Fig. 11. Arrows indicate regions in which a reticular pattern is most apparent in the obliquely cut postjunctional membrane. The fuzzy tangentially cut basement membrane (*B*) is distinguishable in several places. × 70,000.

FIGURE 13 Oblique view of end-plate membrane. At the far right, a convex specialized patch of postjunctional membrane is cut normally and exhibits periodic granules in its outer layer (arrow). Projections to the basement membrane are indistinct. At the center and left of the figure two such patches are cut obliquely. The basement membrane (*B*) between these two has a suggestion of a reticular pattern in it. *Inset:* Detail of obliquely cut end-plate membrane. At this magnification a distinct reticular pattern is visible, probably in the basement membrane. × 90,000. *Inset*, × 150,000.



FIGURES 14 and 15 Amphibian myoneural junctions fixed in glutaraldehyde but not postosmicated. The sections have been stained with both permanganate and uranyl acetate.

FIGURE 14 The nerve ending (*N*) is limited by an indistinct membrane which has a cytoplasmic density applied to it in one region. Synaptic vesicles are not preserved and mitochondria are identifiable only by their dense matrix. The postjunctional membrane of the muscle cell (*M*) in contrast is distinctly doubled, and has a continuous inner dense lamina and a coextensive but discontinuous outer dense lamina. This specialized membrane has several gaps in it and terminates at the edge of the myoneural junction (arrow). The adjacent unspecialized muscle cell membrane appears as an indistinct line. Basement membrane (*B*) is also demonstrated by this method. × 60,000.

FIGURE 15 Postjunctional membrane showing discontinuous specialized patches in which a trilaminar membrane can be resolved (arrow). A reticular pattern is visible in the tangentially cut membrane at the upper right and appears also in the tangentially cut basement membrane (*B*). *N*, nerve ending. *Inset*: Two regions showing discrete granules in the outer dense lamina of the postjunctional membrane. × 80,000. *Inset*, × 120,000.

ized patches of end-plate membrane represent the transmitter receptors, which are reported to have a molecular weight of ~360,000 (14) and dimensions consistent with those of the granules (16). Each such molecule is thought to be capable of binding two α-bungarotoxin molecules (26) and the concentration of granules in the patches is therefore in a range that could account for the number of toxin binding sites found by autoradiography (4).

If at the amphibian junctions, as well as at annelid junctions, some of the end-plate membrane is not covered by receptors, then the significance of the "excess" membrane surface area of the subneural apparatus is unclear. Part of it may be convertible to receptor membrane under some conditions; i.e., there may be plastic changes in the amount of receptor at the junction, depending perhaps on activity or age in addition to the changes that occur on denervation (15). Another possibility is that the junctional folds serve to increase the *volume* of extracellular fluid immediately surrounding the pre- and postjunctional membranes and that this enlarged volume acts as a buffer to minimize local accumulation or depletion of the ions that exchange across the pre- and postjunctional membranes during periods of sustained repetitive activity (cf. reference 8).

If the morphologically specialized membrane described here proves to represent the location of transmitter receptors, then it is implicit that the receptors at amphibian myoneural junctions like those at annelid junctions are segregated into patches whose area is less than the total postjunctional surface area. The size of the real receptive surface may therefore be better assessed by determining the area of the specialized patches in the membrane using the methods described here than by merely assuming that the whole postjunctional membrane is receptive as is often done. The usefulness of this specialization in following developmental and plastic changes at junctions is under investigation.

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