Cytoskeletal Organization at the Postsynaptic Complex

R. L. GULLEY and T. S. REESE

Laboratories of Neuro-otolaryngology and Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT   Postsynaptic densities and the adjacent cytoskeleton were examined in deep-etched, unfixed slices of guinea pig anteroventral cochlear nucleus. The postsynaptic density seen in conventional thin sections corresponds to a meshwork of 4-nm filaments associated with intramembrane particles at the postsynaptic active zone of inhibitory as well as excitatory synapses. These filaments intermesh with a lattice of 8- to 9-nm microfilaments, tentatively identified as F-actin, that is concentrated under the postsynaptic density. We postulate that the meshwork of 4-nm filaments anchors receptors to the adjacent microfilament lattice; this extended postsynaptic complex may limit the mobility of receptors and help maintain the curvature of the postsynaptic membrane.

The cytoplasmic surface of the postsynaptic membrane is lined by a mat of dense material, the postsynaptic density (31). In traditionally prepared thin sections, the postsynaptic density appears as a disk or ring of dense fibrillar material lining the cytoplasmic surface of the postsynaptic membrane (9). Filamentous strands extending from the indistinct boundary of this density into the cytoplasm constitute the subsynaptic web. At certain synapses, this web is attached to cytoplasmic densities of various sizes and shapes (32). The postsynaptic density is thicker at excitatory than at inhibitory synapses, but little else has been revealed about the organization of the postsynaptic density and web.

Biochemical analysis of postsynaptic density fractions has shown that they contain actin, tubulin, calmodulin, proteins Ia and Ib, and other unidentified proteins (3, 4, 13, 22, 28, 36). Isolated postsynaptic densities have also been examined in thin sections (8, 10, 29), with negative staining, and in rotary-shadowed replicas (5). Their structure is generally comparable to that of densities in intact brain, but there are differences between fractions prepared from different regions of the brain (5) and with different detergents (28). Because isolated postsynaptic densities have been subjected to vigorous isolation procedures, fixation and possible contamination with proteins such as neurofilament proteins (26), membrane glycoproteins (17), and receptors (11, 27), it is not clear how comparable they are to postsynaptic densities *in situ*.

Direct freezing and deep etching have been used to visualize the filamentous components of the cytoskeleton of fibroblasts (19) and *Torpedo* electroplaque (18). Here we use a similar approach to study the structure of the postsynaptic specialization at identified synapses in unfixed brain. We identify filamentous constituents of the potsynaptic density and subsynaptic web, and show an association betwen these constituents and intramembrane particles in the postsynaptic membrane.

MATERIALS AND METHODS

18 female National Institutes of Health (NIH) strain guinea pigs (150–250 g) were examined. Each animal was decapitated and its brain rapidly exposed. The rostral pole of the anteroventral cochlear nucleus (AVCN) was removed by undercutting the cochlear nucleus and making a dorsal-ventral cut about 400 μm from the rostral pole of the nucleus. This slice was passed quickly through distilled water and then placed, cut edge up, on the stage of a rapid-freezing device and frozen on a copper block cooled with liquid helium (20). The total time from decapitation to freezing was never >90 s and was usually <75 s. The time from placing the tissue in distilled water until freezing did not exceed 20 s, except for a few pieces that were intentionally left in distilled water for up to 5 min. A few unfixed slices were rapidly frozen without washing in distilled water. All tissue was fractured within the most superficial 10 μm of the slice, where freezing is optimal. The temperature of the tissue during fracturing was held at −195°C or below by introducing liquid helium into the stage of the Balzers 301 freeze-fracture unit (Balzers Corp., Hudson, N.H.). The fractured tissue was then etched for 5–20 min at ~−105°C, rotary shadowed with 1.0–1.5 nm of platinum-carbon at an angle of 25° from horizontal, and supported with a layer of carbon. The replicas were cleaned in bleach, washed in distilled water, and placed on unsupported hexagonal mesh grids.

In our first experiments, which followed procedures described previously (15), animals were fixed by vascular perfusion of buffered aldehydes and then slices were made through the AVCN. The fixed slices were not cryoprotected, but were rinsed for 5 min in distilled water before rapid freezing. These specimens were fractured at −119°C, but were etched and replicated by the procedures described above. Stereomicrographs were taken at ± 6° of tilt from the normal plane. All illustrations are reversal images with platinum deposits appearing white.

RESULTS

The spherical cell in the rostral anteroventral cochlear nucleus was used in this study because the synaptic terminals on it are easily distinguished in thin sections and freeze-fracture replicas by their size and shape as well as the number, size, and shape of their active zones (see references 15 and 34). One type, the end bulb of Held, originates from primary auditory nerve fibers and has an excitatory effect on the spherical cell. Two types of nonprimary terminals have the morphologic characterisics of

inhibitory endings (34). In thin sections of aldehyde-fixed brain, both nonprimary types have elongated synaptic vesicles and a narrow postsynaptic density, ~20 nm wide. These terminals are considerably smaller than end bulbs and are distinguished from them, and from each other, by the size and shape of their active zones. We describe here features of the postsynaptic specialization of these nonprimary boutons that exemplify the common features of postsynaptic specializations. Small differences between specializations at excitatory and inhibitory synapses will be reported separately.

After aldehyde fixation, filamentous components of the cytoskeleton were clumped, making resolution of individual filaments difficult (Fig. 1). In unfixed tissue, the organization of the cytoskeleton was obscured by granular cytoplasmic material (Fig. 2). The most satisfactory approach for visualizing the postsynaptic density and its associated cytoskeleton was to induce slight swelling by briefly immersing unfixed slices of brain stem in distilled water just before freezing (Fig. 3).
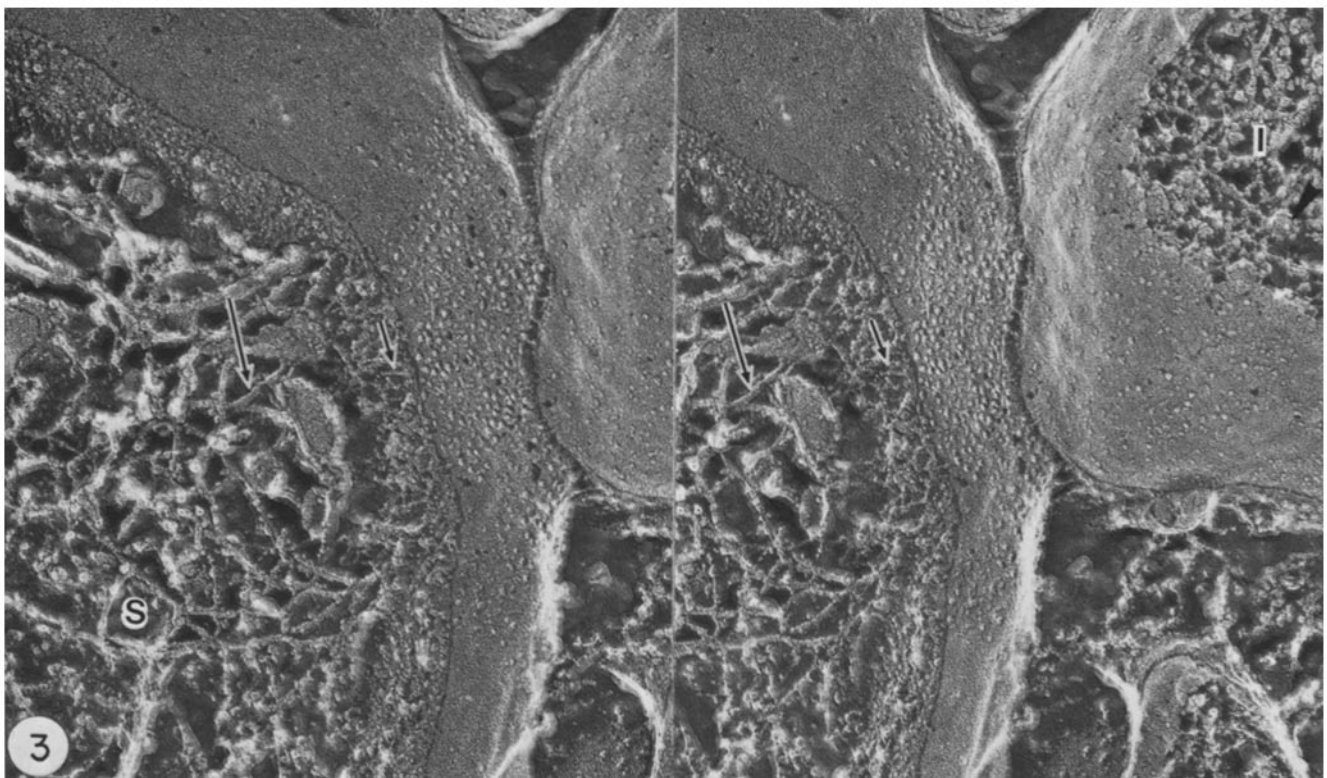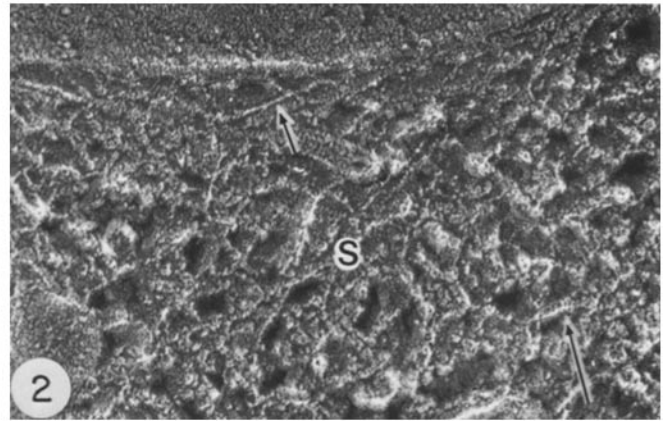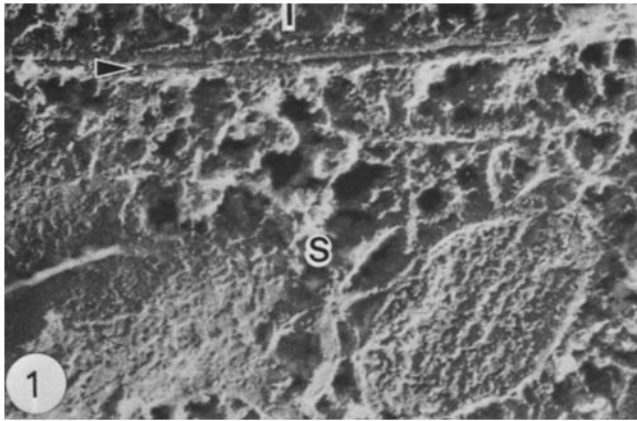

FIGURE 1 Cytoplasm of a spherical cell (S) next to a bouton (I); arrowhead indicates synaptic cleft. After aldehyde fixation, postsynaptic filaments are coarser, less frequent, and more irregular in shape than in unfixed material. × 150,000.

FIGURE 2 Cytoplasm of a spherical cell (S) opposed to a bouton (top) in unfixed tissue prepared without distilled water treatment. Filaments associated with the postsynaptic cytoplasm (arrows) vary in size but are largely obscured by granular cytoplasmic material. A fragment of a microtubule lies just to the right of the upper arrow. × 150,000.

FIGURE 3 An aggregate of small particles defines a postsynaptic active zone on the E face of a spherical cell (S), where it is contacted by a bouton (I, upper right). A meshwork of 4-nm filaments (short arrow) lines the cytoplasmic surface of the postsynaptic plasmalemma adjacent to this particle aggregate. A lattice of 8- to 9-nm microfilaments (long arrow) contacts the meshwork of 4-nm filaments. The cytoplasm of th presynaptic bouton is also exposed; it contains numerous synaptic vesicles and a meshwork of microfilaments which contact them. Nonoverlapping regions are included in this stereo pair in order to enlarge the field of view. × 150,000.

Postsynaptic Intramembrane Specializations

Intramembrane particles aggregate on the E face of the postsynaptic membrane opposite both end bulbs and nonprimary boutons (Figs. 3–5). These particle aggregates are coextensive with the presynaptic active zone. At nonprimary boutons they consist primarily of small intramembrane particles (6 nm in diameter), a few of which are elongated. The identification of particle aggregates at the postsynaptic membrane of these boutons, which are presumably inhibitory, contradicts previous reports that depend on conventional freeze-fracture procedures (15, 34). These aggregates are not visible in tissue fixed by vascular perfusion of aldehydes and then rapid-frozen, fractured at −119°C, deep-etched, and rotary shadowed, but they are found in unfixed tissue that was not rinsed in distilled water. Therefore, aldehyde fixation alone or in conjunction with fracturing at normal temperatures without etching must prevent definition of the postsynaptic aggregates of particles at this and presumably other inhibitory synapses (24).

Postsynaptic Cytoskeletal Specializations

A meshwork of fine filaments, 4 nm in diameter, forms a dense mat ~20-nm thick beneath the postsynaptic membrane. This meshwork is coextensive with the postsynaptic particle aggregate (Figs. 3 and 4), and some filaments in it are associated with intramembrane particles in the aggregate (Figs. 4 and 5). In fractures through the particle aggregates on the postsynaptic E face of the end bulb, intramembrane particles are contiguous to elongated structures 4-nm thick (Fig. 5); these structures are interpreted as fragments of fine filaments that remain attached to the particles during fracturing. The interstices of the fine filament meshwork at nonprimary synapses are filled with coarse, granular material (Figs. 3–5) similar to that which fills the cytoplasm in unfixed tissue that has not been pretreated with distilled water (Fig. 2). Much of this material is removed by long washes in distilled water, leaving only the filamentous skeleton.

A lattice of 8- to 9-nm microfilaments with striations spaced at 5–6 nm lies below the meshwork of fine filaments that lines the postsynaptic membrane (Figs. 3 and 4). This lattice intermeshes with the overlying meshwork and extends ~100 nm into the cytoplasm (Fig. 3). Microtubules and neurofilaments are often enmeshed within the microfilament lattice (Fig. 2), but they do not have a predilection for the postsynaptic area of these synapses.

The microfilaments are part of a larger cortical meshwork of similar filaments that contact nonsynaptic regions of the plasmalemma. However, their concentration beneath the postsynaptic membrane is much greater than in nonsynaptic regions. The microfilaments in nonsynaptic regions approach the plasmalemma at a high angle of incidence and bend to run along its cytoplasmic surface. To which the plasmalemma appears to be attached by delicate transverse strands of unknown nature. In contrast, at the postsynaptic specialization they are enmeshed within the fine filament network adjacent to the membrane and do not contact the plasmalemma directly.

DISCUSSION

The rapid-freezing, deep-etch technique provides a new perspective on the organization of the postsynaptic specialization. Our observations on this structure are unique in that they are made on identified synapses that have not been fixed or stained with heavy metals. Aldehyde fixation aggregates the cytoskeleton and obscures all but the general features of its organization. On the other hand, the organization of the cytoskeleton in unfixed tissue is obscured by granular material thought to be soluble cytoplasmic proteins (19). In the present study, these difficulties were partially avoided when we induced swelling and perhaps some extraction of soluble components by means of a brief treatment with distilled water just before freezing. Where the cytoskeleton could be glimpsed in tissue prepared without distilled water, its shape and organization were similar to that in tissue treated in distilled water. Therefore, we do not believe that this treatment seriously distorts the cytoskeleton.

The postsynaptic density is shown to consist of a meshwork of fine (4-nm) filaments intermeshed with thicker microfilaments. The width of the fine filament meshwork (20-nm) corresponds to that of the postsynaptic density seen in thin sections through the same synapses (34). The granular material within this meshwork presumably represents some of the protein constituents identified biochemically in isolated postsynaptic densities (3, 4, 28).

The fine filament meshwork is found only in conjunction with synaptic regions of the postsynaptic cell. It is coextensive with the postsynaptic active zone, which is characterized by an aggregate of intramembrane particles thought to be receptors (15). Individual filaments in the meshwork contact the individual receptor particles. The observation that remnants of the fine filaments remain associated with intramembrane particles during fracturing is interpreted as a manifestation of this

FIGURE 4   The P face of two nonprimary boutons (1) and the E face and adjacent cytoplasm of a spherical cell (S) from an unfixed slice treated for 20 s in distilled water before rapid-freezing. Portions of the postsynaptic intramembrane particle specialization (large arrowhead) are seen on the E face adjacent to each bouton. Microfilaments (long arrows), 8–9 nm thick, extend toward the postsynaptic membrane and intermesh with a submembranous mat of fine, 4-nm filaments (short arrow). An association of one of these filaments with an intramembrane particle is indicated by a small arrowhead. The 8- to 9-nm microfilaments have regular striations spaced 5–6 nm apart. × 89,000.

FIGURE 5   Upper: Cytoplasm of a nonprimary bouton (1) and adjacent E face and cytoplasm of a spherical cell (S). The 4-nm filaments associated with the underlying cytoplasm contact several particles (arrows) near the juncture of the etched surface and E face. The particles in the aggregate on the postsynaptic E face are smaller and lower than those at end bulbs (below). × 160,000. Middle: A fracture exposing the E face of a spherical cell (S) opposed to the P face of an end bulb (E) at its active zone in an unfixed specimen treated in distilled water for 1 min before rapid-freezing. A portion of a postsynaptic particle aggregate is present on the E face. Fragments of fine filaments (4 nm) appear to originate from some of these particles (arrows; see also Fig. 4). × 125,000. Lower: Postulated organization of the postsynaptic complex of the inhibitory synapse. A fine meshwork of 4-nm filaments is connected to intramembrane particles, presumably receptors, in the postsynaptic membrane. The position of this meshwork corresponds to the postsynaptic density seen in thin sections. A lattice of 8- to 9-nm microfilaments, presumably F-actin, mingles with this meshwork and connects it to membrane sacs under the postsynaptic complex and to the rest of the cytoskeleton.
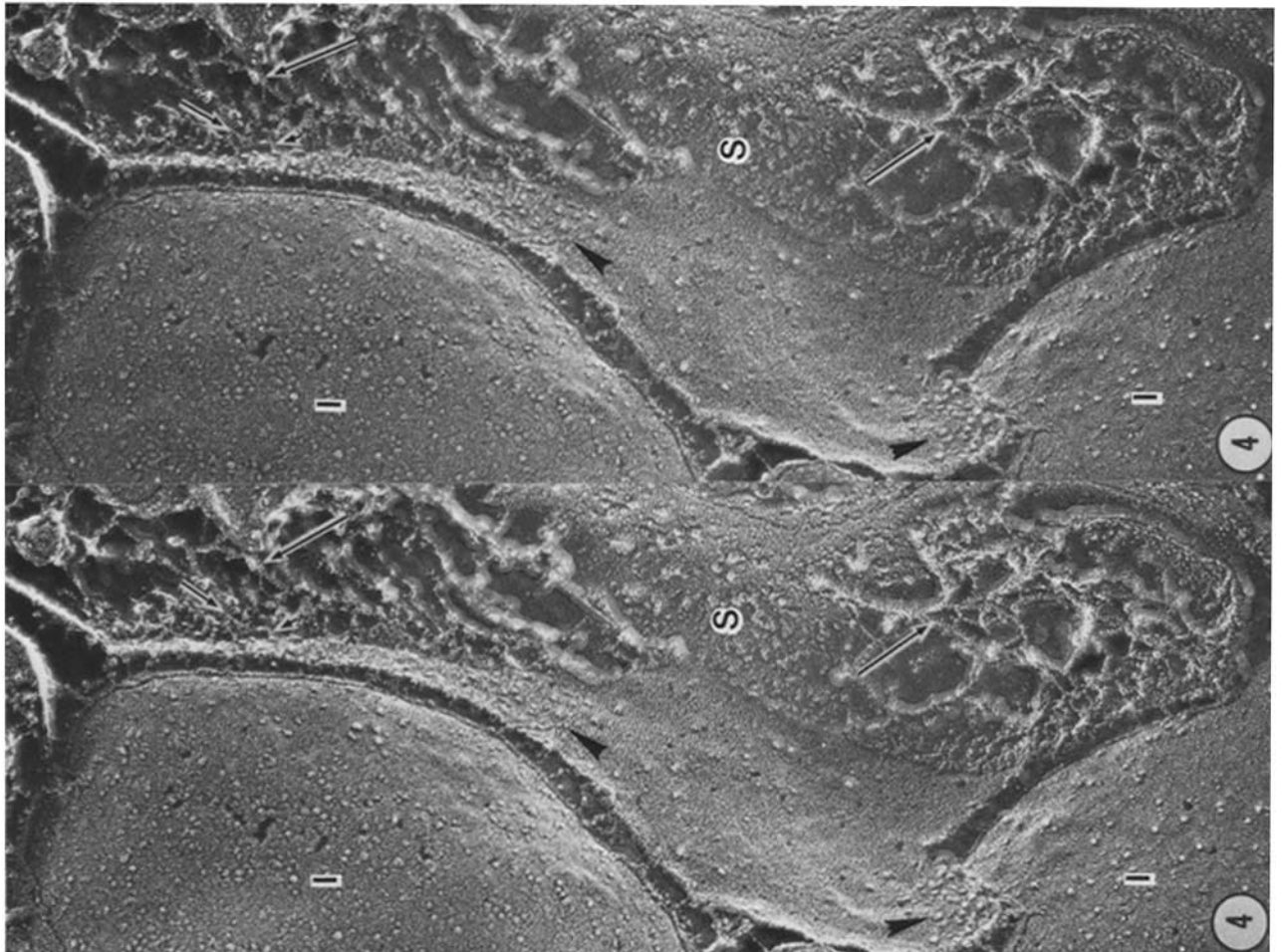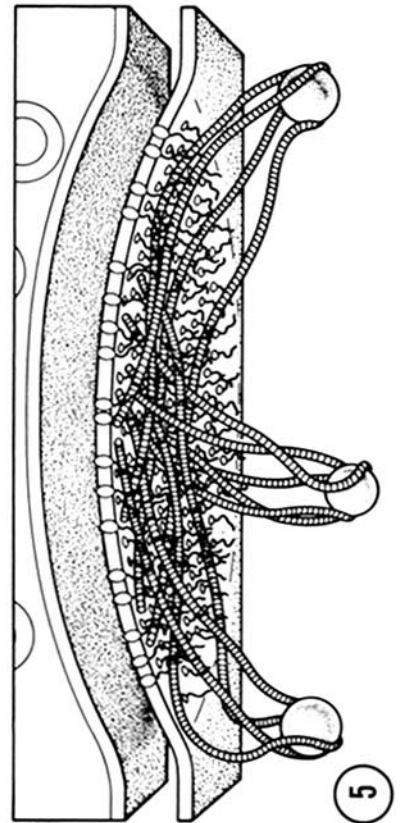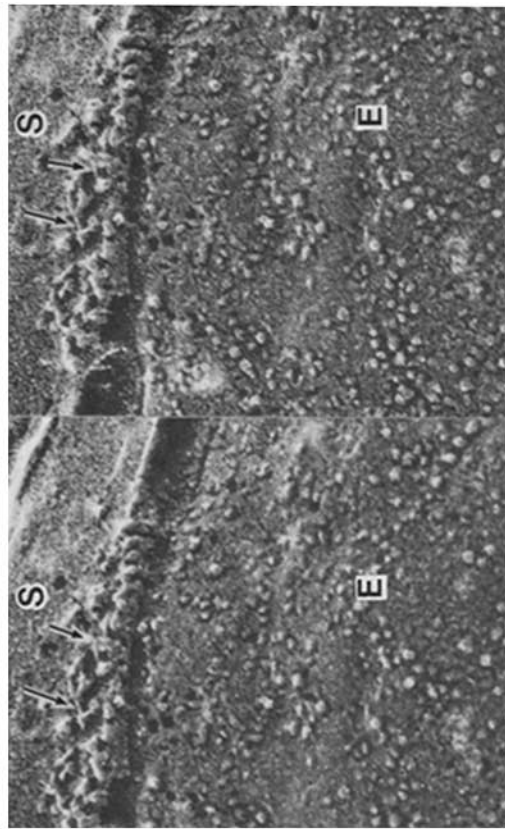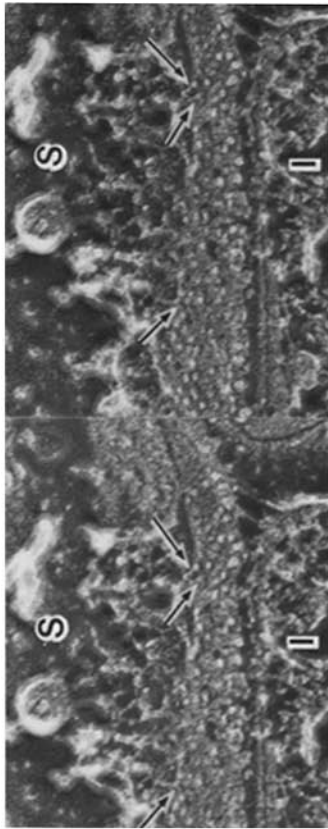
attachment. Similarly, the observation that receptors (11, 27) and lectin-binding glycoproteins (17) are retained in detergent-treated postsynaptic density fractions suggests that they are attached to the filament meshwork. The attached filaments could restrict intramembrane proteins to the postsynaptic active zone, explaining why receptors remain clustered after deafferentation (16) or when a synaptic contact does not form during development (23). In fact, proteins at the active zone exhibit a diminished lateral mobility compared to proteins in nonjunctional regions (21).

Filaments 5–10 nm in diameter are associated with the cytoplasmic side of the postsynaptic membrane of *Torpedo* electroplaque where they contact intramembrane particles (18). However, the distribution of cytoplasmic filaments associated with the postsynaptic membrane in electroplaque does not appear to differ from that in nonsynaptic regions of the plasmalemma (e.g., Figs. 22, 23–25 in reference 18), so this postsynaptic specialization is probably not comparable to that in the cochlear nucleus. In fact, the electroplaque synapse is not considered to have a typical postsynaptic density, and the 5- to 10-nm filaments there are thought to be actin filaments that directly contact the postsynaptic membrane (18).

In the cochlear nucleus, 4-nm filaments appear to link the receptor-rich region of the postsynaptic plasmalemma to an adjacent lattice of 9-nm microfilaments. We tentatively identify these filaments as F-actin on the basis of their size and substructure (19, 33). This identification is also consistent with the presence of actin in isolated postsynaptic densities (3, 4, 22, 28). In the cochlear nucleus neurons, actin filaments only contact the membrane directly in nonsynaptic regions of the plasmalemma.

Actin is bound to plasmalemmal proteins in nonneuronal cells through actin-binding proteins (7, 12, 25). The actin, with its binding protein, may influence the distribution of plasmalemmal proteins (30, 37). The fine filaments at the postsynaptic density may be another example of a protein that links actin to a plasmalemmal protein, in this instance a postsynaptic receptor, to influence its distribution. It must also be considered that the shape of active zones is not fixed (1, 2, 35). The flat active zones of the boutons studied here curve sharply during maximal release of synaptic vesicles (unpublished observations in reference 14). Because actin may also influence cell shape (6), its concentration at the synaptic active zone could reflect, or contribute to, changes in shape during activity.

The improvements in technique that led to the present understanding of cytoskeletal organization at the postsynaptic active zone also permitted observation of characteristic aggregates of small intramembrane particles at inhibitory synapses that had previously been thought to lack intramembrane particles (15, 24). Now that particle aggregates have been identified as a common feature of postsynaptic membranes, the original term "postsynaptic complex" (31) can be extended to include these intramembrane particles, the 4-nm filaments associated with them, and the intermeshed actin lattice (Fig. 5). The fine filament meshwork probably constitutes the classic postsynaptic density, and some aspect of the microfilament lattice produces the subsynaptic web. This postsynaptic complex integrates the active zone with the rest of the cytoplasm, including neurofilaments, microtubules, small vesicles, and the cortex of actin underlying the nonsynaptic plasmalemma.

REFERENCES

1. Bailey, C. H., and E. B. Thompson. 1979. Indented synapses in *Aplysia. Brain Res.* 173:13–20.
2. Bailey, C. H., E. B. Thompson, V. F. Castellucci, and E. R. Kandel. 1979. Ultrastructure of the synapses of sensory neurons that mediate the gill withdrawal reflex in *Aplysia. J. Neurocytol.* 8:415–444.
3. Banker, G., L. Churchill, and C. W. Cotman. 1974. Proteins of the postsynaptic density. *J. Cell Biol.* 63:456–465.
4. Blomberg, F., R. W. Cohen, and P. Siekevitz. 1977. The structure of postsynaptic densities isolated from dog cerebral cortex. II. Characterization and arrangement of some of the major proteins within the structure. *J. Cell Biol.* 74:204–225.
5. Carlin, R. K., D. J. Grab, R. S. Cohen, and P. Siekevitz. 1980. Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J. Cell Biol.* 86:831–843.
6. Clarke, M., and J. A. Spudich. 1977. Non-muscle contractile proteins: the role of actin and myosin in cell mobility and shape determination. *Annu. Rev. Biochem.* 46:797–822.
7. Cohen, C. M., P. L. Jackson, and D. Branton. 1978. Actin-membrane interactions. Association of G-actin with red cell membrane. *J. Supramol. Struct.* 9:113–124.
8. Cohen, R. S., F. Blomberg, K. Berzins, and P. Siekevitz. 1977. The structure of postsynaptic densities isolated from dog cerebral cortex I. Overall morphology and protein composition. *J. Cell Biol.* 74:181–203.
9. Cohen, R. S., and P. Siekevitz. 1978. Form of the postsynaptic density. A serial section study. *J. Cell Biol.* 78:36–46.
10. Cotman, C. W., G. Banker, L. Churchill, and D. Taylor. 1974. Isolation of postsynaptic densities from rat brain. *J. Cell Biol.* 63:441–455.
11. Foster, A. C., E. E. Mena, D. T. Monaghan, and C. W. Cotman. 1981. Synaptic localization of kainic acid binding sites. *Nature (Lond.).* 289:73–75.
12. Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* 77:4127–4131.
13. Grab, D. J., K. Berzins, R. S. Cohen, and P. Siekevitz. 1979. Presence of calmodulin in postsynaptic densities isolated from canine cerebral cortex. *J. Biol. Chem.* 254:8690–8696.
14. Gulley, R. L. 1978. Changes in the presynaptic membrane of the synapses of the cochlear nucleus with different levels of acoustic stimulation. *Brain Res.* 146:373–379.
15. Gulley, R. L., D. M. D. Landis, and T. S. Reese. 1978. Internal organization of membranes at end bulbs of Held in the rostral anteroventral cochlear nucleus. *J. Comp. Neurol.* 180:707–742.
16. Gulley, R. L., R. J. Wenthold, and G. Neises. 1977. Remodeling of neuronal membranes as an early response to deafferentation: freeze-fracture study. *J. Cell Biol.* 75:837–850.
17. Gurd, J. W. 1977. Identification of lectin receptors associated with rat brain postsynaptic densities. *Brain Res.* 126:154–159.
18. Heuser, J. E., and S. R. Salpeter. 1979. Organization of acetylcholine receptors in quick-frozen, deep-etched, and rotary-replicated *Torpedo* postsynaptic membrane. *J. Cell Biol.* 82:150–173.
19. Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86:212–234.
20. Heuser, J. E., T. S. Reese, and D. M. D. Landis. 1976. Preservation of synaptic structure by rapid freezing. *In* The Synapse. *Cold Spring Harbor Symp. Quant. Biol.* 40:17–24.
21. Kelly, P., C. W. Cotman, C. Gentry, and G. L. Nicolson. 1976. Distribution and mobility of lectin receptors on synaptic membranes of identified neurons in the central nervous system. *J. Cell Biol.* 71:487–496.
22. Kelly, P. T., and C. W. Cotman. 1978. Synaptic proteins: characterization of tubulin and actin and identification of a distinct postsynaptic density polypeptide. *J. Cell Biol.* 79:173–183.
23. Landis, D. M. D., and T. S. Reese. 1977. Structure of the Purkinje cell membrane in Stagger and Weaver mutant mice. *J. Comp. Neurol.* 171:247–260.
24. Landis, D. M. D., T. S. Reese, and E. Raviola. 1974. Differences in membrane structure between excitatory and inhibitory components of the reciprocal synapse in the olfactory bulb. *J. Comp. Neurol.* 155:67–92.
25. Lazarides, E., and K. Burridge. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289–298.
26. Matus, A., G. Pehling, M. Ackermann, and J. Maeder. 1980. Brain postsynaptic densities: their relationship to glial and neuronal filaments. *J. Cell Biol.* 87:346–359.
27. Matus, A., G. Pehling, and D. Wilkinson. 1981. α-Aminobutyric acid receptors in brain postsynaptic densities. *J. Neurobiol.* 12:67–73.
28. Matus, A. I, and D. H. Taff-Jones. 1978. Morphology and molecular composition of isolated postsynaptic junctional structures. *Proc. R. Soc. Lond. B. Biol. Sci.* 203:135–151.
29. Matus, A. I., and B. B. Walters. 1975. Ultrastructure of the synaptic junctional lattice isolated from mammalian brain. *J. Neurocytol* 4:369–375.
30. Nicolson, G. L. (1976). Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta.* 457:57–108.
31. Palay, S. L. (1958). The morphology of synapses in the central nervous system. *Exp. Cell Res.* 5(Suppl):275–293.
32. Peters, A., S. L. Palay, and H. deF. Webster. 1976. The Fine Structure of the Nervous System. W. B. Saunders Co., Philadelphia.
33. Ruben, G. C., and N. S. Allen. 1980. Direct structural identification of freeze-etched reconstituted muscle F-actin. *J. Cell Biol.* 87(2, Pt. 2):225 a (Abstr.).
34. Schwartz, A. M., and R. L. Gulley. 1978. Nonprimary afferents to the principal cell of the rostral anteroventral cochlear nucleus of the guinea pig. A correlative thin-section and freeze-fracture study. *Am. J. Anat.* 153:489–508.
35. Streit, P., K. Akert, O. Sandri, R. P. Livingston, and H. Moor. 1972. Dynamic ultrastructure of presynaptic membranes at nerve terminals in the spinal cord of rats. Anesthetized and unanesthetized preparations compared. *Brain Res.* 48:11–26.
36. Ueda, T., P. Greengard, K. Berzins, R. S. Cohen, F. Blomberg, D. J. Grab, and P. Siekevitz. 1979. Subcellular distribution in cerebral cortex of two proteins phosphorylated by a cAMP-dependent protein kinase. *J. Cell Biol.* 83:308–319.
37. Yu, J., and D. Branton. 1976. Reconstitution of intramembrane particles in recombinants of erythrocyte protein band 3 and lipid: effects of spectrin-actin association. *Proc. Natl. Acad. Sci. U. S. A.* 73:3891–3895.