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High-resolution Localization of Clathrin Assembly Protein AP180 in the Presynaptic Terminals of Mammalian Neurons

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ABSTRACT

Synaptic vesicles (SVs) assemble at the presynaptic compartment through a clathrin-dependent mechanism that involves one or more assembly proteins (APs). The assembly protein AP180 is especially efficient at facilitating clathrin cage formation, but its precise ultrastructural localization in neurons is unknown. Using immunoelectron microscopy, we demonstrate the presynaptic localization of AP180 in axon terminals of rat cerebellar neurons. In contrast, the assembly protein AP2 was associated with both the presynaptic plasma membrane and the cytosolic side of the membrane at postsynaptic and extrasynaptic sites. Furthermore, ultrastructural analysis of primate retina showed that AP180 immunoreactivity was preferentially and highly enriched at ribbon synapses, where glutamate is released tonically at high levels and rapid vesicle turnover is essential. To maintain functional synaptic transmission, neurotransmitter-filled SVs must be readily available, and this requires proper reassembly of new vesicles. The expression of AP180, in addition to AP-2, in the clathrin-mediated endocytic pathway might add another level of control to SV reformation for efficient assembly of clathrin, effectively controlling the size of assembled vesicles and faithfully recovering SV-specific components. J. Comp. Neurol. 447:152–162, 2002.

Indexing terms: immunoelectron microscopy; synapse; synaptic vesicle recycling; clathrin-mediated endocytosis

Assembly of synaptic vesicles (SVs) at the nerve terminal following release of neurotransmitter is accomplished by an efficient and local recycling system. Multiple mechanisms work in concert to replenish the supply of SVs (Palfrey and Artalejo, 1998; Zhang and Ramaswami, 1999; Sudhof, 2000), one of which operates using a clathrin-mediated process (Zhang and Ramaswami, 1999; Slepnew and DeCamilli, 2000; Brodin et al., 2000). Clathrin-coated vesicles are involved in various pathways of vesicle trafficking in cells (Brodsky, 1988; Pearse and Robinson, 1990; Schmid, 1997; Pishvaee and Payne, 1998; Kirchhausen, 2000). At the nerve terminal, a major challenge is probably posed by distinct features of SVs and synaptic transmission. First, rapid and frequent synaptic release must not be limited by the number of available SVs, so the recycling process has to be efficient. Second, preservation of a constant postsynaptic response requires that newly formed SVs be highly uniform and small (Katz, 1962). Finally, because the membrane of SVs fuses into the presynaptic plasma membrane during the release of neurotransmitter (Ceccarelli et al., 1979; Heuser et al., 1979; von Wedel et al., 1981), retrieval of SV-membrane components has to be precise to maintain proper SV function. A

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priori one might expect the clathrin-dependent SV recycling system to include mechanisms that provide efficiency, speed, and fidelity to the recovery process.

In addition to clathrin protein, the formation of clathrin-coated vesicles also requires assembly (or adaptor) proteins (APs; for review, see Pearse and Robinson, 1990; Schmid, 1997; Kirchhausen, 1999; Robinson and Bonifacino, 2001). Different APs are specific to particular trafficking pathways (Kirchhausen, 1999; Robinson and Bonifacino, 2001). The role of the tetrametic AP complex AP2 in clathrin-mediated endocytosis has been well characterized (Ahle et al., 1988; Kirchhausen, 1999). AP180, a monomeric assembly protein, appears to be preferentially expressed in brain or neurons (Perry et al., 1991; Sousa et al., 1992; Morris et al., 1993; Takei et al., 1996; Yao et al., 1999). Like the other APs, AP180 is capable of promoting clathrin assembly in vitro, but it is seemingly far more effective (Lindner and Ungewickell, 1992). The presence of AP180 in vitro also contributes to the uniformity of the assembled clathrin cages (Ahle and Ungewickell, 1986; Ye and Lafer, 1995). Recent in vivo studies using species with mutated AP180 (Zhang et al., 1998; Nonet et al., 1999) or disrupted AP180 (Morgan et al., 1999) confirm the in vitro observations that AP180 regulates the size of SVs and therefore is critical to synaptic transmission. Additionally, mutation of the AP180 homolog in *Caenorhabditis elegans* results in the mislocation of an SV-specific component (Nonet et al., 1999). These results in total imply that AP180 contributes to clathrin-mediated endocytosis at synapses through the efficient assembly and sizing of SVs, and probably the recruitment of the proteins necessary for their function.

Despite the strong evidence for a critical role of AP180 in the formation of SVs, its precise subcellular localization *in situ* and whether it is expressed at other neuronal organelles are unknown. Here we show by immunoelectron microscopy (immuno-EM) that AP180 is confined to presynaptic compartments, whereas AP2 is localized to both synaptic and extrasynaptic sites. AP180 is more abundant in some synapses than in others.

MATERIALS AND METHODS

Reagents

Monoclonal anti-AP180 antibody (AP180-I mAb; Ahle and Ungewickell, 1986; Morris et al., 1993) and anti-AP2 antibody (100/2 mAb; Ahle et al., 1988) were obtained from Sigma (St. Louis, MO). Rabbit anti-human synaptophysin antibody was from DAKO (Carpinteria, CA). AlexaFluor conjugate antibodies were from Molecular Probes (Eugene, OR). All other chemicals and reagents used were of the highest quality available.

Tissue preparation

Postmortem human cerebellum tissues were provided by the Alzheimer's Disease Center at the University of Rochester. All cases were well characterized based on clinical and neuropathological criteria (Khachaturian, 1985). All tissues used in this study were from neurologically normal controls, and the mean postmortem delay for the tissues of the five cases used was 8 hours. Tissues were collected at autopsy and fixed in 4% phosphate-buffered paraformaldehyde followed by cryoprotection with graded sucrose.

All methods involving live animals were approved by the University of Rochester Committee on Animal Resources. Sprague-Dawley rats (125–150 g; Taconic, Germantown, NY) were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused through the ascending aorta with 0.9% NaCl followed by fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The cerebellum was removed, postfixed in 4% paraformaldehyde for additional 2 hours, and taken through graded sucrose.

To facilitate the penetration of the antibody, the tissue was freeze-thawed 3 times and then cut into 20–40-μm-thick sections on a Vibratome, collected in buffer, and used for preembedding immuno-EM.

We obtained retinas from adult *Macaca fascicularis* used in unrelated studies at Yerkes Regional Primate Research Center (Atlanta, GA) and at the University of Rochester Medical Center, following guidelines set forth by the National Institutes for Health. After euthanasia of the animal by overdose (sodium pentobarbital, 40 mg/kg), the eyes were removed, and isolated eyecups were cleaned of vitreous and immediately fixed free-floating in 4% paraformaldehyde for 1–2 hours. We isolated and washed the retinas extensively in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and cut small pieces into 20–40-μm-thick vertical sections on a vibratome.

Light microscopic localization of AP180 and synaptophysin

Standard indirect immunofluorescence techniques were employed. After incubation with 0.25% gelatin and 0.5% Triton X-100 in Tris-buffered solution (pH 7.5) for 10 minutes, the sections were blocked with blocking buffer (0.1% goat serum, 0.5% bovine serum albumin [BSA] in PBS, pH 7.4) for 3 hours. The sections were then incubated with anti-AP180 antibody (1:5,000) and anti-synaptophysin antibody (1:100) in the blocking buffer for 16–24 hours at 4°C. After thorough washing, the sections were incubated with AlexaFluor conjugate secondary antibodies at room temperature for 1 hour. The mouse monoclonal antibody to AP180 was detected with an Alexa 488-conjugated goat anti-mouse IgG antiserum (1:1,000). The rabbit antibody to synaptophysin was detected with an Alexa 568-conjugated goat anti-rabbit antiserum (1:2,000; the approximate excitation wavelength for Alexa 488 is 488 nm, and for Alexa 568 it is 568 nm). After another thorough washing, the section was incubated in 0.3% Sudan Black B (w/v, in 70% ethanol) for 10 minutes at room temperature to block autofluoresencent lipofuscin (Schnell et al., 1999; Yao et al., 2000). The sections were then rinsed in 0.05 M Tris-buffered solution (pH 7.4) and mounted with reagents from a ProLong antifade kit (Molecular Probes).

Sections were examined under an Olympus confocal microscope using a krypton-argon laser attached to an Olympus microscope equipped with standard filter sets. The confocal microscope was driven by a computer running Fluoview 2.1 Software. Images taken for AP180- and synaptophysin-labeled tissues were imported into Photoshop 5.0 and were not altered further.

Electron microscopic localization of AP180 and AP2

The sections were placed in blocking solution of 0.8% BSA, 0.1% gelatin, and 5% normal horse serum (NHS) in

0.01 M PBS for 6–12 hours at room temperature. Sections were transferred to primary antibody solution with 0.8% BSA, 0.1% gelatin, and 1% NHS in PBS for 4–6 days at 4°C for immuno-EM. After rinsing in PBS, sections were placed in secondary antibody overnight at 4°C. The secondary antibody solution contained 0.8% BSA, 0.1% gelatin, and 5% NHS in PBS. All solutions contained 0.01% Triton X-100 to enhance penetration of the antibodies.

For visualizing the reaction product for EM, we followed with a few modifications a well-established protocol used for preembedding immuno-EM (Sassoè-Pognetto et al., 1994; Brandstätter et al., 1997). Sections were incubated in Extravidin (1;300; Vector, Burlingame, CA) in PBS + 1% BSA for 1.5–2 hours in the dark at room temperature, rinsed, and transferred to Tris buffer. After conjugation with 3,3′-diaminobenzidine (DAB), sections were fixed for 45 minutes in 2% glutaraldehyde in cacodylate buffer (pH 7.2) and rinsed in cacodylate overnight at 4°C. To enhance the DAB product, sections were incubated in a solution of 2.6% hexamethylenetetramine, 0.2% silver nitrate, and 0.2% disodium tetraborate in double-distilled water (ddH_2O) for 10 minutes at 60°C. Sections were treated with 0.05% gold chloride in ddH_2O for 3 minutes at room temperature, rinsed, and incubated in 2.5% sodium thiosulfate in ddH_2O. Sections were postfixed in 1% osmium in cacodylate buffer for 15–30 minutes, rinsed in cacodylate overnight, dehydrated in graded alcohols, and embedded in Epon-Araldite at 60°C for 48 hours. Serial ultrathin sections were cut at 70 nm and photographed under a Hitachi 7100 electron microscope.

RESULTS

Cellular distribution of AP180 immunoreactivity in human cerebellum

The specificity of the AP180 antibody (AP180-I mAb) has been characterized previously by others (Morris et al., 1993) and by us (Yao and Coleman, 1998a, b; Yao et al., 1999). Here we examined the expression of AP180 in postmortem human cerebellum using immunofluorescent confocal microscopy. The granule cell layer was the strongest immunoreactive area for AP180 (Fig. 1A), with intense labeling in glomeruli appearing as clusters of punctate granules. By contrast, the molecular layer only displayed occasional immunolabeling. Throughout all cortical layers of the cerebellum, no cells were revealed suggesting that the immunoreactivity was not present in cell bodies. These results confirm that AP180 demonstrates the typical staining characteristics for nerve terminal proteins at light microscopy (Sousa et al., 1992; Yao et al., 1999).

To evaluate further the association of AP180 with synapses, we double-labeled the same tissues with an antibody specific to synaptophysin. Synaptophysin is a membrane protein that resides in SVs and therefore has been commonly used as a marker in studies related to synapses (Jahn et al., 1985; Wiedenmann and Franke, 1985; Calhoun et al., 1996). At low-power magnification (Fig. 1A), although immunoreactivity for synaptophysin was also revealed as punctate granules in the neuropil, the intensity of staining was distributed homogeneously among all three layers of cerebellar cortex. At higher magnification of the granule cell layer, immunoreactivity for AP180 and synaptophysin coexisted in many glomeruli, but some structures were labeled with either AP180 alone or synaptophysin alone (Fig. 1B). This double immunolabeling was repeated five times with similar results (five different cases). We do not currently have an explanation as to why AP180 immunoreactivity does not completely overlap with synaptophysin as seen by confocal microscopy.

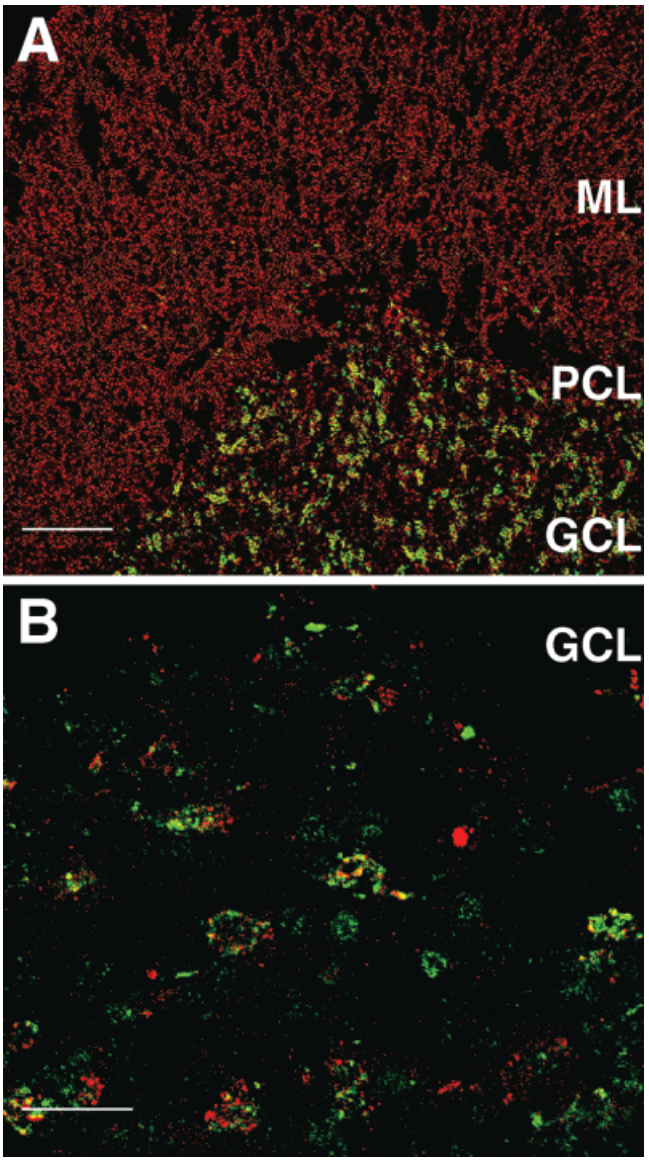

Fig. 1. Expression patterns for AP180 and the synaptic protein synaptophysin are both punctate but not completely overlapping. **A:** Confocal micrograph of the cortical layers of cerebellum double-labeled with antibodies to AP180 (green) and synaptophysin (red) indicates dense distribution of AP180 to the granule cell layer, whereas synaptophysin is more diffuse to all layers. **B:** Higher power confocal micrograph of the granule cell layer in the same material demonstrates partial colocalization of AP180 and synaptophysin (yellow puncta). ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bars = 100 μm in A, 20 μm in B.

Presynaptic localization of AP180 in cerebellar neurons

The subcellular localization of AP180 in the cerebellum was examined further by immuno-EM. Because postmor-

tem human brain tissues usually do not provide sufficient quality for immuno-EM, rat cerebellum was fixed appropriately and used for preembedding immunoperoxidase localization of AP180.

Consistent with the light microscopic analysis (Fig. 1; Yao et al., 1999), AP180 immunoreactivity in the cerebellum was confined to synaptic junctions and was never observed in neuronal cell bodies or their processes (Fig. 2). Glomeruli in the granule cell layer demonstrated prominent AP180 immunoreactivity, whereas the molecular layer contained little immunolabeling. Basket cell terminals that synapse on Purkinje cell soma were labeled with AP180, as demonstrated by the immunoperoxidase product (Fig. 2A). Likewise, the axosomatic contacts between Purkinje cell axonal collaterals and Golgi type II cells were also immunopositive (Fig. 2B).

To determine whether AP180 is expressed in both pre- and postsyaptic compartments, we examined the positively labeled synaptic junctions at higher magnification (Fig. 3). Dense particles representing immunostaining were clearly confined to presynaptic compartments (Fig. 3A–D). The immuno-particles intermingled with SVs, particularly those near presumed synaptic contacts, although specific elements were not easily discerned (Fig. 3A,B). The sparse staining seen occasionally over presynaptic mitochondria is probably nonspecific. No specific staining was found in other parts of neurons including soma (Fig. 3A,B), or dendrites (Fig. 3C,D).

Differential expression of AP180 in cerebellar synapses

In agreement with the light microscopic observations of human cerebellum (Yao et al., 1999; Fig. 1), the molecular layer of rat cerebellum only contained sporadic AP180 immunolabeling, as revealed by low-power immuno-EM (Fig. 2). We surveyed this brain region at higher magnification in at least four different rats and failed to find a synapse unambiguously labeled with AP180. Figure 4A and B shows typical synapses between granule cell parallel fibers and Purkinje cell dendritic thorns (Palay and Chan-Palay, 1974). The postsynaptic density was pronounced, giving the synapses an "asymmetric" appearance. There was no visible immunolabeling for AP180.

To compare the subcelluar distribution of AP180 with other clathrin assembly proteins, we also carried out immunolabeling with an antibody against AP2 (Ahle et al., 1988; Yao et al., 2000). AP2, a member of the clathrin assembly protein family, interacts and forms a complex with AP180 (Hao et al., 1999). Unlike AP180, AP2 immunoreactivity was found in all layers of cerebellar cortex including the molecular layer (Fig. 4C–F). Furthermore, AP2 immunostaining was not restricted to presynaptic terminals, but was found in both pre- and postsynaptic compartments (Fig. 4C,D). The stain was also located on the cytoplasmic face of dendritic spines, even without apparent synapses (Fig. 4E,F).

In the granule cell layer of the cerebellum, although both AP180 and AP2 immunoreactivity was primarily associated with glomeruli, the distribution of the stain was different (Fig. 5). AP180 immunoreactivity was predominantly detected at the periphery of cerebellar glomeruli (Fig. 5A,B), presumed to represent axonal boutons from Golgi cells (Palay and Chan-Palay, 1974). The mossy fiber terminals in the center of the glomeruli demonstrated little, if any, positive stain for AP180. On the other hand,

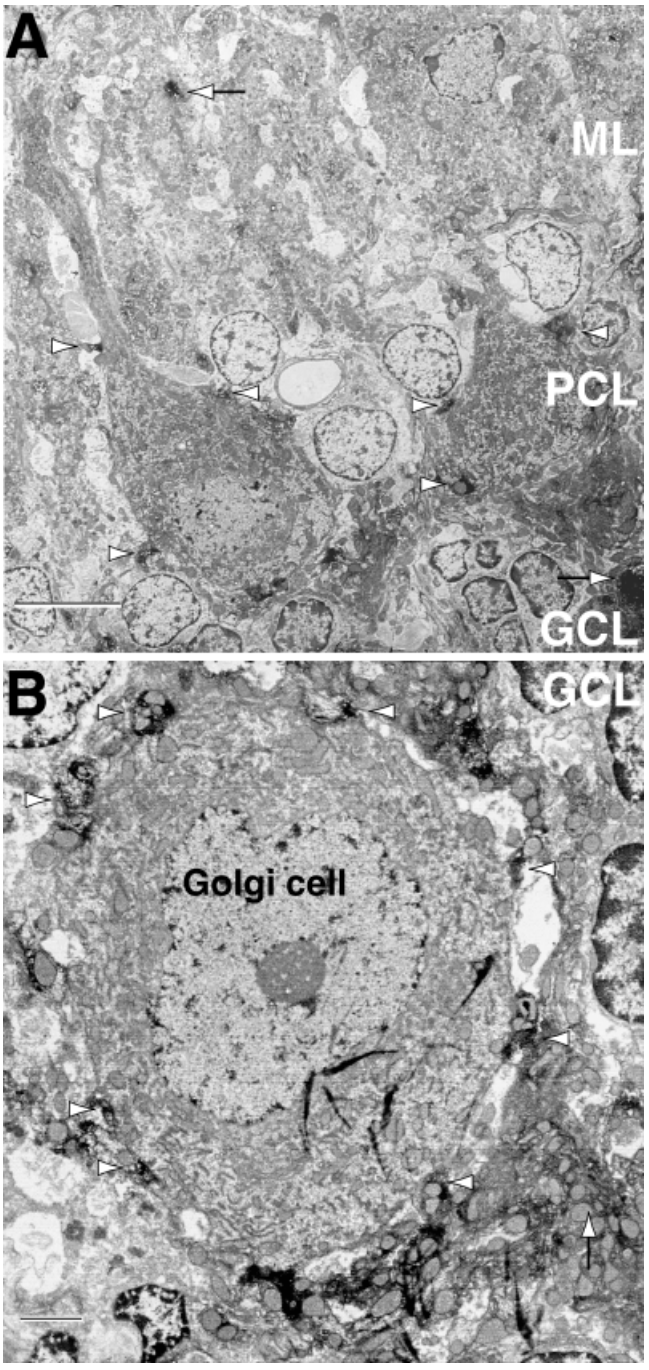

Fig. 2. Preembedding immunoelectron microscopy demonstrates that AP180 is restricted to synaptic junctions. **A:** Cortical layers of rat cerebellum. Dense immunolabel in presumed basket cell axon terminals that contact Purkinje cell bodies (arrowheads). AP180 immunoreactivity is also dense within glomeruli of the granule cell layer and less so in the molecular layer (horizontal arrows). **B:** Immunolabeled synaptic terminals from presumed Purkinje cell axon collaterals (arrowheads) contact a Golgi type II cell that is surrounded by closely packed granule cells. The immunolabel in glomeruli of the granule cell layer is indicated with arrows (lower right corner in both A and B). ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bar = 10 μm in A, 5 μm in B.

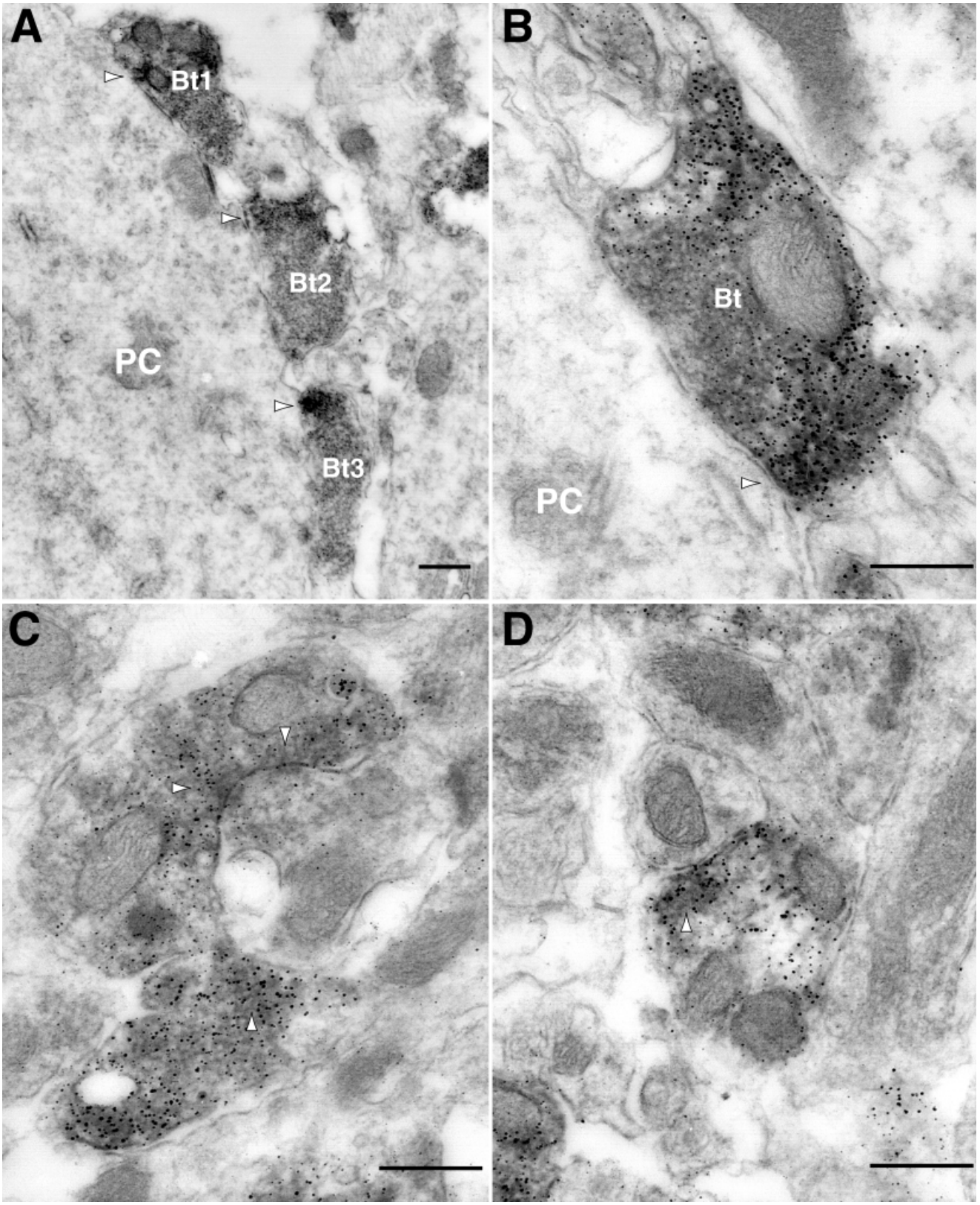

Fig. 3. AP180 is confined to presynaptic terminals. **A:** In three presumed basket cell terminals (Bt1–3) that abut the soma of a Purkinje cell (PC), immunodeposits are associated with densely packed SVs. Distribution of the presynaptic immunodeposits is not homogenous but is denser near one side of the terminals (arrowheads). **B:** Another example at higher magnification of an axosomatic contact between a labeled basket cell terminal (Bt) and a Purkinje cell (PC) Immunodeposits are near synaptic contacts (arrowheads). **C,D:** Micrographs of presumed Golgi-cell axon terminals at the peripheral edge of a glomerulus with dense AP180 localization (arrowheads) presynaptic to what are probably unlabeled dendrites of granule cells. Scale bars = 1 μm.

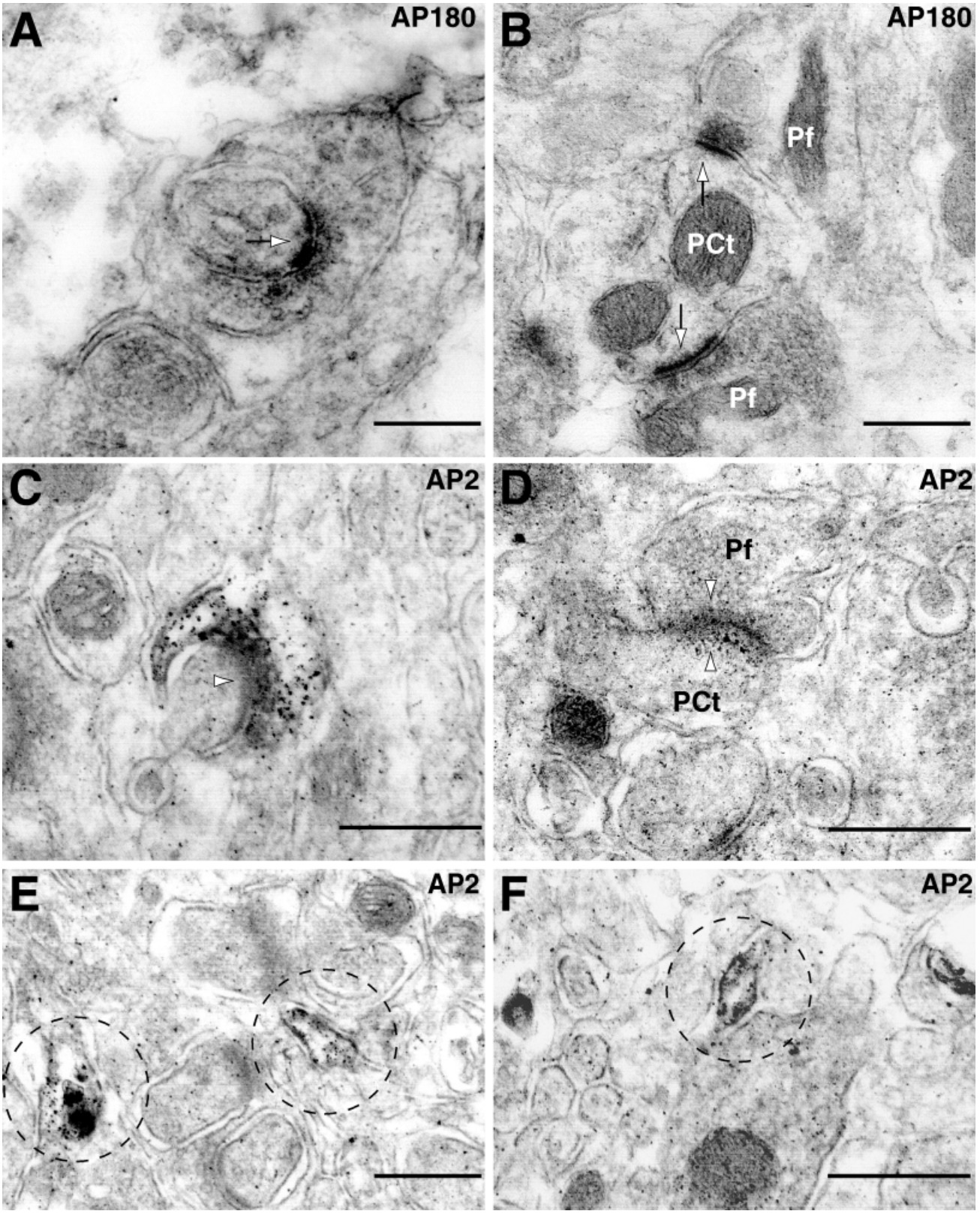

Fig. 4. Comparison of AP180 and AP2 localization in the molecular layer of rat cerebellum. **A:** Example of synapse with pronounced postsynaptic density (arrow) lacking AP180 label. **B:** Similarly, typical synapses (arrows) between ascending granule cell parallel fibers (Pf) and Purkinje cell thorn (PCt) also lack AP180 label. **C,D:** AP2 label is confined to the presynaptic compartment at some synapses (arrowhead in C), but to both the pre- and postsynaptic compartments at other synapses (arrowheads in D). **E,F:** AP2 label is also prevalent in the synapse-free spines of Purkinje cell dendrites (dashed outlines). Scale bars = 1 μm.

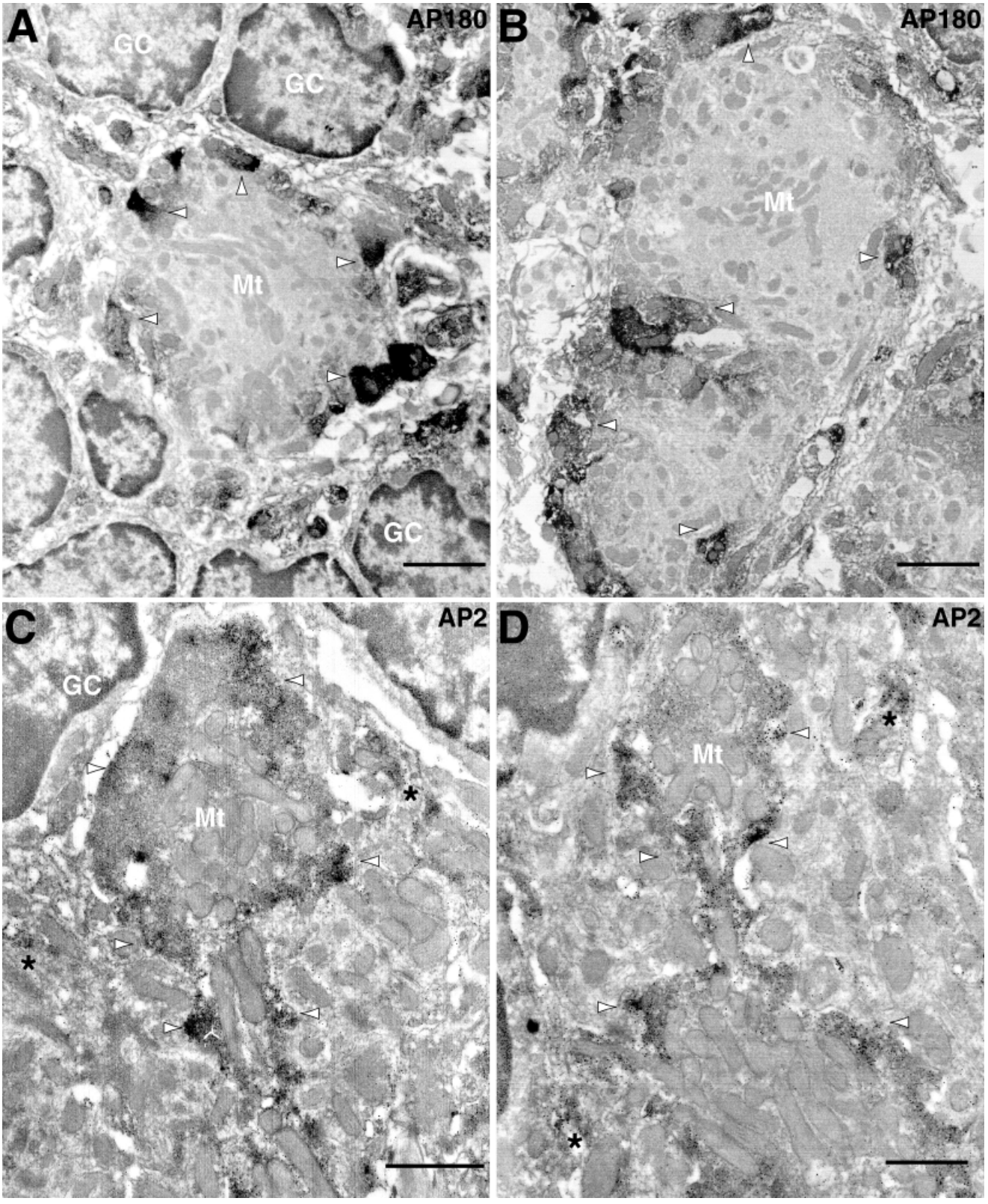

Fig. 5. Comparison of AP180 and AP2 localization in glomeruli of rat cerebellum. **A,B:** Dense immunodeposits of AP180 are distributed along the periphery of the glomerulus (arrowheads), where axons of Golgi cells synapse upon distal dendrites of granule cells, but not within mossy fiber terminals. **C,D:** In contrast, AP2 immunoreactivity is scattered throughout the glomeruli (asterisks) and localized heavily along the cytoplasmic side of the plasma membrane (arrowheads) within the mossy fiber terminal (Mt). GC, granule cell. Scale bars = 1 μm in A–C, 0.5 μm in D.

AP2 labeling in the glomeruli was clearly associated with the mossy fibers (Fig. 5C,D). Peroxidase product depicting AP2 immunoreactivity was in close contact with the plasma membrane outlining the mossy fiber terminals.

Expression of AP180 in retinal synapses

To test whether expression of AP180 at the presynaptic terminals is associated with specific types of neurotransmitters within SVs, we also examined AP180 localization in the retina. Like the cerebellum, both glutamate and γ-aminobutyric acid (GABA) are used as primary neurotransmitters in the retina, but with differing mechanisms of transmitter release (for review, see Kalloniatis and Tomisich, 1999). Photoreceptors release glutamate tonically from specialized structures called *ribbons*. This steady stream of release is greatest in the dark, when photoreceptors are depolarized, and decreases incrementally with increasing levels of light. Thus, the mode of transmission differs significantly from the conventional glutamatergic synapse elsewhere in the brain, where release occurs in transient bursts subsequent to sharp depolarization of the axon terminal. Each ribbon points between a pair of GABAergic lateral processes from inhibitory horizontal-type cells that collect excitation from a large number of photoreceptors (for review, see Sterling, 1998). Although the precise mode of GABA release is not known, horizontal cells do not form conventional synapses at the photoreceptor complex and very likely release GABA via a transporter mechanism (Schwartz, 1987; Haugh-Scheidt et al., 1995). Thus, the release of GABA by horizontal cells also differs from conventional GABAergic synapses in the central nervous system in that transmission does not depend on clustering of SVs at a presynaptic active zone.

Our immuno-EM of primate retina demonstrates several key features of AP180 expression. AP180 immunoreactivity clusters densely in a thin band proximal to the sites of ribbon docking at the photoreceptor axon terminal. Figure 6A demonstrates this tendency at a cone photoreceptor axon terminal, where several presynaptic active zones are generally present in a single section. Although the cone synaptic complex is quite large, with some 250–500 processes postsynaptic to the axon terminal (Calkins, 2000), there is no AP180 expression outside the cone presynaptic membrane. This is consistent with expression of AP180 in cerebellum, which is also confined to the presynaptic active zone. However, at the photoreceptor synaptic complex, AP180 appears to associate strictly with glutamatergic presynaptic ribbons, and not at all with the sites of GABA release from horizontal cell processes (shown at higher magnification in Fig. 6B,C). These same results hold for the rod axon terminal as well, where only one or two synaptic ribbons direct glutamate to far fewer postsynaptic processes (Fig. 6D). Here it is quite evident that the AP180 clusters discretely near the active zone, outlining exclusively the presynaptic aspect of the glutamatergic active zone. We also obtained similar results from rat retina (not shown). Thus, AP180 in the retina, although strictly presynaptic, is associated with the tonic release of glutamate from the photoreceptor, but not with the release of GABA via nonconventional mechanisms (for review, see Kamermans and Spekreijse, 1999).

DISCUSSION

Clathrin-coated vesicles mediate protein and lipid trafficking in various cellular compartments including receptor-mediated endocytosis and the recycling of SVs. Recent biochemical, molecular, and imunocytochemical studies suggest that AP180 is responsible for the regulation of the size of clathrin-coated vesicles and the reformation of SVs (Ahle and Ungewickell, 1986; Ye and Lafer, 1995; Zhang et al., 1998; Nonet et al., 1999; Morgan et al., 1999). In the current study, we use immuno-EM analysis in situ to confirm and expand previous findings that AP180 is localized to the presynaptic compartments of mammalian neurons at conventional synapses. In addition, we also demonstrate that in the retina, AP180 associates closely with synaptic ribbons, in which glutamate is released not in sharp bursts subsequent to an action potential, but in a steady stream that is modulated in grades dependent on the level of presynaptic depolarization. In contrast, in the retina, AP180 is not detected in horizontal cell processes where GABA is released via a specialized transport mechanism (Schwartz, 1987). Thus, AP180 is not likely to associate strictly with a particular transmitter, but with the mechanism of SV exo/endocytosis.

The basic structure of the clathrin coat is a three-legged complex called a triskelion consisting of three pairs of heavy-chain and light-chain proteins (for review, see Kirchhausen, 2000). Clathrin triskelia polymerize into cages to capture plasma membrane during coated vesicle formation. The association of clathrin with plasma membrane and the assembly of clathrin into clathrin-coated vesicles is mediated and promoted by APs (for review, see Kirchhausen, 1999; Robinson and Bonifacino, 2001). Our electron microscopy demonstrates AP2 immunoreactivity in cerebellum on both pre- and postsynaptic sides of synaptic junctions (Fig. 4C,D), as well as at extrasynaptic sites (such as the dendritic spines in Fig. 4E,F). This finding is consistent with the dogma that AP2 is associated with general clathrin-mediated endocytosis at the plasma membrane, which includes but is not restricted to the route of SV recycling (Ahle et al., 1988; Gonzalez-Gaitan and Jackle, 1997; Kirchhausen, 1999; Slepnev and DeCamilli, 2000; Robinson and Bonifacino, 2001). In addition, the present report provides further evidence demonstrating that SV endocytosis in the presynaptic compartment requires both AP180 and AP2, whereas clathrin-mediated endocytosis of neurotransmitter receptors (Kittler et al., 2000; Man et al., 2000; Wang and Linden, 2000; Vissel et al., 2001) at the postsynaptic membrane is dependent only on AP2

Why does the clathrin-mediated machinery for SV reformation need AP180 in addition to AP2? Possibly the SV recycling, albeit depending on clathrin, is a unique process that is not identical to the clathrin-mediated endocytosis of other purposes. It is conceivable that the re-supply of SVs has to be fast and efficient; the size of newly formed SVs needs to remain uniform, and the specific identity of SVs must be maintained. Nowhere is this more true than at the tonic glutamatergic retinal synapse (Rao-Mirotznik et al., 1995). This idea of linking AP180 with specialized clathrin-mediated endocytosis at synapses is in agreement with the following findings.

First, AP180 is more efficient at assembling clathrin than other APs (Lindner and Ungewickell, 1992), and when AP180 interacts with AP2, the clathrin assembly

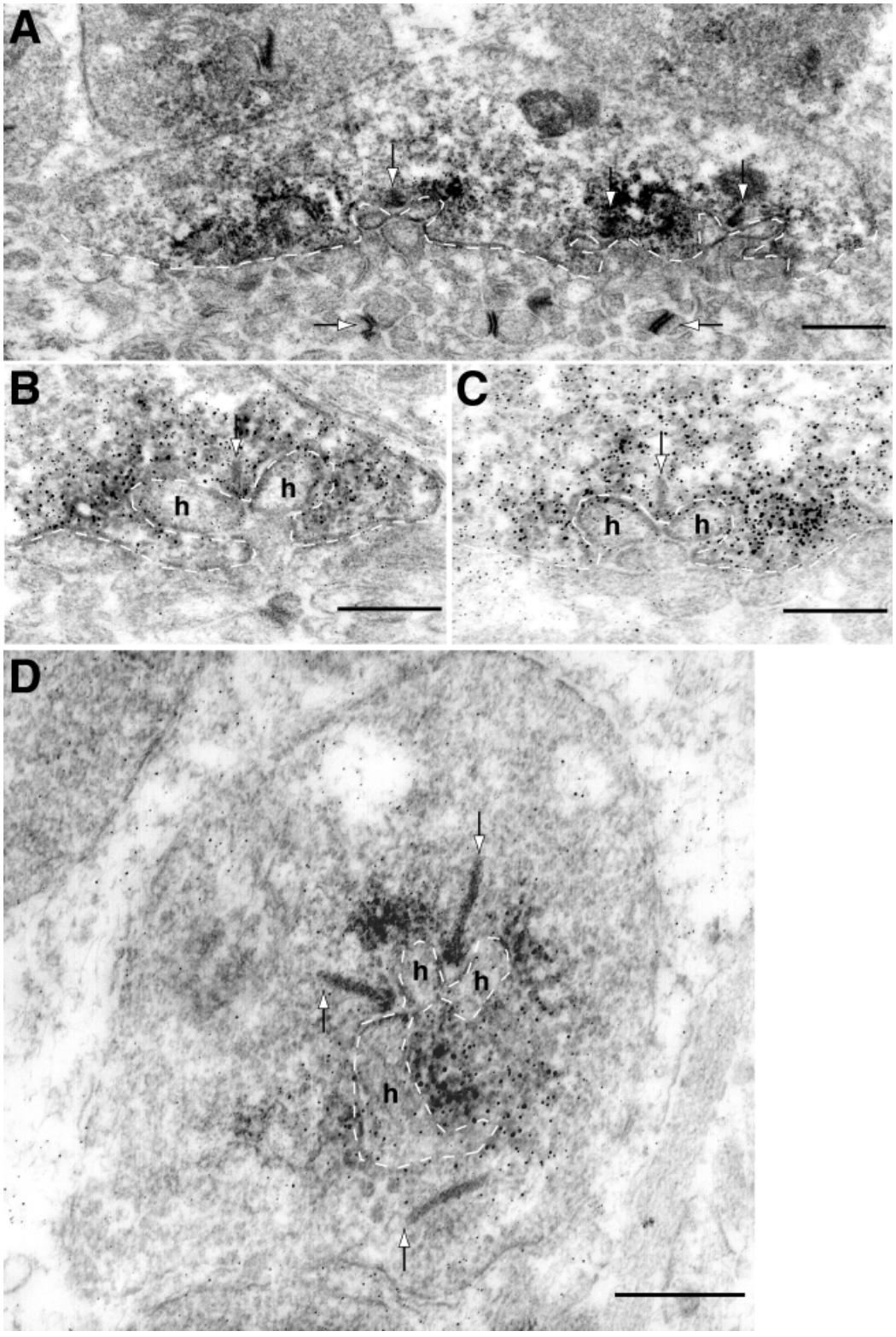

Fig. 6. AP180 expression at tonic synapses in primate retina. **A:** Electron micrograph of the axon terminal of a cone photoreceptor with AP180 label confined to the presynaptic compartment (dotted outline). Label is most dense near each glutamatergic ribbon synapse (vertical arrows) and absent from the postsynaptic cleft that includes desmosomal junction connections between the GABAergic processes of horizontal cells (horizontal arrows). **B,C:** Higher magnification micrographs of two ribbon synapses at the cone terminal (vertical arrows) illustrating dense localization of AP180 at the presynaptic active zone and absence of label to GABAergic horizontal cell processes (h). **D:** Electron micrograph of the axon terminal of a rod photoreceptor with AP180 label again localized selectively to the presynaptic active zone proximal to synaptic ribbons (vertical arrows). Label is absent from horizontal cells (h). Scale bars = 0.5 μm in A, 1 μm in B–D.

activity is even more effective than either AP180 or AP2 alone (Hao et al., 1999). The combined strength in their assembly capability may be critical in providing the available SVs in the presynaptic compartments even at high intensity of synaptic transmission.

Second, both AP180 and AP2 reveal their ability to control the size of clathrin cages assembled in vitro (Ahle and Ungewickell, 1986; Ye and Lafer, 1995). A series of molecular and genetic perturbation experiments clearly demonstrates a role for AP180 in regulation of SV size (Zhang et al., 1998; Nonet et al., 1999; Morgan et al., 1999). Conceivably, the extra control and fine tuning of SV size is crucial for maintaining a constant level of neurotransmitter release and subsequent postsynaptic response. Third, *unc*-11, a relative of AP180 in *C. elegans*, demonstrates a role in recruiting synaptobrevin, an SV-specific component (Nonet et al., 1999). AP180 might have a similar role in recovering SV-specific components at the mammalian synapse, but this possibility needs to be examined.

It remains unclear why granule cell parallel fiber and mossy fiber terminals have shown little immunolabeling for AP180 in our microscopic analyses of both human and rat cerebellar tissues (Yao et al., 1999; Figs. 1, 2, 4A,B). The detection of AP180 mRNA in cerebellar neurons nondiscriminatively (Sousa et al., 1992) implies that all neurons in cerebellum probably contain AP180 protein, but levels may vary. We hypothesize that the expression level of AP180 is regulated specifically and is perhaps tailored to its respective functional roles in different synapses. Because AP180 and AP2 are both associated with clathrin-mediated SV recycling and also have overlapping functions (Lindner and Ungewickell, 1992; Hao et al., 1999; Kirchhause, 1999; Slepnev and DeCamilli, 2000; Robinson and Bonifacino, 2001), it is possible that AP2 serves as a housekeeping AP in SV endocytosis, whereas AP180 works to enhance or a fine-tune vesicle turnover. Whether there is a true correlation between the level of AP180 and the particular type of synapse and whether the level of AP180 is regulated differentially in different synapses need to be examined in greater detail.

The level of AP180 is reduced in the brains of Alzheimer's disease (AD), and the loss is consistent with AD pathology (Yao and Coleman, 1998a,b). Similarly, AP2 is also reduced in AD although the reduction is more prominent in the areas enriched with synaptic contacts (Yao et al., 2000). The loss of these proteins may be merely a reflection of neuronal or synaptic destruction that occurs in AD. Alternatively, AP180/AP2 may be involved in the pathological cascade leading to synaptic dysfunction. Localization of AP180/AP2 in the high resolution studies presented here will help us to understand better their functions in clathrin-mediated SV recycling and will allow us to examine their connection with AD pathology in future studies.

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