# The Neuronal Form of Adaptor Protein-3 Is Required for Synaptic Vesicle Formation from Endosomes

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Heterotetrameric adaptor complexes vesiculate donor membranes. One of the adaptor protein complexes, AP-3, is present in two forms; one form is expressed in all tissues of the body, whereas the other is restricted to brain. Mice lacking both the ubiquitous and neuronal forms of AP-3 exhibit neurological disorders that are not observed in mice that are mutant only in the ubiquitous form. To begin to understand the role of neuronal AP-3 in neurological disease, we investigated its function in *in vitro* assays as well as its localization in neural tissue. In the presence of GTPγS both ubiquitous and neuronal forms of AP-3 can bind to purified synaptic vesicles. However, only the neuronal form of AP-3 can produce synaptic vesicles from endosomes *in vitro*. We also identified that the expression of neuronal AP-3 is limited to varicosities of neuronal-like processes and is expressed in most axons of the brain. Although the AP-2/clathrin pathway is the major route of vesicle production and the relatively minor neuronal AP-3 pathway is not necessary for viability, the absence of the latter could lead to the neurological abnormalities seen in mice lacking the expression of AP-3 in brain. In this study we have identified the first brain-specific function for a neuronal adaptor complex.

*Key words: adaptor protein; synaptic vesicle; AP-3; endosome; brain; neuronal isoforms*

Membrane trafficking in neurons appears to be more complex than in most other cell types (Morris and Schmid, 1995). Although neurons use basically the same machinery as nonneuronal cells, they also express forms of trafficking proteins unique to nerve cells (Hirst and Robinson, 1998). Many membrane-trafficking proteins have neuronally expressed splice isoforms or separate gene products, including AP180 (Morris et al., 1993), auxilin (Ahle and Ungewickell, 1990; Maycox et al., 1992), intersectin (Hussain et al., 1999), dynamin (Faire et al., 1992; Altschuler et al., 1998), and the clathrin light chains LCa and LCb (Jackson et al., 1987; Kirchhausen, 2000).

One class of proteins that plays a large role in trafficking is the adaptor protein complexes. The adaptor complexes bind to cargo proteins that get sorted from donor membranes into vesicles. These complexes also interact with other proteins that help to regulate the process of vesiculation (Pearse and Robinson, 1990; Kirchhausen, 1999). The adaptor protein complexes, AP-1, AP-2, AP-3, and AP-4, are heterotetrameric complexes composed of a large variable subunit (γ, α, δ, or ϵ, respectively), a large subunit that shares higher homology among the complexes (β1, β2, β3, or β4, respectively), a medium-sized subunit (μ1, μ2, μ3, or μ4), and a small subunit (σ1, σ2, σ3, or σ4). Although all of the adaptor protein complexes function similarly to vesiculate membranes, their specificity may be attributable to their proper targeting to the donor compartment. For instance, AP-1 is involved in trafficking from the *trans*-Golgi network (TGN), whereas AP-2 is involved in endocytosis from the plasma membrane. AP-1 is localized predominantly to the TGN, whereas AP-2 is primarily at the plasma membrane. Both the AP-1 and AP-2 adaptor complexes also associate with the coat protein clathrin. Additional complexity exists in that the adaptor complex AP-2 has alternatively spliced brain isoforms of the subunits β2 and αA, yet their specific functions remain unknown (Ball et al., 1995; Hirst and Robinson, 1998). The other adaptor complexes, AP-3 and AP-4, have been implicated in traffic from the TGN and/or endosomal compartments. Our work focuses on the AP-3 adaptor complex. This complex, which consists of the subunits δ, β3A, μ3A, and σ3, is expressed ubiquitously. Yet similarly to AP-2, there are two neuronally expressed subunits of the AP-3 complex that are referred to as β3B [β-NAP (Newman et al., 1995)] and μ3B. Until now, no brain-specific role for neuronal isoforms of the adaptor complexes has been identified. We have chosen to study the adaptor complex AP-3, with its two neuronally expressed subunits, to ask whether it performs a brain-specific function.

Most work done on the AP-3 complex until now has focused on the ubiquitously expressed form. This complex appears to be localized to the TGN and/or endosomal compartments and participates in trafficking to the vacuole/lysosome in yeast (Cowles et al., 1997; Stepp et al., 1997), flies (Ooi et al., 1997; Mullins et al., 1999; Kretzschmar et al., 2000), and mammals (Le Borgne et al., 1998; Yang et al., 2000). Several mouse mutants in AP-3 have been characterized previously. Two AP-3 mutant mice, the *pearl*

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mouse (β3A mutant; Feng et al., 1999, 2000; Richards-Smith et al., 1999) and the *mocha* mouse (δ mutant; Kantheti et al., 1998) are members of the platelet storage pool deficiency (SPD) class of mutants (Swank et al., 2000). The defects observed in melanosomes, platelet dense granules, and lysosomal traffic in the mutant mice have been linked to defects in ubiquitous AP-3 (Kantheti et al., 1998; Zhen et al., 1999). Although the *pearl* and *mocha* mice have some characteristics in common, such as coat and eye color dilution and bleeding disorders, the *mocha* mouse has neurological defects that the *pearl* mouse does not share. This suggests that neuronal AP-3 functions separately from ubiquitous AP-3. The *mocha* mouse, the δ mutation of which leads to a virtual null of all AP-3 expression in all tissues including brain, has balance problems and hearing problems leading to deafness, is hyperactive, undergoes seizures, and has abnormal theta rhythms (Kantheti et al., 1998; Miller et al., 1999; Vogt et al., 2000). In addition, a knock-out of one of the neuronal AP-3 subunits, μ3B, shares some of the neurological defects seen in the *mocha* mouse (F. Nakatsu and H. Ohno, unpublished data). These data suggest that the absence of neuronal AP-3 alone, and not ubiquitous AP-3, causes such deficiencies.

Other work has implicated the AP-3 complex as well as the ADP ribosylating factor (ARF; Faundez et al., 1997) in the biogenesis of a class of synaptic vesicles, often called synaptic-like microvesicles (SLMVs), from endosomes (Faundez et al., 1998). *In vivo* ARF and possibly AP-3 have been linked to the formation of the class of synaptic vesicles that can release neurotransmitter along developing axons (Zakharenko et al., 1999). These data, in addition to the result that liver and yeast cytosol could not replace brain cytosol in the reconstitution of vesicle budding from endosomes (Faundez et al., 1998), suggested that synaptic vesicle budding from this compartment may be a function exclusive for neuronal AP-3. The loss of this pathway could lead to the neurological defects observed in the AP-3 mutant mice. Consequently, we have taken advantage of our *in vitro* assays to determine the function of neuronal AP-3. To test our hypothesis, we needed a way to remove the function of neuronal AP-3. Therefore, we made an antibody to β3B, one of the neuronal AP-3 subunits, which we used to immunodeplete the neuronal complex from cytosol. This cytosol, which now lacked the neuronal AP-3 complex, as well as cytosol from a recently constructed mouse that lacks expression of the μ3B subunit of AP-3 then could be tested in our biochemical assays. We also used our antibody as a tool to examine the localization of the complex in differentiated PC12 cells as well as in wild-type brain tissue. Our results reveal that the biogenesis of SLMVs requires neuronal AP-3. The pattern of neuronal AP-3 expression in the brain also provides hints to the neurological defects observed in its absence. This is the first characterization of neuronally expressed isoforms of adaptor protein complexes, and our work has suggested a new function within neurons.

## MATERIALS AND METHODS

*Reagents.* [$^{125}$I]Na, ECL reagents, and protein G-Sepharose were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). ATP, creatine phosphate, and creatine kinase were obtained from Boehringer Mannheim (Indianapolis, IN). Geneticin and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Life Technologies (Gaithersburg, MD). Superfrost/Plus slides and Lab-Tek chamber slides were received from Fisher Scientific (Pittsburgh, PA). The Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA). Rat and mouse brains were obtained from Pel-Freez Biologicals (Rogers, AR). Female Sprague Dawley rats were obtained from Bantin and Kingman (Fremont, CA). Cell culture media and reagents were purchased from the University of California, San Francisco (UCSF) Cell Culture Facility. Collagen was purchased from Collaborative Biomedical Products (Bedford, MA). GTPγS, glutathione agarose, diaminobenzidine (DAB) tablets, $H_2O_2$, and other reagent grade chemicals were obtained from Sigma (St. Louis, MO).

*Cell culture.* Wild-type and stably transfected N49A vesicle-associated membrane protein–T-antigen (VAMP–TAg) PC12 cells were grown in DME H-21 culture media supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Media for the stably transfected cells also contained 0.25 mg/ml Geneticin. Cells were grown in 10% $CO_2$ at 37°C. N49A VAMP–TAg PC12 cells were treated 12–18 hr before experiments with 6 mM sodium butyrate to induce VAMP–TAg expression. Differentiated PC12 cells were grown on Lab-Tek (Naperville, IL) chambers coated with collagen (75 μg/ml) and poly-L-lysine (50 μg/ml). They were grown in low serum medium (DME H-21 containing 1% horse serum, 0.5% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml Geneticin for N49A cells) supplemented with nerve growth factor (50 ng/ml). Cells were differentiated on average between 8 and 11 d.

*Production of glutathione S-transferase fusion proteins.* To prepare a glutathione *S*-transferase (GST) fusion protein containing a segment of the β3B hinge domain, we annealed complementary oligonucleotides containing the sequence from the hinge domain with overhanging restriction sites and ligated them into the pGEX-2T vector (Amersham Pharmacia Biotech). The inserts were cloned in-frame into the *Bam*HI–*Eco*RI cloning sites of the vector. The DNA sequence was confirmed from sequencing by the UCSF Biomolecular Resource Center sequencing facility. The fusion protein was expressed in *Escherichia coli* cells and then purified by using glutathione agarose beads according to the manufacturer's instructions.

*Antibodies.* Polyclonal antibodies to β3B were raised in rabbits by immunization with the GST–β3B hinge (Alpha Diagnostics, San Antonio, TX). Polyclonal pan-μ3 and pan-σ3 antibodies were prepared similarly but against GST fusion proteins composed of residues 393–404 of rat p47a (μ3A) and residues 16–180 of σ3B, respectively. Monoclonal antibodies to synaptophysin (SY38) were purchased from Boehringer Mannheim. The monoclonal antibody to the clathrin light chain (neuronal variant) was purchased from Synaptic Systems (Göttingen, Germany). Monoclonals to δ, μ3 (p47A), and σ3 were purchased from Transduction Laboratories (Lexington, KY). Biotinylated goat anti-rabbit IgG (H+L) was purchased from Vector Laboratories. KT3 monoclonal antibody against the TAg epitope tag was prepared as described. The polyclonal synaptophysin antibody was from Zymed (San Francisco, CA). The monoclonal synaptotagmin antibody was purified from hybridoma cell lines obtained from Dr. Reinhard Jahn (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany). Affinity-purified donkey anti-rabbit IgG (H+L) horseradish peroxidase (HRP) and affinity-purified donkey anti-mouse IgG (H+L) HRP were purchased from Jackson Laboratories (Bar Harbor, ME). The secondary antibodies, Texas Red-conjugated goat anti-mouse IgG, and fluorescein-conjugated goat anti-mouse used for immunofluorescence were purchased from Cappel (West Chester, PA).

*Cytosol preparations, immunoprecipitations, and immunodepletions.* Rat and mouse brain cytosol and rat liver cytosol were prepared as described. Immunoprecipitations and immunodepletions were performed with anti-β3B or anti-σ3 antibodies bound to protein G-Sepharose beads as described previously by Faundez et al. (1997).

*Cell-free synaptic vesicle biogenesis assay.* PC12 N49A cells were labeled at 15°C with iodinated anti-TAg antibodies as described previously (Desnos et al., 1995). Next the cells were washed with uptake buffer and additionally were washed by pelleting in uptake buffer and then in bud buffer. Cells were homogenized, and the homogenate was spun at 1000 × *g* for 5 min. The S1 membranes were used for the budding reaction (ratio of 1.0 mg of membrane to 1.5 mg/ml final concentration of brain cytosol). They were incubated with an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 5 μg/ml creatine kinase) and either mock-depleted cytosol or immunodepleted cytosols at 37°C for 30 min. Reactions were stopped on ice. They were spun at 27,000 × *g* for 35 min. The S2 was loaded onto 5 ml velocity gradients of 5–25% glycerol in bud buffer. They were spun at 218,000 × *g* for 1.5 hr. Then 17 fractions were collected from the bottoms of the tubes and counted in the gamma counter.

*Synaptic vesicle coating assay.* Cell-free synaptic-like microvesicle coating assays were performed in 250 μl total volume in intracellular buffer,

using N49A VAMP–TAg PC12 vesicles as described previously by Faundez et al. (1998) and Faundez and Kelly (2000).

*Immunofluorescence.* Differentiated PC12 cells were washed three times in PBS and fixed in 4% paraformaldehyde for 20 min. Then the slides were washed in 25 mM glycine/PBS and blocked for 1 hr in 2% BSA, 1% fish skin gelatin, and 0.02% saponin in PBS (block solution). Next the slides were incubated in their respective primary antibodies for 90 min at room temperature and subsequently were washed three times in block solution, after which they were incubated in secondary antibody for 1 hr at room temperature. Last, they were washed three times in block solution and then two times in PBS.

*Immunohistochemistry.* Adult rat brain sections were generously provided by Dr. Matt Troyer (University of California, San Francisco). The perfused tissue (4% paraformaldehyde) was cut into 40-$\mu$m-thick sections. Sections were washed in PBS (calcium- and magnesium-free; cmf) and then incubated in 0.3% $H_2O_2$/cmf PBS for 15 min at room temperature. Then the tissue was washed in cmf PBS and blocked in buffer B (0.2% Triton X-100, 10% normal goat serum, cmf PBS) for 1 hr at room temperature. Sections were incubated overnight at 4°C in primary antibody diluted in buffer C (0.2% Triton X-100, 1% normal goat serum, cmf PBS). Sections were washed thoroughly in buffer C for 60 min between five and seven times and then once for 60 min in buffer B. Sections were incubated overnight at 4°C in secondary antibody diluted in buffer C. The next day the sections again were washed six times for 60 min in buffer C and then washed twice in cmf PBS. Sections were incubated in the ABC Vectastain mix (according to the manufacturer's instructions) for 30 min at room temperature. Fresh DAB was prepared, and the sections were incubated in the mixture. The reaction was stopped by washing the tissues in cmf PBS. Sections were transferred to slides, air dried overnight, and dehydrated the next day in EtOH, followed by xylene.

*Transgenic mouse.* The $\mu$3B knock-out mouse used here expresses no detectable $\mu$3B mRNA (for the homozygote mutant) in brain or spinal cord. A complete description of the construction of this mouse and its characterization is in progress (F. Nakatsu and H. Ohno, unpublished data).

## RESULTS

### AP-3 is required for SLMV formation

It has been demonstrated previously that AP-3 has a role in the budding of SLMVs from endosomes (Faundez et al., 1998). To establish a requirement for AP-3 in this pathway, we have taken advantage of the naturally occurring SPD mutant *mocha* mouse, which lacks all AP-3 expression, in the *in vitro* reconstitution of SLMV biogenesis (Desnos et al., 1995). For this *in vitro* reconstitution a PC12 cell line has been used that is transfected with a construct (N49A VAMP–TAg) encoding an epitope-tagged form of VAMP/synaptobrevin mutated in its sorting domain to enhance its targeting to SLMVs (Clift-O'Grady et al., 1998). So that the endosomes can be labeled, the cells are incubated with an antibody [$^{125}$I]-KT3, which recognizes the TAg, at 15°C before homogenization. A membrane fraction enriched in endosomes is incubated in the presence of an ATP-regenerating system and brain cytosol. This fraction generates SLMVs that are recognized as a peak of radioactivity that co-migrates with synaptic vesicle markers after velocity sedimentation. SLMVs also are produced when the brain cytosol is replaced with purified AP-3 and recombinant ARF1 (Faundez et al., 1998). When we used brain cytosol from *mocha* mice in our budding assay, SLMV biogenesis from endosomes was reduced to 50% of wild type (Fig. 1*A,B*). Adding back brain-purified AP-3 to *mocha* cytosol rescued the defect in budding (Fig. 1*A,B*). We compared the activity of *mocha* cytosol with the activity in cytosol that was immunodepleted of all AP-3 by using an antibody to $\sigma$3. Immunodepleting $\sigma$3 should remove all AP-3 activity, neuronal and ubiquitous. As with *mocha*, budding activity of this cytosol also was reduced to 50% of wild type (Fig. 1*C,D*). These results verify that SLMV biogenesis from endosomes is dependent on AP-3 but show that other soluble factors facilitate vesicle biogenesis from the endosomal compartment. Contribution from such factors could contribute to the 50% vesicle biogenesis that remains in the absence of AP-3. Two of these, ARF1 and phosphorylation by a casein kinase 1$\alpha$-like activity, have been described previously (Faundez et al., 1997; Faundez and Kelly, 2000), but others may exist. Our results confirm a role for AP-3 in synaptic vesicle biogenesis. However, because the $\sigma$3 depletion as well as the *mocha* mutation removes all AP-3 complexes, neuronal and ubiquitous, the form or forms of the AP-3 complex that functions in SLMV biogenesis are unclear.

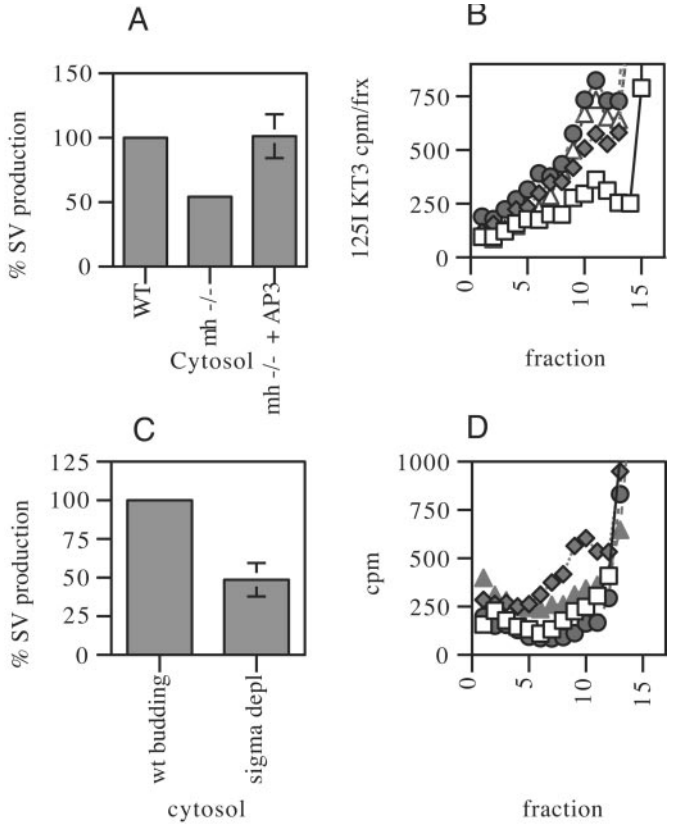


*Figure 1.* The *in vitro* budding of synaptic vesicles requires AP-3. PC12 N49A cells were labeled with [$^{125}$I]-KT3 at 15°C. Endosomal membranes were incubated with *mocha* cytosol and an ATP-regenerating system. Budding reactions were performed at 37°C for 30 min. *A*, *Mocha* mice brain cytosol shows a 50% reduction in the production of synaptic vesicles from the donor endosome compartment compared with wild-type brain cytosol. *Mocha* cytosol supplemented with brain-purified AP-3 rescued the defect in budding, returning vesicle production to wild-type levels. The data shown represent an average ± SEM ($n$ = 3). *B*, A representative example of the budding assay in which the fractions from the gradient, shown along the $x$-axis, have been collected from the bottom and counted. The no-cytosol control (□), *mocha* cytosol (♦), wild-type brain cytosol (●), and *mocha* brain cytosol plus brain-purified AP-3 (▲) were tested in this assay. The peak is at fractions 10 and 11 and represents the newly budded pool of synaptic vesicles; the label on the *right* is free antibody. *C*, The *in vitro* budding assays were performed with brain cytosol that was depleted for the $\sigma$3 subunit. The results show a 50% reduction in synaptic vesicle biogenesis compared with wild-type budding production ($n$ = 3). *D*, A representative assay in which cytosol is depleted of $\sigma$3. The fractions collected from the gradient are shown along the $x$-axis. Here, a no-cytosol control (□), wild-type brain cytosol (♦), 4° rat brain cytosol (●), and brain cytosol that was immunodepleted by using the $\sigma$3 antibody (▲) were tested. When AP-3 was removed, the height of the peak was reduced, indicating reduced vesicle production.

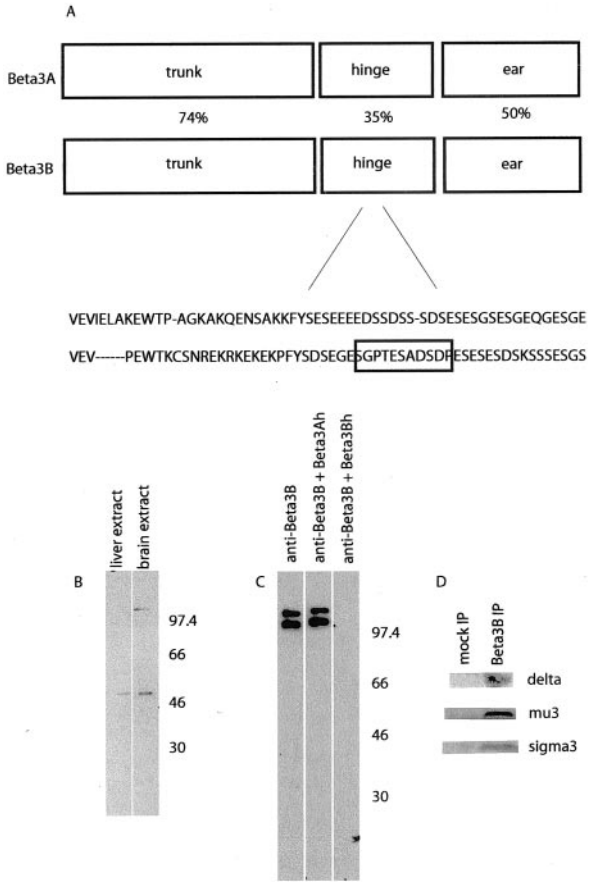


*Figure 2.* Production of β3B-specific antibody. *A*, The AP-3 subunits β3A and β3B are highly homologous. Within the hinge domain, the least homologous region between the ubiquitously and neuronally expressed β3 subunits, we chose a stretch of amino acids within β3B as our antigen. The GST fusion protein was used as the immunogen. *B*, Liver and brain extracts were run on SDS-PAGE gels and analyzed via immunoblot by antisera. This antiserum recognized a band of the approximate molecular weight of the β3B subunit, present only in brain extract. The antibody also nonspecifically recognized a lower-molecular-weight band present in both liver and brain extracts. *C*, Purified brain AP-3 was run on SDS-PAGE gels and probed with this antiserum. It recognized a protein of the correct molecular weight. Antisera also were preincubated with either purified β3A hinge (*β3Ah*) or with purified β3B hinge (*β3Bh*) and then used for Western blots. Anti-β3B recognized the neuronal subunit as well as antibody preincubated with the β3A hinge. Antibody preadsorbed with β3B hinge no longer could bind the neuronal subunits on blots, showing its specificity. The β3B subunit often appears as a doublet in purified AP-3, perhaps because of limited proteolysis during purification. *D*, The β3B antibody was also used to immunoprecipitate the other subunits of the AP-3 complex. Mock immunoprecipitations did not bring down any of the AP-3 subunits.

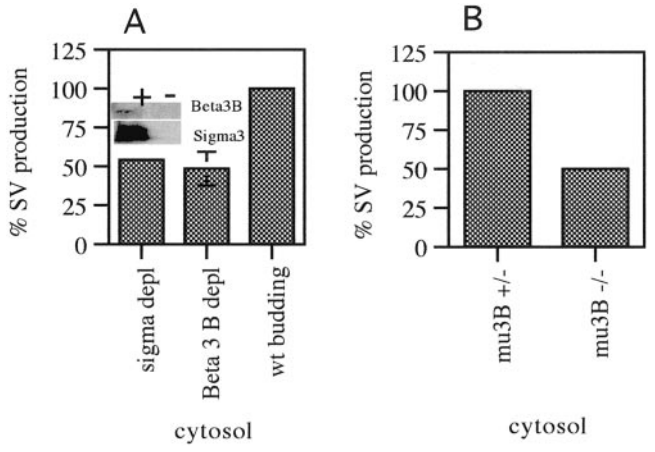


*Figure 3.* *In vitro* budding of synaptic vesicles depends on the neuronal form of AP-3. *A*, *In vitro* budding assays were performed as described. Budding assays were performed by using either mock-depleted cytosol (wild-type budding) or cytosol that was immunodepleted for β3B or for σ3. The *inset* shows immunoblots of either mock (+) or (−) immunodepleted cytosols. The *top* blot was probed for β3B in either mock or immunodepleted cytosol, and the *bottom* blot was probed for the σ3 subunits in either mock or immunodepleted cytosol. Cytosol that was immunodepleted showed essentially complete depletion. The depleted cytosols both showed a similar 50% reduction in the biogenesis of SLMVs compared with wild-type levels of synaptic vesicle production. *B*, *In vitro* budding assays also were performed by using cytosol from mice heterozygous for μ3B and for mice that lacked all expression of the μ3B subunit. Although the heterozygote cytosol showed robust SLMV biogenesis, the knock-out mouse cytosol showed a 50% reduction in budding compared with cytosol from its heterozygous littermate.

## Production of β3B-specific antibody

We generated a tool to immunodeplete neuronal AP-3 as well as to determine its localization by making an antibody to the β3B subunit. We compared the protein sequence of the ubiquitous β3A subunit versus the neuronal-specific β3B subunit and focused on regions that are not highly similar or identical. Although the two proteins share a high degree of homology (74%) within their core/trunk regions, the hinge and ear of the proteins are less homologous, 35 and 50%, respectively (Dell'Angelica et al., 1997a,b). We therefore made a rabbit polyclonal antibody to a GST fusion protein containing a small stretch of the β3B hinge domain not present in the hinge of β3A (Fig. 2*A*). By Western blot, anti-β3B recognized a band of ~140 kDa present in brain but not in liver (Fig. 2*B*) as well as in brain-purified AP-3 (Fig. 2*C*). When the antibody was preincubated with the GST–β3A hinge region, there was no effect on the binding of the anti-β3B antibody to brain AP-3. This suggests that our antibody does not recognize β3A, the subunit to which β3B is most similar. However, when we preincubated the antibody with GST–β3B hinge, our antibody could no longer recognize brain AP-3 by Western blot (Fig. 2*C*) because it had been competed away by GST–β3B. The low-molecular-weight band that occasionally was detected in Western blots was a result of nonspecific binding (Fig. 2*B*). GST–β3B did not inhibit binding to the nonspecific low-molecular-weight band. These data establish that our antibody is specific for only the β3B subunit. In addition, we could use our antibody to the β3B subunit to immunoprecipitate the other subunits of the AP-3 complex (Fig. 2*D*).

## Formation of synaptic vesicles from an endosome is dependent on neuronal AP-3

To identify the specific role the neuronal complex itself plays in SLMV biogenesis from early endosomes, we used our β3B antibody to immunodeplete rat brain cytosol of the neuronal AP-3 complex (Fig. 3*A*, *inset*). This cytosol that lacked only neuronal AP-3 was used then in our *in vitro* budding assays and was compared with cytosol that was immunodepleted with the σ3 antibody, which removes all AP-3 complexes, in our assays. We found that cytosol that was depleted only of neuronal AP-3 complexes showed the same 50% reduction in SLMV biogenesis as cytosol that was depleted of all AP-3 (Fig. 3*A*). We also tested brain cytosol from μ3B knock-out mice compared with the heterozygote littermates. The cytosol from the mice that lacked μ3B also showed a 50% reduction in SLMV biogenesis (Fig. 3*B*).

Together, these data strongly suggest that synaptic vesicle budding from endosomes is attributable solely to the neuronal form of the AP-3 complex, because the removal of all AP-3 complexes led to the same reduction of SLMV production as specific removal of the neuronal form. To examine the specificity for the neuronal complex further, we performed the same budding assays with the use of brain cytosol from the *pearl* mice (mutant for ubiquitous AP-3 only), which showed wild-type vesicle production from endosomes (data not shown). Hence, neuronal AP-3 is required for this budding event, with little or no contribution coming from the ubiquitous complex that is present in the cytosol.

## Neuronal AP-3 is not the predominant form of AP-3 in the brain

The results in Figure 3 could be explained if only neuronal AP-3 could execute budding or if neuronal AP-3 performed the same function as ubiquitous AP-3 but was much more abundant in the brain than the ubiquitous form. Neuronal-specific isoforms could be performing the same role as their ubiquitous counterparts, but they would need to be in great abundance in brain to enhance the function they both perform, in this case to vesiculate endosomes into SLMVs. To examine whether the requirement for neuronal AP-3 reflects its specificity or its abundance, we asked whether neuronal AP-3 was the predominant species of AP-3 in the brain. If it was, depleting it would inhibit SLMV formation from endosomes *in vitro* even if the ubiquitous form were active in SLMV biogenesis. To determine the relative abundance of neuronal AP-3 in brain, we measured ubiquitous AP-3 levels in wild-type brain cytosol compared with brain cytosol lacking the neuronal form. The levels of δ and σ3, components of both ubiquitous and neuronal AP-3, were compared in cytosol either lacking neuronal AP-3 or having both neuronal and ubiquitous forms. In both the μ3B knock-out and the β3B depletions in which neuronal AP-3 is removed, the levels of δ (Fig. 4*A*) and σ3 (Fig. 4*B*) essentially were unchanged. This indicates that most AP-3 in the brain is the ubiquitous form. A pan-μ3 antibody that recognizes both ubiquitous μ3A and neuronal μ3B detected essentially the same levels of μ3 in brain cytosol from heterozygotes as well as homozygotes of μ3B knock-out mice (Fig. 4*A*). If there is a reduction of μ3 in the homozygote, it is only a slight reduction. This also suggests that most of the AP-3 in brain is in the ubiquitous complex. Our data are in agreement with published work that examined the levels of AP-3 in brains of a β3A knock-out mouse (Yang et al., 2000). In the β3A knock-out, there was a great reduction of AP-3 subunit levels in the brain, which also supports the concept that most AP-3 in the brain is in the ubiquitous complex. Therefore, neuronal AP-3 is the minor form in the brain and has a function that is not shared by ubiquitous AP-3. Although it is unusual for a neuronal-specific isoform to be a minor component in the brain, perhaps in this case ubiquitous AP-3 has to be present in abundance to take care of the extensive amounts of endosomal and lysosomal traffic in brain.

## Coat recruitment to SLMVs is independent of neuronal AP-3 under GTPγS

To determine whether or not neuronal AP-3 is necessary for coat recruitment onto membranes, we took advantage of an *in vitro* coating assay. In this assay PC12 synaptic-like microvesicles are recovered at a higher buoyant density when incubated with brain cytosol and an ATP-regenerating system (Faundez et al., 1998; Salem et al., 1998). Briefly, in the assay the vesicles were purified by velocity sedimentation from homogenates of cells (N49A

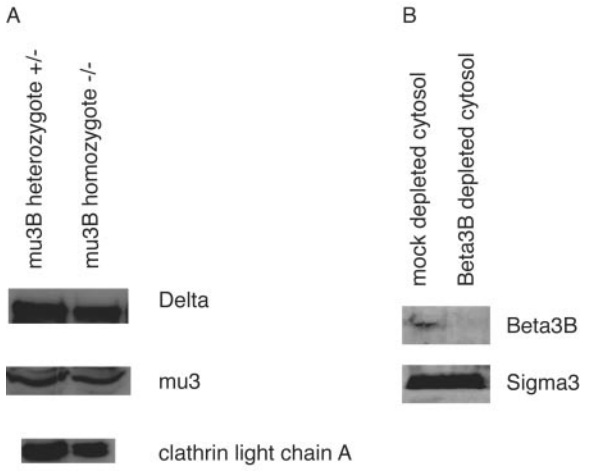


*Figure 4.* Neuronal AP-3 is not the major form of AP-3 in brain. *A*, Brain cytosol from heterozygotes of μ3B (+/−) and knock-outs for the neuronal μ3B subunit were run on SDS-PAGE gels. To determine whether there was significantly less AP-3 remaining in brains that lack the neuronal form, we probed for the δ subunit, present in all forms of AP-3. The levels of δ appear to be unchanged in the knock-out compared with the heterozygote, suggesting that the majority of brain AP-3 is in the ubiquitous form. We also probed with a pan-μ3 antibody that recognizes both μ3A and μ3B. The levels seen in the heterozygote of both ubiquitous and neuronal forms appeared to be no more than those in the knock-out, which contained only the ubiquitous form. Protein levels were standardized to levels of a variant of clathrin light chain A. *B*, Levels of the σ3 subunit, the other ubiquitously expressed subunit in all AP-3 complexes, also were compared in mock-depleted cytosol versus cytosol that was immunodepleted for β3B. Equal amounts of protein were run in each lane. Although β3B was removed in the depleted cytosol, levels of σ3 were unchanged from the amount in mock-depleted cytosol.

VAMP–TAg PC12) labeled with [$^{125}$I]-KT3 at 15°C. Then they were incubated at 37°C with an ATP-regenerating system, GTPγS, and rat brain cytosol. The recruitment of adaptor complexes onto vesicles was detected as an increase in the rate of sedimentation in sucrose gradients. N49A PC12 vesicles that have not recruited coat are recovered at 22% sucrose, whereas vesicles that have recruited coat from the cytosol sediment to 30–32% sucrose. We also titrated the levels of cytosol to ensure we were not saturating the system (data not shown).

This assay can be used to determine the role of AP-3 in coating synaptic vesicles. *Mocha* brain cytosol, which lacks all AP-3, cannot provide coat to these vesicles (Fig. 5*D*), indicated by their failure to change in density. This demonstrates that AP-3 is necessary to provide the coat. To determine whether or not the only coat that could be recruited to vesicles was the neuronal form of AP-3, we tested whether cytosol that had been depleted of β3B could coat purified vesicles. We showed that vesicles incubated with such cytosol still sedimented at 30–32% sucrose, consistent with complete coating with the remaining ubiquitous AP-3 (Fig. 5*A,B*). We also tested the μ3B knock-out mouse cytosol in the assay. Cytosols from both the heterozygote and the knock-out mice could provide coat to the vesicles (Fig. 5*C*).

Ubiquitous AP-3 can bind purified vesicles only under conditions in which neuronal AP-3 is removed from brain cytosol. If we use normal brain cytosol in which both forms of AP-3 are present, ubiquitous AP-3 does not bind (data not shown), demonstrating that neuronal AP-3 competes effectively with the ubiquitous form for binding. Although we can get ubiquitous AP-3 to bind to purified synaptic vesicles, the ubiquitous complex cannot function

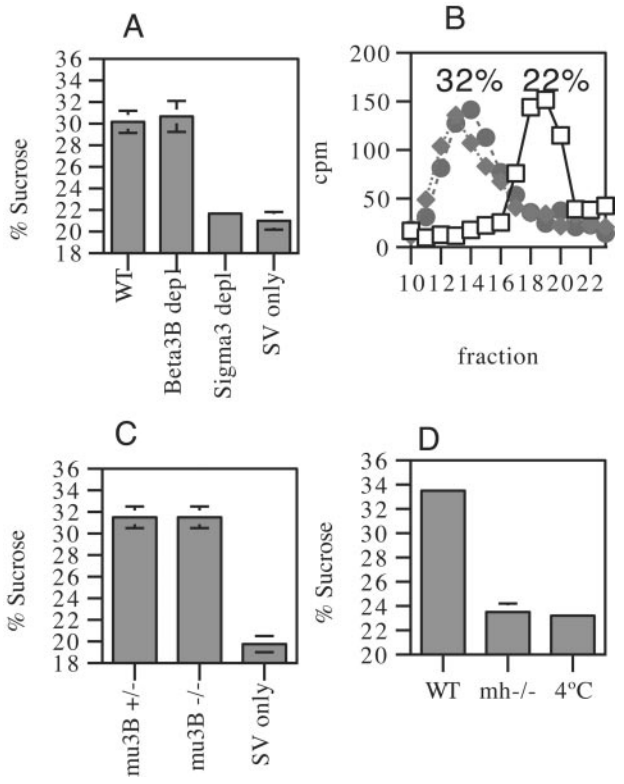


*Figure 5.* AP-3 is necessary to coat synaptic vesicles. *A*, Purified synaptic vesicles that are run over sucrose gradients sediment at ~22% sucrose. The same vesicles that are incubated with wild-type brain cytosol, ATP-regenerating system, and GTPγS recruit coat and sediment at 30–32% sucrose. Cytosol that has been depleted for σ3-containing AP-3 complexes could not coat synaptic vesicles fully. Cytosol that had been depleted for β3B-containing AP-3 complexes, however, could provide coat to vesicles, which sedimented at 30–32% sucrose. *B*, A representative example of a coating assay analyzed on sucrose gradients showing the magnitude of the change in sedimentation properties. The fractions collected from the bottom of the gradient are shown along the *x*-axis. Conditions tested in the assay were synaptic vesicles without cytosol (□), mock-depleted rat brain cytosol (♦), and anti-β3B immunodepleted brain cytosol (●). Synaptic vesicles incubated without a source of coat, as in brain cytosol, did not undergo a density shift. Vesicles incubated with either mock-depleted rat brain cytosol or β3B-depleted rat brain cytosol did undergo a density shift. *C*, Synaptic vesicles could be coated fully after incubation in GTPγS with either brain cytosol that lacked μ3B or cytosol that did contain μ3B. *D*, Without any AP-3 in brain, as in the *mocha* mice ($mh^{-/-}$), vesicles could not be coated. *In vitro* coating assays kept at 4°C, instead of incubation at 37°C, also could not recruit coat.

to bud a synaptic vesicle from an endosome. It thus appears that binding assays can conceal specificity that is revealed by the more physiological budding assays. Both the budding and the coating assays require the activity of a casein kinase (Faundez and Kelly, 2000). Yet the specificity of neuronal AP-3 does not lie in its ability to bind casein kinase, because immunoprecipitation of ubiquitous AP-3 from human embryonic kidney cells contains this kinase activity (data not shown).

### Localization of β3B

To determine where neuronal AP-3 functions, we examined the subcellular localization of neuronal-specific β3B-containing AP-3 complexes within differentiated PC12s. Our β3B antibody shows staining in differentiated PC12 cells and neuronal cells, although we saw no staining in non-neuronal cells (data not shown). Thus our antibody appears to be specific for neuronal, or neuroendocrine, cells. The staining for β3B was blocked when our antibody was preadsorbed with GST–β3B hinge, but not with GST–β3A hinge. We saw a similar staining for nAP-3 along varicosities in primary cultures of cortical neurons (data not shown). Neuronal AP-3 is found predominantly in varicosities of the processes (Fig. 6*A,F*) and is primarily absent from tips (Fig. 6*A,E*), whereas synaptotagmin, a good marker for the AP-2/clathrin pathway (Fig. 6*B,D*), was found predominantly at tips. In addition, active endocytosis of synaptotagmin at the tip of the process was enriched over uptake at the varicosities in differentiated PC12 cells (N. Jarousse and R. Kelly, unpublished observations). These data are consistent with previous work that showed that the AP-3 pathway of synaptic vesicle production is separate from the AP-2/clathrin pathway of synaptic vesicle biogenesis from the plasma membrane (Shi et al., 1998).

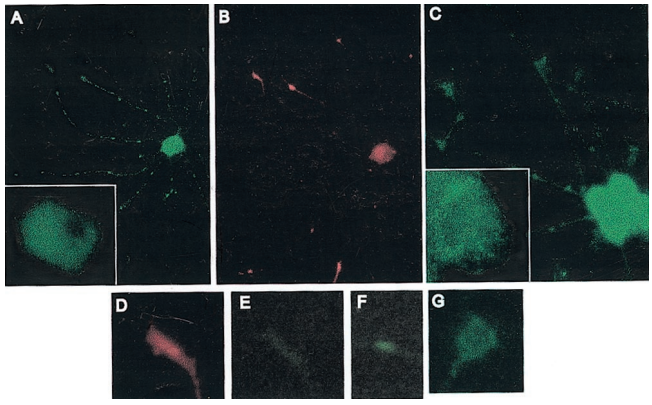


*Figure 6.* Neuronal AP-3 is localized to varicosities of neuronal-like processes. *A*, Differentiated PC12 cells were stained with the β3B antibody. Although there was no specific staining in the cell body (*inset*), we observed staining in the varicosities along the processes, yet it was absent at the tips. *B*, Differentiated cells were double stained for synaptotagmin; the staining was in contrast to that seen with the β3B antibody. Synaptotagmin staining was most intense at the tips of the processes. *C*, Differentiated PC12 cells also were stained for the δ subunit of AP-3, a subunit present in all AP-3 complexes. δ Staining is seen in varicosities, as with β3B, but in addition there is punctate staining in the cell body. *D*, A representative tip of a differentiated PC12 cell stained with synaptotagmin antibody. *E*, The same tip, which is enriched for synaptotagmin, lacks expression of β3B. *F*, A representative varicosity of a differentiated PC12 cell process enriched in β3B expression. *G*, A representative varicosity of a differentiated PC12 cell enriched in δ expression.

Our data are also supported by previous work that examined neurotransmitter release along processes of developing axons. Although release at the terminals was not Brefeldin A-sensitive (BFA-sensitive), suggestive of an AP-2 mechanism, release along the process was inhibited, indicative of an AP-3-like mechanism (Zakharenko et al., 1999).

We also examined the localization of both forms of AP-3 by using an antibody to the δ subunit. Although neuronal AP-3 appears to be localized to varicosities and shows no specific organelle staining in the cell body (Fig. 6*A*, *inset*), the δ subunit also exhibits punctate staining in the cell body (Fig. 6*C*, *inset*) in addition to its localization at varicosities (Fig. 6*G*). This suggests that ubiquitous AP-3 is enriched in organelles in cell bodies, whereas the neuronal complex is targeted preferentially to varicosities. Neuronal AP-3 appears not only to have a separate function from ubiquitous AP-3 but also to be localized separately and only to neuronal processes.

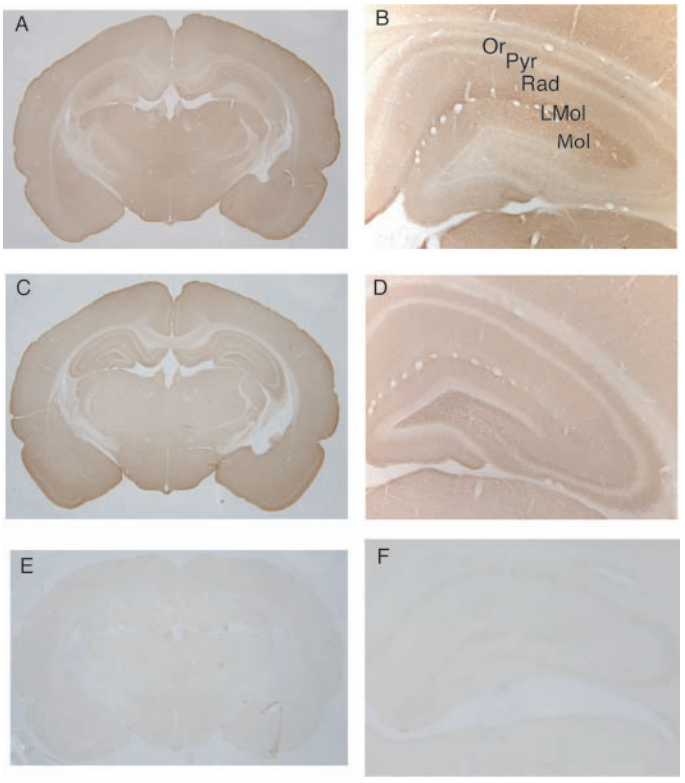


*Figure 7.* Neuronal AP-3 is expressed throughout axons in the brain. *A*, Adult rat brain sections were stained for β3B immunoreactivity. Neuronal AP-3 is seen in axons in most regions of the brain. *B*, A close-up view of β3B staining in the hippocampus shows intense staining in the lacunosum moleculare (*LMol*) as well as in the stratum oriens (*Or*), stratum radiatum (*Rad*), and the molecular layer of the dentate gyrus (*Mol*). *C*, Adjacent adult rat brain sections were stained for synaptophysin immunoreactivity. Synaptophysin also is expressed in most axonal pathways of the brain. *D*, A close-up view of synaptophysin staining in the hippocampus shows a different pattern of expression than that seen for neuronal AP-3. Synaptophysin has a more even level of expression throughout the hippocampus, although it appears to label the mossy fiber pathway more intensely than neuronal AP-3. *E*, Adult rat brain sections were stained with the β3B antiserum that had been preadsorbed with the GST fusion protein against which the antibody was made. No immunoreactivity was observed. *F*, A close-up view of the hippocampus also shows that no staining was observed in the negative control.

### Neuronal AP-3 distribution

We wanted next to examine the distribution of neuronal AP-3 in intact brain tissue compared with a cell culture system. Mutants that do not express any AP-3 are viable, yet they do display neurological defects. One hypothesis was that neuronal AP-3 expression was limited to one particular region/pathway of the brain that is not essential for viability. To address where neuronal AP-3 is expressed, we used our β3B antibody to stain 40 μm sections of adult rat brains. Although β3B was not expressed in all regions of the brain, it was expressed widely and appeared predominantly in processes rather than in cell bodies (Fig. 7*A,B*; data not shown). Its staining was in general similar to that of synaptophysin, a synaptic vesicle marker (Fig. 7*C,D*), although differences were noted. If we compare staining in the hippocampus, for example, β3B is enriched in the molecular layer of the dentate gyrus and lacunosum moleculare layer along with the stratum radiatum and stratum oriens (Fig. 7*B*), whereas synaptophysin staining is more even throughout the hippocampus. Staining for β3B could be blocked by preadsorbing the antibody with either the GST fusion protein that is used to generate the antibody (Fig. 7*E,F*) or with a GST fusion protein to the β3B hinge (data not shown). In addition, when we preadsorbed the antibody with GST alone, we saw no change in the staining pattern of our antibody (data not shown). Our results overlap quite well with the staining pattern seen in the brain with the use of antibodies against β-NAP, identified from a human patient with autoimmune neurological degeneration (Newman et al., 1995). This suggests that, whereas AP-3 knock-outs are viable, nAP-3 plays a global, although nonessential, role in the brain and is enriched in certain pathways.

## DISCUSSION

Although multiple isoforms of adaptor complex subunits have been identified (Takatsu et al., 1998; Folsch et al., 1999; Ohno et al., 1999; Meyer et al., 2000), ours is the first characterization of an adaptor complex containing neuronally expressed subunits. We have examined the role of neuronal AP-3 by looking at the steps it can perform *in vitro*, at its subcellular localization, and at its cellular distribution within brain. Our results establish a role for neuronal AP-3 in the biogenesis of one type of synaptic vesicle or synaptic-like microvesicle. This pathway of synaptic vesicle biogenesis is separate and distinct from the AP-2 pathway of synaptic vesicle biogenesis as well as from the pathway in which ubiquitous AP-3 is involved.

The four major types of adaptor complexes, AP-1, AP-2, AP-3, and AP-4, perform distinct targeting functions within a cell and are localized to different cellular compartments (Robinson, 1993; Seaman et al., 1993; Page and Robinson, 1995). AP-2 normally is associated with plasma membranes and AP-1 with the TGN. Ubiquitous AP-3 also has been linked to the TGN. In contrast to the association of AP-3 with the TGN, *in vitro* reconstitution demonstrated that AP-3 could facilitate budding from a particular class of endosomes (Faundez et al., 1998; Lichtenstein et al., 1998). One possible explanation for this apparent discrepancy is that only the neuronal form of AP-3 is specialized for budding from the endosomal intermediate. Although AP-3 is expressed throughout differentiated PC12 cells, the neuronal complex is targeted to varicosities, suggesting that the organelles to which they are localized are different. Our results, therefore, are consistent with the idea that the differences between adaptor complexes target them to different donor organelles.

An unexpected result was the binding of ubiquitous AP-3 to vesicles. In previous work the results obtained by using the synaptic vesicle binding assay have always been in agreement with those obtained by using the vesiculation assay. Both assays share temperature sensitivity (Faundez et al., 1998), require a casein kinase 1α-like activity (Faundez and Kelly, 2000), and are inhibited by tetanus toxin (Salem et al., 1998). Both work well with brain cytosol from *pearl* mice, which is deficient in the ubiquitous form of AP-3, and not at all with cytosol from *mocha*, which lacks both forms of AP-3. It was thus no surprise when ubiquitous AP-3 was not found on SLMVs coated in the presence of brain cytosol (V. Faundez and R. Kelly, unpublished observations). Only when the brain cytosol was depleted of neuronal AP-3 was there an apparent disparity between the vesiculation and coating assays. One explanation might be that studying adaptor binding in the presence of GTPγS conceals a mechanism that normally regulates binding specificity (Seaman et al., 1993). First the AP-3s may bind reversibly to a receptor, and then a second step occurs that is irreversible in the presence of GTPγS. Neuronal AP-3 could bind more tightly than ubiquitous AP-3 to the receptor or partic-

ipate more readily in the second irreversible step. At present little is known about the molecular details of the coating step except that binding to synaptobrevin/VAMP is involved (Salem et al., 1998).

Knowing that neuronal AP-3 is required specifically for vesicle formation from endosomes allows us to connect it to specific processes within neurons. Making synaptic vesicles from endosomes, for example, could be a mechanism for recovering such vesicles that have escaped the conventional recycling path. A variety of experiments support the conclusion that the AP-3-mediated pathway of synaptic vesicle formation is usually a minor one and that the major one uses AP-2 and clathrin to form synaptic vesicles directly from the plasma membrane (Murthy and Stevens, 1998; Shi et al., 1998; Vogt et al., 2000). Supporting evidence for two populations of synaptic vesicles comes primarily from developmental studies. Synaptic vesicle recycling reportedly is blocked by tetanus toxin at synapses, whereas vesicle recycling before synaptogenesis is not (Verderio et al., 1999), suggesting a change in vesicle composition. Quantal release of neurotransmitter from synaptic sites also was distinguished from nonsynaptic release by Popov and colleagues (Zakharenko et al., 1999). Vesicular release along the axons of developing frog motoneurons in culture were sensitive to Brefeldin A, whereas quantal release from the nerve termini was BFA-insensitive. Because the AP-3-mediated production of SLMVs is inhibited by Brefeldin A also, the latter results link nonsynaptic production of synaptic vesicles to neuronal AP-3. Consistent with these observations, the tips of processes lack AP-3 although they are rich in synaptotagmin. One possibility is that synaptic vesicle proteins that escape the normal, nonendosomal route of recapture are internalized into axonal endosomes and are retrieved by the AP-3 route (Fig. 8). In this scheme most synaptic vesicles in PC12 cells are recycled by the AP-3 pathway because the cells have not differentiated sufficiently to have a significant nonendosomal mechanism. In neurons AP-3-mediated retrieval would be into specialized endosomes in the axons around exocytotic sites but not immediately adjacent to them, explaining both our morphology and the results of Popov's lab.

Spillover of synaptic vesicle membranes into a second pathway can be seen readily in *Drosophila* neuromuscular junctions, especially in *shibire* mutants at temperatures that prevent vesicle membrane recapture. Synaptic vesicle proteins diffuse out of the varicosities and along axons (Ramaswami et al., 1994). When preparations are returned to permissive conditions, the membranes use an endosomal-like internalization route that is not seen under more normal conditions (Kuromi and Kidokoro, 1998). If this backup retrieval mechanism is absent when neuronal AP-3 is missing, we might see deficiencies in synaptic transmission when synaptic demands are high.

Another potential function for endosome-derived synaptic vesicles is in the recovery of membrane components of large dense core vesicles (LDCVs) that have just undergone exocytosis (Fig. 8). Membrane retrieval of this type has been detected in PC12 cells transfected with a chimeric P-selectin (Blagoveshchenskaya et al., 1998). A mutant membrane protein that could not be targeted to the SLMVs was degraded rapidly by lysosomes. Thus neuronal AP-3 could recapture protein components of LDCV proteins, which release their contents at regions of the plasma membrane distant from sites of synaptic vesicle exocytosis. A recapture step could sequester selected LDCV proteins from a degradative pathway and allow them to be incorporated into the standard synaptic vesicle recycling mode.

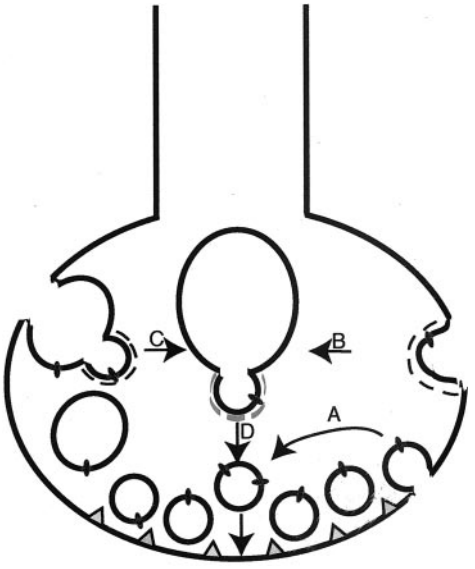


*Figure 8.* Neuronal AP-3-mediated pathway of synaptic vesicle biogenesis from endosomes. Synaptic vesicles that cluster in the active zone (the *triangles* at the plasma membrane) undergo a cycle of exocytosis and recycling. Synaptic vesicle proteins normally recycle via the AP-2/clathrin pathway of endocytosis (*arrow A*) but escape recovery at the plasma membrane and may recycle via the AP-3 pathway. Such synaptic vesicle proteins may be retrieved into specialized axonal endosomes that use neuronal AP-3 to bud synaptic vesicles (*arrow B*). The endosomal pathway of synaptic vesicle production also may function to recycle components of large dense core vesicles (LDCVs). LDCV proteins recycle via an endosomal intermediate, and some proteins may get sorted into synaptic vesicles. Neuronal AP-3 could recognize and bud such cargo into SLMVs from this endosomal intermediate (*arrow C*). Axonal endosomes that contain synaptic vesicle, as well as some LDCV membrane proteins, use neuronal AP-3 to produce synaptic vesicles, which are competent to fuse with the plasma membrane and release their contents (*arrow D*).

The distribution of neuronal AP-3 in the brain shows that, whereas there is some overlap in its expression with synaptophysin, it is not identical. A backup retrieval pathway or LDCV membrane recycling could be used more frequently in some neuronal pathways than others. The distribution of neuronal AP-3 showed some resemblance to that reported for chromogranin A, a marker of dense core granules, particularly in the stratum oriens and the molecular layer of the dentate gyrus (Munoz, 1990). This is interesting not only as a link between two vesicle pathways but also because it has been suggested that this chromogranin expression may offer resistance to epileptic brain damage (Munoz, 1990). The *mocha* mice as well as the $\mu$3B knock-out mice have neurological defects, which include epileptic seizures. Additional work may provide further insight into why separate populations of synaptic vesicles exist and why the absence of one generates neurological defects.

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