Characterization of a neuronal adaptor complex

by

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This thesis is dedicated to my father.

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Regis B. Kelly directed and supervised the research that forms the basis for this thesis dissertation.

Involvement of Co-authors:

Thesis Advisor's Statement

The majority of Jessica Blumstein's thesis work is evidenced in a published manuscript in the Journal of Neuroscience. Jessica has also contributed to other projects in the lab, as well as other experiments regarding her work on neuronal AP-3.

The work presented in Chapter Two has been published as Blumstein J, Faundez V, Nakatsu F, Saito T, Ohno H, Kelly RB (2001) The Neuronal Form of Adaptor Protein-3 is Required for Synaptic Vesicle Formation from Endosomes. J Neurosci 21(20):8034-8042. For this first-author paper, Jessica was responsible for the vast majority of work.

The afore-mentioned manuscript is included as a chapter in this thesis dissertation. Proper permission has been obtained from the journal.

The work described in this thesis meets the standards required for completion of the Ph.D. program.

Regis B. Kelly, Ph.D.

Thesis Advisor

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Characterization of a Neuronal Adaptor Protein Complex

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Abstract

Adaptor protein complexes function to sort and traffic protein cargo from one cellular location to another. The APs, which are heterotetrameric complexes, have been well studied. AP-1 and AP-2 function to sort cargo from the trans-golgi network (TGN) and the plasma membrane respectively. The AP-3 complex has also been identified to sort proteins from the TGN, similarly to the AP-1 complex, albeit, routing proteins in an alternate way. Yet other studies implicated the AP-3 complex in the biogenesis of synaptic-like microvesicles from an endosomal compartment in PC12 neuroendocrine cells.

How can adaptors which are required to bind and sort proteins at specific times and places, perform more than one distinct function? All of the AP complexes, which are composed of four subunits, can apparently be composed

of more than one type of each of the subunits. Alternate isoforms have been identified for most of the subunits to date and more may be found. Some are generated by alternative splicing of a single gene product, while others are coded for by separate genes. AP-3 is a protein complex that has at least two isoforms expressed for three of its four subunits, and expression for two of the subunits is limited to neuronal cells. By characterizing the role of a neuronal AP-3 complex, as compared to the function of the ubiquitously expressed AP-3 complex, one could begin to understand how multiple functions for AP complexes are ascribed, as well as why a need exists for alternate isoforms of AP subunits.

This work has been able to distinguish a unique role for the neuronal AP-3 complex, as compared to the ubiquitous complex. While many neuronal isoforms of proteins involved in membrane traffic have been identified, this is the first characterization of a neuronal adaptor complex. Such work will hopefully provide insight into why neuronal isoforms in general exist, and perhaps their function and expression can provide greater information about different regions of the brain.

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Chapter One: Introduction

All cells contain organelles. Throughout the life of a cell, during almost all cellular events, membrane traffic from one compartment to another is occurring. Such traffic requires carrier vesicles to form off of one membrane and fuse with another. How do these events occur and how are they regulated? Components involved in such processes have been described. Yet many of these components are highly similar to one another, and appear to overlap in their binding capacity of protein cargo based on studies using *in vitro* assays. How is it that such machinery acts so precisely within the cell?

About thirty years ago, it was observed by looking at cells under a microscope that coated pits could form at the plasma membrane, with vesicles also observed around the area (Hirst and Robinson 1998). Such vesicle formation could explain how proteins destined for one organelle started off at another compartment. Through the years that have followed, different protein and lipid components have been identified that play a role in vesicle trafficking. Probably the most studied component of vesicle traffic has been the coat protein clathrin.

The clathrin coat is involved in several types of vesicle formation and is easily recognizable visually based upon its well known lattice-like structures. The coat itself is composed of two forms of clathrin, the clathrin heavy chains (a 180 kDa protein) and the clathrin light chains (33 and 36 kDa proteins) (Pearse and Robinson 1990; Schmid 1997; Kirchhausen 2000). Three clathrin heavy chains

and three clathrin light chains come together to form a triskelion. The ability of clathrin to self-assemble into triskelions which go on to associate with one another to form a lattice, has also been demonstrated in vitro. The lattice that associates with a membrane consists of hexagonal clathrin structures. Then it is believed some of the hexagons may be altered into pentagons in order to drive the curvature of the membrane. Once the clathrin lattice has shifted shape and formed coated pits, vesicles can bud off from the donor compartment, destined for a new location within the cell.

Yet how is clathrin, a coat that is known to form at more than one location in the cell, such as at both the plasma membrane and at the trans-golgi network (TGN), as well as self-assemble (Lemmon, Lemmon et al. 1988; Liu, Marks et al. 1998; Ybe, Greene et al. 1998; Ybe, Brodsky et al. 1999; Greene, Liu et al. 2000), directed to such membranes? It is apparently though an interaction with another set of key players in membrane trafficking known as the adaptor proteins.

The adaptor proteins were originally identified within clathrin coated vesicles. Further studies showed that these proteins were found in between the clathrin and the vesicle membrane, hence the term "adaptor" for a protein that could link the two together. The most well characterized adaptor proteins are the heterotetrameric adaptor protein complexes, composed of two large subunits, a medium subunit, and a small subunit. As of now, four of these adaptor protein complexes have been identified (not all appear to be expressed in all organisms), yet others that are less classical adaptors, such as the GGAs and the stonins,

which I will discuss later, (Black and Pelham 2001; Robinson and Bonifacino 2001) appear to play similar roles to the more traditional adaptor complexes. These complexes, known as AP-1, AP-2, AP-3, and AP-4, are highly homologous to one another, especially within higher organisms. When the adaptor complexes were first identified, it appeared the role they played would be straightforward, as linkers of clathrin to protein cargo found at membranes. Yet not all adaptors appear to function in this way, which has complicated the picture. It appears the roles adaptors play may actually be straightforward (just not necessarily the role first identified), and it is membrane trafficking itself which is more complicated than originally thought.

AP-1 and AP-2 were the first adaptor complexes to be identified from clathrin coated vesicles. At steady state, AP-1 is predominantly localized to the trans-golgi network, while AP-2 is mainly localized to the plasma membrane. These complexes appeared to serve as links between the donor compartment and the coat. Obvious questions were what does the adaptor bind to at the membrane and how? What are the roles of the individual subunits in the complex? How does the adaptor bind clathrin? With time, such questions have begun to be answered (although as with any interesting set of questions and answers, more questions appear).

AP-1 and AP-2 are very similar to one another at the sequence and structure level. They each have two large subunits (γ and $\beta1$ for AP-1 and α and $\beta2$ for AP-2), a medium subunit ($\mu1$ or $\mu2$) and a small subunit ($\sigma1$ or $\sigma2$). The functions attributed to each of the subunits are similar as well. Both the $\beta1$ and

β2 subunits bind the coat clathrin (Gallusser and Kirchhausen 1993), and do so through a well characterized clathrin binding motif known as the clathrin box found in the hinge domain (Shih, Gallusser et al. 1995; Dell'Angelica, Klumperman et al. 1998). These boxes are composed of short consensus sequences, LLNLD. The motifs found within the hinge regions bind to the N-terminal domains of clathrin trimers. The appendage domain of β2 also binds clathrin to some extent, without promoting clathrin cage formation (Owen, Vallis et al. 2000). This subunit has also been implicated in binding to protein cargo that gets sorted through what is known as a dileucine based sorting signal (Rapoport, Chen et al. 1998).

The other large subunits, α and γ, are referred to as the large variable subunits because there is less homology among these subunits in the adaptor complexes. They also seem to have similar functions at first glance. Both appear to bind accessory proteins that help regulate the vesiculation event [Rapoport, 1997 #1198][Owen, 1999 #1193]. The α subunit binds such regulatory proteins as amphiphysin (Slepnev, Ochoa et al. 2000), epsin (Chen, Slepnev et al. 1999), AP180 (Hao, Luo et al. 1999), and auxilin (Scheele, Kalthoff et al. 2001). The ear of the α subunit has also been shown to bind to clathrin but does not appear to stimulate clathrin cage formation (Peeler, Donzell et al. 1993), but it is possible there is another clathrin binding site within the α subunit.

The only potential accessory protein identified that the γ subunit binds is γ-synergin (Page, Sowerby et al. 1999). Yet the γ subunit has recently been found to bind clathrin (Doray and Kornfeld 2001; Yeung and Payne 2001) and aid in

clathrin coat formation. Until very recently, only the β subunits of adaptor protein complexes had been shown to promote clathrin cage formation. The hinge domains of β subunits contain motifs within them termed clathrin boxes. Yet recent work from the Kornfeld group has identified another version of the clathrin binding motif, LLDLL, within the hinge domain of the γ subunit (Doray and Kornfeld 2001). And both the γ hinge and appendage (or ear) domains are important in recruiting and polymerizing clathrin. Previous work (Schmid 1997) showed that γ adaptin increased the efficiency of β1 induced clathrin coat formation; these results may now begin to explain why. So both large subunits of the adaptor complexes appear to play a role in coat formation. This theme of cooperativity and multifinctional roles for the adaptor subunits appears increasingly important.

The role of the large variable subunit has also begun to be clarified by work done constructing a γ-adaptin knockout. When the γ subunit is knocked-out, the free β1, μ1, and σ1 subunits are not stable without the large variable subunit- they do not form partial adaptor complexes (Zizioli, Meyer et al. 1999). These data suggest that the large variable subunit functions as a scaffold for adaptor complex formation. These γ-adaptin knockout mice are also not viable. This is in contrast with work in Saccharomyces cerevisiae where knockouts of the AP-1 subunits do not lead to any serious phenotype (Yeung, Phan et al. 1999) (this may now be explained by the GGAs, to be discussed later). While yeast is a very genetically tractable system to work in, the roles of their adaptors

and coats does appear quite different than in higher organisms (and obviously no work on neuronal adaptors can be performed).

In addition to the role the adaptor complexes play in binding to cargo, clathrin, and their associated proteins, the large variable subunits also seem to be involved in targeting to the proper membrane compartment. Work by Page and Robinson in 1995 began to map out the domains within the adaptor complexes AP-1 and AP-2 that were responsible for targeting the complex to donor compartments as well as which regions of the subunits interacted with each other (Page and Robinson 1995). They found that much of the targeting information was contained within the N-terminal trunk portion of the large variable subunit, in approximately 200 amino acids within the core. Some targeting information also appeared encoded within the ear domain and potentially as well by the medium and small subunits that assembled in the complex. The medium and small chains that assembled in a complex, either of AP-2 or of AP-1, went to their proper target, either the plasma membrane or the TGN, respectively.

The function of the medium subunit, known as μ1 or μ2 depending on the complex, is also very well characterized. The μ subunits bind to the complex through an interaction with the β subunit, and the carboxy terminus is free to bind what are known as sorting signals that are found within the cytoplasmic domains of cargo protein. The μ subunit binds to a tyrosine based sorting signal (Aguilar, Ohno et al. 1997; Owen and Evans 1998; Bonifacino and Dell'Angelica 1999; Aguilar, Boehm et al. 2001), YXXϕ or NPXY and perhaps other sorting signals like the dileucine motif. Structural studies have been done showing how YXXϕ

based peptides fit into a binding pocket present in the medium sized subunit. The role of σ subunits has not been identified, but it may contribute in targeting (Page and Robinson 1995) and the σ1 subunit has been shown to bind PACS-1 (Wan, Molloy et al. 1998), a protein involved in trafficking other proteins to the TGN.

It may have appeared at first glance that this would be a very simple system- one adaptor complex per membrane, recruiting the clathrin coat. The system may actually have an adaptor for each membrane, but it appears there are more sorting events than originally thought. There are many trafficking steps within a cell, beyond just simple endocytosis or TGN budding. There is more than one pathway of internalization from the cell surface, different types of endosomal compartments, specialized lysosomes like melanosomes or platelet dense granules, and specialized routes of sorting as in polarized epithelial cells or in neurons. To further complicate things, the same or similar organelles or vesicles can apparently be formed in more than one way. This is something that is of particular interest in my work. And while there are many pathways of membrane traffic within the cell, up until very recently, it appeared there were very few adaptors or coats that were involved in vesicle formation. Several years ago a third adaptor complex, AP-3,(Dell'Angelica, Ohno et al. 1997; Simpson, Peden et al. 1997; Stepp, Huang et al. 1997) was identified in yeast as well as *higher o*rganisms. AP-3 studies implicated it in another trafficking route from the TGN (yeast) or TGN/endosomal structures (mammalian). This observation alone was interesting. Why would there need to be more than one pathway from the

TGN to the vacuole? As of now, only two cargo proteins, Vam3p and ALP, have been identified to sort through the AP-3 pathway in yeast. Why do they not sort through the predominant AP-1 pathway? More recently another AP complex, AP-4 has been identified and while it also seems perinuclear or TGN-like in its localization, as well as being Brefeldin A sensitive, its trafficking function is unclear.

Work in the past few years has started to change the picture of membrane traffic in the cell. It was believed that adaptor protein complexes, along with clathrin, were going to be the only players in vesicular traffic. Yet the picture has gotten clouded. Not all of the adaptor complexes seem to utilize a clathrin coat (AP-3 does not, for example), and some monomeric adaptors have recently been identified that are quite different from the AP complexes. As more studies are done, a picture emerges that is much more complex. It seems there will be an adaptor for every pathway within the cell, some adaptors will be specialized versions of other adaptors, some will be altogether unique, and some adaptors may sort a limited set of cargo. Why do so many routes of traffic exist? Why must there be more than one adaptor per pathway/set of cargo? How can these adaptors, including the GGAs and stonins, recognize similar sorting signals present within the tails of cargo proteins, and act specifically (at the correct time and with only the proper adaptor binding)?

Before discussing the AP-3 studies, there are other adaptors that have recently been identified, although little is known about them. These adaptors include AP-4, the GGAs, and the stonins. AP-4 resembles the other adaptor

complexes in structure, composed of two large subunits, a medium subunit, and a small subunit. It is not found in yeast, C. elegans, or Drosophila, yet it is present in mammals, plants, and certain protists (Dictyostelium discoideum) (Robinson and Bonifacino 2001). AP-4 appears to have a similar localization to AP-1 and ubiquitous AP-3 (TGN-like) and its membrane association appears to rely on similar factors like the small GTPase ARF (i.e. it is Brefeldin A sensitive) (Dell'Angelica, Mullins et al. 1999; Hirst, Bright et al. 1999). AP-4, if it utilizes a coat, probably does not associate with clathrin based on the lack of identified clathrin boxes in the hinge and ear domains of its large subunits, ε and β4. It is also present in very low abundance in most cells. Yeast two-hybrid assays have been performed using the μ4 subunit, as has been done for all of the μ chains, and its residue preferences for sorting signals have been mapped. It, like every μ chain binds YXXϕ motifs, but the binding is stronger when certain residues are within and surround the signal (Ohno, Aguilar et al. 1998; Aguilar, Boehm et al. 2001). But, as I will discuss, two-hybrid analysis (as well as other binding assays) can fall short in identifying true physiological preferences.

The GGAs (Golgi-localized, γ ear-containing, ARF-binding proteins) are a new class of adaptor molecules, that unlike the AP complexes, function as a single monomer (Dell'Angelica, Puertollano et al. 2000; Hirst, Lui et al. 2000). The GGAs contain four domains designated the VHS, GAT, hinge, and ear regions. There is some homology to the γ-adaptin ear at the C-terminus of the GGA. While the ear of γ-adaptin has been found to bind the protein γ-synergin, which as of now has an unknown function, the ear domain of the GGAs may also

be important in binding proteins that could play a regulatory role. The GGA hinge domain may be a flexible linker as it is in the adaptor protein complexes, and it may also bind clathrin as it contains potential clathrin binding sequences. The GGAs have been shown to interact with clathrin in vitro and colocalization with the coat in yeast has also been observed (Puertollano, Randazzo et al. 2001) (Costaguta, Stefan et al. 2001). If the GGAs could serve as clathrin adaptors, this may explain why deleting all the AP complexes in yeast does not effect clathrin coated vesicle formation. The GAT domain found within the GGAs binds ARF, and this binding is required for membrane recruitment of the GGA protein. The VHS domain is found at the N-terminus of the protein. This domain binds to acidic cluster di-leucine motifs (Takatsu, Katoh et al. 2001) and the cargo it has been shown to bind though this interaction so far are sortilin (Nielsen, Madsen et al. 2001), the cation-independent mannose 6-phosphate receptor, and the cation-dependent mannose 6-phosphate receptor (Puertollano, Aguilar et al. 2001). A dominant negative GGA mutant blocks transport of the mannose 6-phosphate receptors from the TGN. These recent data on the GGAs, plus the results suggesting that AP-1 mediates transport from endosomes to TGN, are changing our current picture of membrane traffic within the cell. AP-1 may be functioning differently than anyone had previously thought, and a new type of adaptor (GGA) has emerged that is fulfilling the role it was originally attributed to having.

Another recent class of adaptors to be uncovered is the stoned B family. The stoned gene encodes two separate gene products, stoned A and stoned B

(Andrews, Smith et al. 1996). The function of Stoned A has not been worked out, although it contains five DPF motifs that could interact with the ear domain of α-adaptin (AP-2). Stoned B, on the other hand, contains proline-rich domains at its amino terminus and its carboxy terminus shares homology with the AP complex μ domains, however it does not bind tyrosine or di-leucine sorting signals. Homologues for stoned B have been identified in nematodes, humans, and mice (none in *S. cerevisiae*). Human stonin 2 interacts directly with eps15 and intersectin1 (NPF-EH interactions) (Martina, Bonangelino et al. 2001). This links stonin 2 to AP-2. The μ-homology domains (MHD) bind C2B domains of the synaptotagmin family (Phillips, Smith et al. 2000). Synaptotagmin, which binds AP-2, may recruit stonins to the plasma membrane which could promote recruitment of eps15 and intersectin which would recruit their respective binding partners (dynamin, clathrin, etc.) and carry out clathrin coated vesicle formation. Other work in Drosophila could suggest a reverse role of sorts, that stoned B recruits synaptotagmin I at hot spots of endocytosis because overexpression of synaptotagmin in stoned flies rescues their defect (Fergestad and Broadie 2001).

Also complicating this increasingly complex picture is the fact that some of these adaptors, if not all, can be composed of more than one kind of subunit isoform. Many years ago neuronal specific isoforms of the AP-2 subunits α2 and β2 were identified (the α subunit is encoded by one of two genes, αA or αC, which are 80% identical and presumed functionally equivalent). The αA subunit is alternatively spliced in neurons (Robinson 1989), and so is the β2 subunit. While there has been postulation that the extreme demands of trafficking in

neurons (as opposed to other types of cells) could require additional machinery to aid the system, no functions for the neuronal specific subunits of AP-2 have been identified. But the alternative isoform picture grows. In recent years, "alternative" isoforms for subunits of AP-1, AP-2 (mentioned above), and AP-3 have been identified. What is the purpose of these other subunits? Do the complexes they are in serve a unique function? Why isn't there just a separate adaptor complex to serve a new function?

Recently, multiple isoforms of the γ and μ1 subunits of the AP-1 complex have been identified and studied, as well as an additional isoform of the σ1 subunit, yet no work has been published about its function or expression.

The γ subunit of AP-1 is present in more than one form. γ1-adaptin, as it is now referred to, is ubiquitously expressed. A new form, γ2-adaptin, is 61.7% identical to γ1-adaptin (Lewin, Sheff et al. 1998). Structurally they appear quite similar, yet the γ2 subunit has a truncated ear domain. Although no information has yet been reported, it seems there may also be a neuronal specific form of the γ2-adaptin subunit. The function of this alternate AP-1 complex is unknown, as well as whether or not the γ2 subunit associates with the other ubiquitously expressed AP-1 subunits. Both AP-1 subunits are localized similarly- to perinuclear, Golgi-like structures. However, some differences have been observed. Looking at localization of both forms of γ adaptins by immunoelectron microscopy show that while both are localized to Golgi membranes, and may be in close proximity to one another, they were never found on the same membranes (Takatsu, Sakurai et al. 1998). Also while the γ1-adaptin-containing

ubiquitous AP-1 complex shows sensitivity to the fungal metabolite Brefeldin A (it becomes cytosolic), expression of the γ2 subunit is unchanged and its remains membrane bound.

Such work on the AP-1 complex may be able to help us understand some of the reasons a cell needs specialized adaptors/subunits. Another specialized AP-1 complex has also been identified and its function has been characterized. The medium sized subunit of AP-1 is found in more than one form. Polarized epithelial cells express two types of μ1 subunit, μ1A and μ1B (Folsch, Ohno et al. 1999; Ohno, Tomemori et al. 1999). These subunits are 79% identical and while the μ1A subunit is expressed ubiquitously, the μ1B isoform expression is limited to polarized epithelial cells. The two complexes, now commonly referred to as AP-1A and AP-1B are involved in TGN and endosome trafficking and TGN to basolateral plasma membrane trafficking respectively. In cell lines that lack expression of μ1B, the LDL receptor is incorrectly sorted to apical membranes. The LDL receptor normally sorts to basolateral membranes by virtue of the basolateral sorting signals found within its cytoplasmic tail. This suggests that the basolateral sorting signals are recognized specifically by the μ1B subunit, and in its absence μ1A can perhaps bind to an apical signalling determinant. In addition, both forms of the AP-1 complexes, AP-1A and AP-1B can coexist within the same cell; the two complexes could be eluted by gel filtration. As well, the μ1B containing AP-1B complex could not recognize and sort cargo proteins that did not have basolateral sorting signals (apically destined proteins). Yet, AP-1B may not be required for the sorting of all basolateral proteins. One example has

been examined thus far. The IgG Fc receptor FcRII-B2 contains a dileucine type of sorting signal and does not appear to require AP-1B to be sorted properly. Dileucine type sorting signals are believed, however, to interact not with the μ subunits but with the β chains of adaptor complexes. Either there is another complex that is involved in basolateral sorting or perhaps there is an as yet unidentified β1 isoform involved in basolateral trafficking.

The same group in Peter Schu's lab that produced a γ-adaptin knockout has also made a μ1A-adaptin deficient mouse (Meyer, Zizioli et al. 2000). True μ1A knockouts, like the γ1 knockouts were lethal. Yet, one difference between the two AP-1A mutants, was that while no partial complex formation could be observed in the absence of the large variable γ subunit, there was partial complex formation in the absence of the μ1A subunit. The medium sized subunit, known predominantly to be important for its binding to sorting signals in the cytoplasmic domains of cargo proteins, and slightly for membrane targeting, clearly does not play the same structural scaffolding role as the γ chain. Cells that were deficient in μ1A expression showed no clathrin localization at the TGN. Yet, while AP-1 may recruit clathrin to the TGN, work from this lab and others suggests that the role of AP-1A may not actually be in traffic from the TGN to endosomal structures as it has previously been assumed. MPR46 and MPR300 were improperly localized in μ1A deficient cells. Normally, these two mannose-6-phosphate receptors are predominantly perinuclear or TGN-like in localization. In the μ1A mutant cells, staining for the MPRs becomes redistributed to vesicular structures throughout the cytoplasm. The compartments the MPRs are now

localized to also contain the early endosome associated protein, EEA1. Further experiments this group performed also support the idea that AP-1A is involved in retrograde transport of the MPRs from endosomal compartments to the TGN, and not anterograde transport from the TGN as long believed. This becomes a more intriguing possibility in light of the GGA research in transport of the MPRs from the TGN to endosomes.

Further evidence for multiple roles for μ1 subunits comes from work done in the nematode Caenorhabditis elegans. In the nematode, two μ1 subunits are encoded for by two separate genes, unc-101 and apm-1. Both of these gene products are expressed ubiquitously and throughout development and appear to have overlapping yet somewhat distinct functions (Shim, Sternberg et al. 2000). After examination of the genomic sequences for both apm-1 and unc-101, it appears that one may have arisen from a duplication, yet have acquired some changes in that they do not share completetly redundant functions. They appear to be partially redundant and both are found in complexes with the other AP-1 subunits (only one of each identified in nematodes). More recent work has shown that in unc-101 mutants, dendritic vesicles are absent (Dwyer, Adler et al. 2001). It seems this μ1 subunit is important in sorting a receptor from the TGN into dendritic transport vesicles destined for the cilia plasma membrane. This is an analogous role to that played by AP-1B in polarized epithelia.

Other intriguing evidence to explain specialized adaptors such as AP-1A and AP-1B comes from work done by the Hirokawa lab examining motor proteins involved in the traffic of transport vesicles from the TGN. If there is more

than one way to transport proteins from an organelle, to an organelle, or to separate cargo within that organelle, there needs to be specific ways to recognize each class of vesicles. All of the adaptor complexes (AP-1, AP-2, A-3, and AP-4) participate in budding from a membrane compartment to form a vesicle, yet no motor proteins involved in the transport of these specific vesicles has been identified until recently. This work identified a motor protein involved in the transport of AP-1B derived vesicles bound for the plasma membrane. The motor protein identified is KIF13A (Nakagawa, Setou et al. 2000). The C-terminal tail domain of KIF13A binds directly to the ear domain of the β1 subunit. The interaction is also specific for AP-1 and KIF13A does not show any interaction with the other adaptor complexes. There is partial overlap by immunofluorescence between KIF13A and AP-1 staining (only partial overlap could be due to the two forms of AP-1 complexes). Overexpression of this motor protein also disrupts normal localization of AP-1B cargo such as Mannose-6-Phosphate Receptor. M6PR is normally found at the TGN at steady state. When KIF13A is overexpressed, the motor protein, as well as M6PR and AP-1B are now relocalized to the cell periphery. There are several interesting points that arise from this work. One is that vesicles arising from compartments such as the TGN must be segregated somehow to only include one type of adaptor and cargo. For example, here the KIF13A vesicles only contained AP-1B, not AP-1A (which may be steady state localized to endosomes) nor AP-3 which would be localized to a similar donor compartment. Another interesting issue is whether or not this implies that there is another isoform of the β1 subunit, as previously

suggested (proteins that get sorted basolaterally but without the requirement for μ1B). If KIF13A binds to the ear of the β1 subunit, how does it not affect AP-1A traffic? Perhaps there is another β1 present, or *in vivo* there are accessory proteins involved in specific binding of a motor protein to an adaptor.

Further work characterizing the differences between AP-1A and AP-1B have begun to highlight differences in sorting properties, as well as reflect some of the limitations in assays such as the yeast two hybrid. The Schu group in collaboration with the Mellman lab has continued the work on understanding the role of AP-1B in polarized epithelial cells (Folsch, Pypaert et al. 2001). As mentioned earlier, apical sorting signals are quite different than basolateral sorting signals. Apical sorting is believed to be aided by N- or O- linked carbohydrate moieties and/or transmembrane domain properties. Basolateral sorting signals generally are found within the cytoplasmic domain of the protein and are usually dominant over apical sorting signals. These basolateral signals appear to be degenerate, but some do contain tyrosines or di-leucines. This group made HA-tagged μ1A subunits and μ1B subunits (internal tags based on previous work done on the μ2 subunit by Nesterov et al. and Owen and Evans) and then studied incorporation into AP-1 complexes and localization. It is relatively unexpected that there would be such selectivity of cargo binding between AP-1A and AP-1B because the μ1 subunits are so nearly identical, yet this group showed that μ1B directly binds to basolateral targeting signals on proteins like LDLR. This is surprising also based on work from Ohno et al. using the yeast two-hybrid assay to examine binding of μ subunits to the canonical

YXXϕ sorting signals (Ohno, Aguilar et al. 1998). LDLR does not sort basolaterally through such a signal, and both μ1A and μ1B are so similar that is unexpected that their binding capacities would be so different. When the basolateral sorting signal of LDLR was mutated, the AP-1B interaction was gone. They also found both forms of AP-1 localized to some degree to endosomal structures. When they examined localization by immunoelectron microscopy, they found that both AP-1A and AP-1B associate with clathrin coats at the TGN. Yet, they observed that the two different AP-1 complexes appear to localize to different regions of the TGN (by immunofluorescence AP-1A or AP-1B was compared to furin localization). But μ1B could partially rescue localization of AP-1 in the previously described μ1A deficient cell line. In this line, the γ subunit staining is diffuse. When this group transfected in the μ1B subunit, the γ subunit is now properly localized to the TGN suggesting that μ1B can substitute in the AP-1A complex. (This is reminiscent of the unpublished result that the β3B subunit of AP-3 can rescue pearl/β3A mutant fibroblasts.) Yet, the μ1A mutant cells that were transfected with μ1B could still not sort AP-1A cargo properly. Furin, for example, was still localized to endosomes, when it should normally be found at the TGN. Even though AP-1A/B was localized properly, it could not transport the AP-1A cargo. These studies further implicate the μ chains in targeting, as well as cargo recognition, but show them to be different and separable.

There are other clear differences between AP-1A and AP-1B. Similarly to their separate cargo binding capabilities, or specific motor protein binding, the

PACS-1 protein has also been identified to bind the AP-1A complex (Wan, Molloy et al. 1998; Crump, Xiang et al. 2001). PACS-1 binds the cytosolic domain of furin. The TGN localization of furin and M6PR is dependent on PACS-1. When PACS-1 expression is removed by antisense, furin localization dispersed from its TGN-like localization. How does PACS-1, involved in the transport of a select subset of proteins that are trafficked by the AP-1A complex, only interact with this AP-1A complex and not AP-1B? (PACS-1 has also been suggested to bind to AP-3.) PACS-1 has been shown to bind directly to μ1 as well as σ1, but not β1 or γ. Perhaps PACS-1 only binds to μ1A containing complexes, and this creates a ternary complex composed of cargo, adaptor, and PACS-1 serving as a linker and providing a means of specificity.

Complexity in adaptor complex formation and function is not limited to the AP-1 adaptor complex(es). AP-2, the complex known to function in endocytosis from the plasma membrane also has similar complexity associated with it. AP-2 is also a heterotetrameric complex composed of two large subunits, α and β2, a medium subunit, μ2, and a small subunit, σ2. Like the AP-1 complex, some of the AP-2 subunits have been identified to be found in more than one form- alternate isoforms of the subunits. Yet unlike AP-1, the AP-2 alternate isoforms have had no function ascribed to them and no role for another (or more than one type) of AP-2 complex has been determined. The other AP-2 subunit isoforms are for the α and β chains. The large variable subunit, α, is known to bind accessory proteins which aid in regulation of budding from the plasma membrane. This subunit, in mammalian cells, is encoded for by two different

genes, αA and αC. The gene products are around 80% identical to one another, and both appear to form AP-2 complexes with the other subunits. While no functional differences have been distinguished between the two forms of AP-2's large variable subunit, some differences have been observed nonetheless. The αA protein is slightly larger than the αC subunit (Robinson 1989), and its expression is very high in brain, while the αC subunit is highly expressed in liver. Both forms have been shown to localize to clathrin coated pits, sometimes even in the same pits. In addition, the αA subunit also gets alternatively spliced in neurons to form a subunit that contains an extra 22 amino acids in its hinge domain. The other large subunit of AP-2, the β2 subunit has been very well studied. Its role in binding to clathrin through its clathrin box as well as its ability to bind to dileucine sorting signals or cargo proteins has been documented. What has not been further clarified, is that the β2 subunit, as apparently every adaptor complex subunit it seems, also is expressed in more than one form. β2 can undergo alternative splicing in neurons, like the αA subunit can. An additional 14 amino acids remain in the neuronal isoform of β2. Even the coat proteins, such as the clathrin light chains, that the APs bind, also undergo alternative splicing in neurons (Jackson, Seow et al. 1987; Wong, Ignatius et al. 1990; Stamm, Casper et al. 1992; Uriu-Mikami, Omori et al. 1995).) While there has been speculation as to why alternate isoforms of subunits exist in neurons, (Morris and Schmid 1995) for example that there are high demands of traffic in neurons under extreme stimulation, no definite role has been determined.

This brings us to the major question: why are there alternate isoforms for subunits of adaptor complexes? The question has now been partially answered by work done on the AP-1 complexes, yet a unique opportunity existed in the Kelly lab to address this question by working on the AP-3 adaptor complex.

AP-3, like AP-1, AP-2, and AP-4, is composed of two large subunits, a medium one, and a small one. Originally the medium chain was identified, and then one of the large subunits was identified and shown to interact with it. The AP-3 complex, including the other large subunit and the small subunit which were subsequently identified, began to be characterized in many organisms from yeast to flies as well as in mammalian systems (Simpson, Bright et al. 1996). While the work in yeast has clearly established a role for the AP-3 complex in traffic to the vacuole, from what seems like the TGN (Cowles, Odorizzi et al. 1997; Stepp, Huang et al. 1997), work in mammals has shown greater complexity (Dell'Angelica, Ohno et al. 1997; Simpson, Peden et al. 1997). Like AP-1 and AP-2, there are alternate isoforms for subunits of the AP-3 complex. Specifically, there are brain-specific isoforms of AP-3 subunits.

The ubiquitous AP-3 complex, which I refer to as the complex composed of the subunits expressed in all cell types, which is the presumed equivalent in yeast, is composed of δ, β3A, μ3A, and σ3. In yeast, the AP-3 complex is quite similar to that in higher organisms, except that the β3 subunit lacks the ear domain found in all large subunits. The function of the AP-3 complex in yeast is apparently to provide an alternate pathway to traffic certain proteins from the TGN to the vacuole. The most studied pathway of traffic from the TGN to the

vacuole in yeast relies upon the AP-1 complex, or perhaps the GGAs. This pathway, referred to as the CPY (carboxypeptidase Y) pathway, named for a protein identified to sort through this route, transports cargo from the TGN to a prevacuolar compartment (PVC)/endosomal compartment, and from there cargo are transported to the vacuole. Two proteins, Vam3p, a vacuolar t-SNARE, and alkaline phosphatase, ALP, are known to sort from the Golgi through this alternate pathway. This pathway bypasses the PVC and goes to the vacuole through another intermediate. Why there is another means to sort proteins from one organelle to another, is unclear. The pathway facilitated by AP-3 in yeast, and indeed all organisms is not essential. Yeast are viable when any (or all) subunit of AP-3 is removed.

In Drosophila melanogaster, mutants in the AP-3 complex have also provided clues as to the function of the complex in membrane traffic. The first AP-3 mutant identified, in the δ subunit, is known as garnet in flies (Ooi, Moreira et al. 1997). These flies have eye pigmentation defects, as well as pigmentation defects in other organs, due to defects in the number of pigment granules formed, as well as less total pigment in the organ. The garnet gene product was found to associate with the other AP-3 subunits. Since the original identification of AP-3 in flies, pigmentation mutants in each of the subunits have been identified (Kretzschmar, Poeck et al. 2000). They are carmine (μ3), orange (σ3), and ruby (β3A). Pigmentation defects, are also associated with the light gene (Warner, Sinclair et al. 1998), which is a homologue of Vps41p, a protein in yeast that has been shown to interact with the δ subunit (Rehling, Darsow et al. 1999),

and may serve as a coat protein for the AP-3 complex (Darsow, Katzmann et al. 2001). This set of AP-3 defects fits with the yeast AP-3 mutations, suggesting a role in the sorting to and/or biogenesis of lysosomes or lysosome-related organelles, that is not required for viability.

More data exist in mammalian systems to support the role of ubiquitous AP-3 in lysosomal traffic. Naturally occurring mouse mutants, pearl, mutant in the β3A subunit, and mocha, mutant in the δ subunit, (Kantheti, Qiao et al. 1998) were identified as AP-3 mutants. Both of these mice have pigmentation defects that affect coat color, hence their names, and have problems associated with defects in lysosome or lysosome-related organelle biogenesis. Humans are also known to have defects in the β3A subunit, causing a disorder called Hermansky-Pudlak Syndrome (Shotelersuk and Gahl 1998), a syndrome in a larger class of Storage Pool Disorder diseases. These patients suffer from oculocutaneous albinism, platelet storage defects, and ceroid lipofuscinosis. Recent work has shown that certain lysosomal membrane proteins, such as Lamp-1 (Le Borgne, Alconada et al. 1998), LIMP-II (Honing, Sandoval et al. 1998), and tyrosinase (Huizing, Sarangarajan et al. 2001), sort to lysosomes by an AP-3 pathway.

The β3A chain has been suggested to bind to dileucine sorting signals, as well as to binding to clathrin *in vitro* (Dell'Angelica, Klumperman et al. 1998), although no evidence supports this *in vivo*. The δ subunit seems to play a similar scaffolding role that the large variable subunit γ plays for the AP-1 complex. As with γ adaptin knockouts, the mocha mutants which are null for δ expression, the other subunits of the adaptor complex become destabilized and these mutants

are virtual nulls for expression of the entire complex. The μ3A subunit, like the other μ subunits of the other adaptor complexes (as well as the neuronal μ3B subunit), likely binds to tyrosine based sorting motifs of cargo destined for the lysosome or related organelles. The σ3 subunit, like the small subunits of the other adaptor complexes, has not been implicated in any functions thus far, but it has been identified to interact with IRS-1, the insulin receptor substrate-1 (VanRenterghem, Morin et al. 1998).

AP-3 has a clear and established role in traffic from the Golgi to lysosomes. Yet work by Faundez and Kelly complicated the picture by suggesting another function that the AP-3 complex could perform in producing small vesicles that resembled synaptic-like microvesicles from endosomes (Faundez, Horng et al. 1998). Because these experiments used AP-3 purified from brain, the complication arose that perhaps only the neuronal subunit isoforms of AP-3 were functioning in such assays. AP-3, like AP-1 and AP-2, has subunits present in more than one form. The large β3 subunit was originally identified as β-NAP, a protein recognized by a patient's own antisera (Newman, McKeever et al. 1995). This isoform of the large subunit, β3B, is a brain-specific form of the ubiquitously expressed β3 (referred to as β3A) subunit. The medium chain, μ3, is also present in more than one form. The ubiquitously expressed subunit is referred to as μ3A, and the isoform whose expressed is limited to neurons is called μ3B. There are also two forms of the small subunit, σ3A and σ3B, both of which are ubiquitously expressed and currently no clear distinctions in function have as of yet been drawn between the two. The work of Faundez et

al. showed that brain purified AP-3, containing both ubiquitous and neuronal forms of AP-3, played a role in SLMV biogenesis from early endosomes and was important in coating purified synaptic-like microvesicles. One piece of evidence was that in the *in vitro* budding assay from the endosomal compartment, mocha brain cytosol which lacks all forms of AP-3 does not allow SLMV formation.

Here was a good opportunity to further examine the alternative roles the AP-3 complexes play, which in turn might tell us why there are alternate isoforms and what is unique about trafficking in the brain. By focusing on a neuronal subunit of the AP-3 complex, we believed several questions could be addressed. Is AP-3 found in more than one form or are all the complexes in the brain mixed of ubiquitous and neuronal subunits? If there is more than one AP-3 complex, are their functions distinct? How and why can AP-3 perform two very separate functions? Why should there be a specialized adaptor to play an additional quite different role instead of an entirely different adaptor complex? What is unique about trafficking in neurons that requires specialized machinery? Can assays in current use, like the yeast two hybrid assay, which did not elucidate differences between binding preferences for μ1A and μ1B, or liposome binding studies (Drake, Zhu et al. 2000) which contained no protein on membranes to which an adaptor or coat could specifically bind answer these questions?

The work I have pursued in the Kelly lab was to address these issues. By studying the role and expression of an adaptor complex that consists of neuronal-specific isoforms, clarifying its functional requirement, and where it is localized, we have begun to get a handle on these questions.

Aguilar, R. C., M. Boehm, et al. (2001). "Signal-binding Specificity of the {mu}4 Subunit of the Adaptor Protein Complex, AP-4." J Biol Chem **98**: 2.

Aguilar, R. C., H. Ohno, et al. (1997). "Functional domain mapping of the clathrin-associated adaptor medium chains mu1 and mu2." J Biol Chem **272**(43): 27160-6.

Andrews, J., M. Smith, et al. (1996). "The stoned locus of Drosophila melanogaster produces a dicistronic transcript and encodes two distinct polypeptides." Genetics **143**(4): 1699-711.

Black, M. W. and H. R. Pelham (2001). "Membrane traffic: How do GGAs fit in with the adaptors?" Curr Biol **11**(12): R460-2.

Bonifacino, J. S. and E. C. Dell'Angelica (1999). "Molecular bases for the recognition of tyrosine-based sorting signals." J Cell Biol **145**(5): 923-6.

Chen, H., V. I. Slepnev, et al. (1999). "The interaction of epsin and Eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation-dependent dephosphorylation in nerve terminals." J Biol Chem **274**(6): 3257-60.

Costaguta, G., C. J. Stefan, et al. (2001). "Yeast gga coat proteins function with clathrin in golgi to endosome transport." Mol Biol Cell **12**(6): 1885-96.

Cowles, C. R., G. Odorizzi, et al. (1997). "The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole." Cell **91**(1): 109-18.

Crump, C. M., Y. Xiang, et al. (2001). "PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic." Embo J **20**(9): 2191-201.

Darsow, T., D. J. Katzmann, et al. (2001). "Vps41p Function in the Alkaline Phosphatase Pathway Requires Homo-oligomerization and Interaction with AP-3 through Two Distinct Domains." Mol Biol Cell **12**(1): 37-51.

Dell'Angelica, E. C., J. Klumperman, et al. (1998). "Association of the AP-3 adaptor complex with clathrin." Science **280**(5362): 431-4.

Dell'Angelica, E. C., C. Mullins, et al. (1999). "AP-4, a novel protein complex related to clathrin adaptors." J Biol Chem **274**(11): 7278-85.

Dell'Angelica, E. C., H. Ohno, et al. (1997). "AP-3: an adaptor-like protein complex with ubiquitous expression." Embo J **16**(5): 917-28.

Dell'Angelica, E. C., R. Puertollano, et al. (2000). "GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex." J Cell Biol **149**(1): 81-94.

Doray, B. and S. Kornfeld (2001). "gamma Subunit of the ap-1 adaptor complex binds clathrin: implications for cooperative binding in coated vesicle assembly." Mol Biol Cell **12**(7): 1925-35.

Drake, M. T., Y. Zhu, et al. (2000). "The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes." Mol Biol Cell **11**(11): 3723-36.

Dwyer, N. D., C. E. Adler, et al. (2001). "Polarized dendritic transport and the AP-1 mu1 clathrin adaptor UNC-101 localize odorant receptors to olfactory cilia." Neuron **31**(2): 277-87.

Faundez, V., J. T. Horng, et al. (1998). "A function for the AP3 coat complex in synaptic vesicle formation from endosomes." Cell **93**(3): 423-32.

Fergestad, T. and K. Broadie (2001). "Interaction of stoned and synaptotagmin in synaptic vesicle endocytosis." J Neurosci **21**(4): 1218-27.

Folsch, H., H. Ohno, et al. (1999). "A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells." Cell **99**(2): 189-98.

Folsch, H., M. Pypaert, et al. (2001). "Distribution and Function of AP-1 Clathrin Adaptor Complexes in Polarized Epithelial Cells." J Cell Biol **152**(3): 595-606.

Gallusser, A. and T. Kirchhausen (1993). "The beta 1 and beta 2 subunits of the AP complexes are the clathrin coat assembly components." Embo J **12**(13): 5237-44.

Greene, B., S. H. Liu, et al. (2000). "Complete reconstitution of clathrin basket formation with recombinant protein fragments: adaptor control of clathrin self-assembly." Traffic **1**(1): 69-75.

Hao, W., Z. Luo, et al. (1999). "AP180 and AP-2 interact directly in a complex that cooperatively assembles clathrin." J Biol Chem **274**(32): 22785-94.

Hirst, J., N. A. Bright, et al. (1999). "Characterization of a fourth adaptor-related protein complex." Mol Biol Cell **10**(8): 2787-802.

Hirst, J., W. W. Lui, et al. (2000). "A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome." J Cell Biol **149**(1): 67-80.

Hirst, J. and M. S. Robinson (1998). "Clathrin and adaptors." Biochim Biophys Acta **1404**(1-2): 173-93.

Honing, S., I. V. Sandoval, et al. (1998). "A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3." Embo J **17**(5): 1304-14.

Huizing, M., R. Sarangarajan, et al. (2001). "Ap-3 mediates tyrosinase but not trp-1 trafficking in human melanocytes." Mol Biol Cell **12**(7): 2075-85.

Jackson, A. P., H. F. Seow, et al. (1987). "Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments." Nature **326**(6109): 154-9.

Kantheti, P., X. Qiao, et al. (1998). "Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles." Neuron **21**(1): 111-22.

Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**(9): 699-727.

Kretzschmar, D., B. Poeck, et al. (2000). "Defective pigment granule biogenesis and aberrant behavior caused by mutations in the Drosophila AP-3beta adaptin gene ruby." Genetics **155**(1): 213-23.

Le Borgne, R., A. Alconada, et al. (1998). "The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins." J Biol Chem **273**(45): 29451-61.

Lemmon, S., V. P. Lemmon, et al. (1988). "Characterization of yeast clathrin and anticlathrin heavy-chain monoclonal antibodies." J Cell Biochem **36**(4): 329-40.

Lewin, D. A., D. Sheff, et al. (1998). "Cloning, expression, and localization of a novel gamma-adaptin-like molecule." FEBS Lett **435**(2-3): 263-8.

Liu, S. H., M. S. Marks, et al. (1998). "A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway." J Cell Biol **140**(5): 1023-37.

Martina, J. A., C. J. Bonangelino, et al. (2001). "Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery." J Cell Biol **153**(5): 1111-20.

Meyer, C., D. Zizioli, et al. (2000). "mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors." Embo J **19**(10): 2193-203.

Morris, S. A. and S. L. Schmid (1995). "Synaptic vesicle recycling. The Ferrari of endocytosis?" Curr Biol **5**(2): 113-5.

Nakagawa, T., M. Setou, et al. (2000). "A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex." Cell **103**(4): 569-81.

Newman, L. S., M. O. McKeever, et al. (1995). "Beta-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein." Cell **82**(5): 773-83.

Nielsen, M. S., P. Madsen, et al. (2001). "The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein." Embo J **20**(9): 2180-90.

Ohno, H., R. C. Aguilar, et al. (1998). "The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals." J Biol Chem **273**(40): 25915-21.

Ohno, H., T. Tomemori, et al. (1999). "Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells." FEBS Lett **449**(2-3): 215-20.

Ooi, C. E., J. E. Moreira, et al. (1997). "Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the Drosophila eye color mutant garnet." Embo J **16**(15): 4508-18.

Owen, D. J. and P. R. Evans (1998). "A structural explanation for the recognition of tyrosine-based endocytotic signals." Science **282**(5392): 1327-32.

Owen, D. J., Y. Vallis, et al. (2000). "The structure and function of the beta 2-adaptin appendage domain." Embo J **19**(16): 4216-27.

Page, L. J. and M. S. Robinson (1995). "Targeting signals and subunit interactions in coated vesicle adaptor complexes." J Cell Biol **131**(3): 619-30.

Page, L. J., P. J. Sowerby, et al. (1999). "Gamma-synergin: an EH domain-containing protein that interacts with gamma-adaptin." J Cell Biol **146**(5): 993-1004.

Pearse, B. M. and M. S. Robinson (1990). "Clathrin, adaptors, and sorting." Annu Rev Cell Biol **6**(9): 151-71.

Peeler, J. S., W. C. Donzell, et al. (1993). "The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits." J Cell Biol **120**(1): 47-54.

Phillips, A. M., M. Smith, et al. (2000). "The Products of the Drosophila stoned Locus Interact with Synaptic Vesicles via Synaptotagmin." J Neurosci **20**(22): 8254-8261.

Puertollano, R., R. C. Aguilar, et al. (2001). "Sorting of mannose 6-phosphate receptors mediated by the GGAs." Science **292**(5522): 1712-6.

Puertollano, R., P. A. Randazzo, et al. (2001). "The GGAs promote ARF-dependent recruitment of clathrin to the TGN." Cell **105**(1): 93-102.

Rapoport, I., Y. C. Chen, et al. (1998). "Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site." Embo J **17**(8): 2148-55.

Rehling, P., T. Darsow, et al. (1999). "Formation of AP-3 transport intermediates requires Vps41 function." Nat Cell Biol **1**(6): 346-53.

Robinson, M. S. (1989). "Cloning of cDNAs encoding two related 100-kD coated vesicle proteins (alpha-adaptins)." J Cell Biol **108**(3): 833-42.

Robinson, M. S. and J. S. Bonifacino (2001). "Adaptor-related proteins." Curr Opin Cell Biol **13**(4): 444-53.

Scheele, U., C. Kalthoff, et al. (2001). "Multiple interactions of auxilin 1 with clathrin and the AP-2 adaptor complex." J Biol Chem **24**: 24.

Schmid, S. L. (1997). "Clathrin-coated vesicle formation and protein sorting: an integrated process." Annu Rev Biochem **66**: 511-48.

Shih, W., A. Gallusser, et al. (1995). "A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes." J Biol Chem **270**(52): 31083-90.

Shim, J., P. W. Sternberg, et al. (2000). "Distinct and redundant functions of mu1 medium chains of the AP-1 clathrin-associated protein complex in the nematode Caenorhabditis elegans." Mol Biol Cell **11**(8): 2743-56.

Shotelersuk, V. and W. A. Gahl (1998). "Hermansky-Pudlak syndrome: models for intracellular vesicle formation." Mol Genet Metab **65**(2): 85-96.

Simpson, F., N. A. Bright, et al. (1996). "A novel adaptor-related protein complex." J Cell Biol **133**(4): 749-60.

Simpson, F., A. A. Peden, et al. (1997). "Characterization of the adaptor-related protein complex, AP-3." J Cell Biol **137**(4): 835-45.

Slepnev, V. I., G. C. Ochoa, et al. (2000). "Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of

clathrin coat function by amphiphysin fragments comprising these sites." J Biol Chem **275**(23): 17583-9.

Stamm, S., D. Casper, et al. (1992). "Clathrin light chain B: gene structure and neuron-specific splicing." Nucleic Acids Res **20**(19): 5097-103.

Stepp, J. D., K. Huang, et al. (1997). "The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole." J Cell Biol **139**(7): 1761-74.

Takatsu, H., Y. Katoh, et al. (2001). "Golgi-localizing, gamma -Adaptin Ear Homology Domain, ADP-ribosylation Factor-binding (GGA) Proteins Interact with Acidic Dileucine Sequences within the Cytoplasmic Domains of Sorting Receptors through Their Vps27p/Hrs/STAM (VHS) Domains." J Biol Chem **276**(30): 28541-5.

Takatsu, H., M. Sakurai, et al. (1998). "Identification and characterization of novel clathrin adaptor-related proteins." J Biol Chem **273**(38): 24693-700.

Uriu-Mikami, T., K. Omori, et al. (1995). "Distribution of neurone-specific clathrin light chain b between clathrin-coated vesicle subclasses." Neuroreport **6**(14): 1922-6.

VanRenterghem, B., M. Morin, et al. (1998). "Interaction of insulin receptor substrate-1 with the sigma3A subunit of the adaptor protein complex-3 in cultured adipocytes." J Biol Chem **273**(45): 29942-9.

Wan, L., S. S. Molloy, et al. (1998). "PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization." Cell **94**(2): 205-16.

Warner, T. S., D. A. Sinclair, et al. (1998). "The light gene of Drosophila melanogaster encodes a homologue of VPS41, a yeast gene involved in cellular-protein trafficking." Genome **41**(2): 236-43.

Wong, D. H., M. J. Ignatius, et al. (1990). "Neuron-specific expression of high-molecular-weight clathrin light chain." J Neurosci **10**(9): 3025-31.

Ybe, J. A., F. M. Brodsky, et al. (1999). "Clathrin self-assembly is mediated by a tandemly repeated superhelix." Nature **399**(6734): 371-5.

Ybe, J. A., B. Greene, et al. (1998). "Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges." Embo J **17**(5): 1297-303.

Yeung, B. G. and G. S. Payne (2001). "Clathrin Interactions with C-Terminal Regions of the Yeast AP-1 beta and gamma Subunits are Important for AP-1 Association with Clathrin Coats." Traffic **2**(8): 565-76.

Yeung, B. G., H. L. Phan, et al. (1999). "Adaptor complex-independent clathrin function in yeast." Mol Biol Cell **10**(11): 3643-59.

Zizioli, D., C. Meyer, et al. (1999). "Early embryonic death of mice deficient in gamma-adaptin." J Biol Chem **274**(9): 5385-90.

Chapter Two

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

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The Neuronal Form of AP-3 is Required for Synaptic Vesicle Formation From Endosomes

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Abstract

Heterotetrameric adaptor complexes vesiculate donor membranes. One of the adaptor complexes, AP-3, is present in two forms; one form is expressed in all tissues of the body, while another is restricted to brain. Mice lacking both the ubiquitous and neuronal forms of AP-3 exhibit neurological disorders which are not observed in mice 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. While 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 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 more complex than in most other cell types (Morris and Schmid 1995). While neurons use basically the same machinery as non-neuronal 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, Schroder et al. 1993), auxilin (Ahle and Ungewickell 1990; Maycox, Link et al. 1992), intersectin (Hussain, Yamabhai et al. 1999), dynamin (Faire, Trent et al. 1992; Altschuler, Barbas et al. 1998), and the clathrin light chains, LCa and LCb, (Jackson, Seow 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 which help 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 ($\beta1$, $\beta2$, $\beta3$, or $\beta4$, respectively), a medium-sized subunit ($\mu1$, $\mu2$, $\mu3$, or $\mu4$), and a small subunit ($\sigma1$, $\sigma2$, $\sigma3$, or $\sigma4$). While all the adaptor protein complexes function similarly to vesiculate membranes, their specificity may be

due to their proper targeting to the donor compartment. For instance, AP-1 is involved in trafficking from the TGN while AP-2 is involved in endocytosis from the plasma membrane. AP-1 is predominantly localized to the TGN while AP-2 is mainly 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, Hunt et al. 1995; Hirst and Robinson 1998). The other adaptor complexes AP-3 and AP-4 have both 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 which are referred to as β3B (β-NAP, (Newman, McKeever 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 if 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, Odorizzi et al. 1997; Stepp, Huang et al. 1997), flies (Ooi, Moreira et al. 1997; Mullins, Hartnell et al. 1999; Kretzschmar, Poeck et al. 2000), and mammals (Le Borgne, Alconada et al. 1998; Yang, Li et al. 2000). Several mouse mutants in AP-3 have previously been characterized.

Two AP-3 mutant mice, the *pearl* mouse (β3A mutant) (Feng, Seymour et al. 1999; Richards-Smith, Novak et al. 1999; Feng, Rigatti et al. 2000) and the *mocha* mouse (δ mutant) (Kantheti, Qiao et al. 1998) are members of the platelet storage pool deficiency (SPD) class of mutants (Swank, Novak 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, Qiao et al. 1998; Zhen, Jiang et al. 1999). While 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, whose δ mutation leads to a virtual null of all AP-3 expression in all tissues including brain, has balance problems, hearing problems leading to deafness, is hyperactive, undergoes seizures, and has abnormal theta rhythms (Kantheti, Qiao et al. 1998; Miller, Burmeister et al. 1999; Vogt, Mellor et al. 2000). In addition, a knockout of one of the neuronal AP-3 subunits, μ3B, shares some of the neurological defects seen in the *mocha* mouse (manuscript in preparation, Nakatsu and Ohno). 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 ADP ribosylating factor (ARF) (Faundez, Horng et al. 1997) in the biogenesis of a class of synaptic vesicles, often called synaptic-like microvesicles (SLMVs), from endosomes (Faundez, Horng 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, Chang 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, Horng 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, could then 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 provided 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.

Results

AP-3 is required for SLMV formation

It has previously been demonstrated that AP-3 has a role in the budding of synaptic-like microvesicles (SLMVs) from endosomes (Faundez, Horng et al. 1998). In order to establish a requirement for AP-3 in this pathway, we took advantage of the naturally occurring SPD mutant *mocha* mouse, which lacks all AP-3 expression, in the *in vitro* reconstitution of SLMV biogenesis (Desnos, Clift-O'Grady et al. 1995). For this *in vitro* reconstitution a PC12 cell line is 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, Desnos et al. 1998). To label endosomes the cells are incubated with an antibody, [^{125}I]-KT3 which recognizes the TAg, at 15 °C prior to 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 comigrates with synaptic vesicle markers after velocity sedimentation. SLMVs are also produced when the brain cytosol is replaced with purified AP-3 and recombinant ARF1 (Faundez, Horng 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 (Figures 1A and 1B). Adding back brain purified AP-3 to *mocha* cytosol rescues the defect in budding (Figures 1A and 1B). We compared the activity of *mocha* cytosol to the activity in cytosol immunodepleted of all AP-3 by using an antibody to σ3. Immunodepleting σ3 should remove all

AP-3 activity, neuronal and ubiquitous. As with *mocha*, budding activity of this cytosol is also reduced to 50% of wild type (Figures 1C and 1D). These results verify that SLMV biogenesis from endosomes is dependent upon 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, ARF 1 and phosphorylation by a casein kinase 1 α-like activity, have already been described (Faundez, Horng 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 σ3 depletion as well as the *mocha* mutation removes all AP-3 complexes, neuronal and ubiquitous, the form(s) of the AP-3 complex that functions in SLMV biogenesis was unclear.

Production of β3B specific antibody

We generated a tool to immunodeplete neuronal AP-3, as well as 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. While 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, Ohno et al. 1997; Dell'Angelica, Ooi et al. 1997). 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 (Figure 2A). By Western blot, anti-β3B recognized a band of approximately 140 kDa present in brain and not in liver (Figure 2B), as well as in brain purified AP-3 (Figure 2C). When the antibody was pre-incubated with 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 pre-incubated the antibody with GST-β3B hinge, our antibody could no longer recognize brain AP-3 by Western blot (Figure 2C) because it had been competed away by GST-β3B. The low molecular weight band that occasionally was detected in Westerns was a result of non-specific binding (Fig 2B). GST-β3B did not inhibit binding to the non-specific 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 (Figure 2D).

Formation of synaptic vesicles from an endosome is dependent upon 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 (Figure 3A inset). This cytosol which lacked only neuronal AP-3 was then used in our *in vitro* budding assays,

and compared with cytosol immunodepleted with the σ3 antibody, which removes all AP-3 complexes, in our assays. We found that cytosol depleted only of neuronal AP-3 complexes showed the same 50% reduction in SLMV biogenesis as cytosol depleted of all AP-3 (Figure 3A). We also tested brain cytosol from μ3B knockout mice, as compared to the heterozygote littermates. The cytosol from the mice that lacked μ3B also showed a 50% reduction in SLMV biogenesis (Figure 3B). Together, these data strongly suggest that synaptic vesicle budding from endosomes is due solely to the neuronal form of the AP-3 complex, since the removal of all AP-3 complexes led to the same reduction of SLMV production as specific removal of the neuronal form. To further examine the specificity for the neuronal complex, the same budding assays were performed using 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 perhaps be performing the same role as their

ubiquitous counterparts but 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 if 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 as compared to 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 knockout and the β3B depletions where neuronal AP-3 is removed, the levels of δ (Figure 4A) and σ3 (Figure 4B) were essentially 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 knockout mice (Figure 4A). 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 is in agreement with published work that examined the levels of AP-3 in brains of a β3A-knockout mouse (Yang, Li et al. 2000). In the β3A-knockout, there was a great reduction of AP-3 subunit levels in the brain, which also supports 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. While 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

In order 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, Horng et al. 1998; Salem, Faundez et al. 1998). Briefly, in the assay, the vesicles were purified by velocity sedimentation from homogenates of cells (N49A VAMP-TAg PC12) labeled with [^{125}I]-KT3 at 15°C. They were then 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, while 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 (Figure 5D), indicated by their failure to change in density. This demonstrates that AP-3 is necessary to provide the coat. In order to determine whether or not the only coat that could be recruited to vesicles was the neuronal form of AP-3, we tested whether or not 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 (Figures 5A and 5B). We also tested the μ3B knockout mouse cytosol in the assay. Cytosols from both the heterozygote and the knockout mice could provide coat to the vesicles (Figure 5C).

Ubiquitous AP-3 can only bind purified vesicles under conditions where neuronal AP-3 is removed from brain cytosol. Using normal brain cytosol where 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. While we can get ubiquitous AP-3 to bind to purified synaptic vesicles, the ubiquitous complex cannot function 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 since 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, while we saw no staining in non-neuronal cells (data not shown). Thus our antibody appears specific for neuronal, or neuroendocrine, cells. The staining for β3B was blocked if 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 6A, 6F) and largely absent from tips (Figure 6A, 6E), while synaptotagmin, a good marker for the AP-2/clathrin pathway (Figure 6B, 6D), 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 (Jarousse and 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, Faundez et al. 1998).

Our data is also supported by previous work that examined neurotransmitter release along processes of developing axons. While release at the terminals was not Brefeldin A (BFA) sensitive, suggestive of an AP-2

mechanism, release along the process was inhibited, indicative of an AP-3-like mechanism. (Zakharenko, Chang et al. 1999)

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

Neuronal AP-3 distribution

We wanted next to examine the distribution of neuronal AP-3 in intact brain tissue, as compared to 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. While β3B was not expressed in all regions of the brain, it was widely expressed and appeared predominantly in processes rather than in cell bodies (Figures 7A,

7B, and data not shown). Its staining was in general similar to that of synaptophysin, a synaptic vesicle marker (Figures 7C and 7D), although differences were noted. Comparing 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 (Figure 7B), while synaptophysin staining is more even throughout the hippocampus. Staining for β3B could be blocked by preadsorbing the antibody with either the GST fusion protein used to generate the antibody (Figures 7E and 7F), 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 using antibodies against β-NAP, identified from a human patient with autoimmune neurological degeneration (Newman, McKeever et al. 1995). This suggests that while AP-3 knockouts are viable, nAP-3 plays a global, though non-essential, role in the brain and is enriched in certain pathways.

Discussion

While multiple isoforms of adaptor complex subunits have been identified (Takatsu, Sakurai et al. 1998; Folsch, Ohno et al. 1999; Ohno, Tomemori et al. 1999; Meyer, Zizioli 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 (SLMV). 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 ubiquitous AP-3 is involved in.

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, Ball et al. 1993; Page and Robinson 1995). AP-2 is normally associated with plasma membranes and AP-1 with the TGN. Ubiquitous AP-3 has also 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, Horng et al. 1998; Lichtenstein, Desnos 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. While 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 results obtained using the synaptic vesicle binding assay have always been in agreement with those obtained using the vesiculation assay. Both assays share temperature sensitivity (Faundez, Horng et al. 1998), require a casein kinase 1α –like activity (Faundez and Kelly 2000) and are inhibited by tetanus toxin (Salem, Faundez et al. 1998). Both work well with brain cytosol from *pearl* mice, deficient in the ubiquitous form of AP-3, and not at all with cytosol from *mocha*, lacking 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 (Faundez and 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, Ball et al. 1993). The AP-3s may first 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 participate 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, Faundez et al. 1998).

Knowing that neuronal AP-3 is specifically required 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 the major one uses AP-2 and clathrin to form synaptic vesicles directly from the plasma membrane (Murthy and Stevens 1998; Shi, Faundez et al. 1998; Vogt, Mellor et al. 2000). Supporting evidence for two populations of synaptic vesicles comes mainly from developmental studies. Synaptic vesicle recycling is reportedly blocked by tetanus toxin at synapses, whereas vesicle recycling before synaptogenesis is not (Verderio, Coco et al. 1999), suggesting a change in vesicle composition. Quantal release of neurotransmitter from synaptic sites was also distinguished from non-synaptic release by Popov and colleagues (Zakharenko, Chang et al. 1999). Vesicular release along the axons of developing frog motoneurons in culture were sensitive to Brefeldin A while quantal release from the nerve termini was BFA insensitive. Since the AP-3-mediated production of SLMVs is also inhibited by Brefeldin A , the latter results link non-synaptic production of synaptic vesicles to neuronal AP-3. Consistent with these observations we find that the tips of processes lack AP-3 although they are rich in synaptotagmin. One possibility is that synaptic vesicle proteins that escape the normal, non-endosomal route of recapture are internalized into axonal endosomes and retrieved by the AP-3 route (Figure 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 non-endosomal 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 readily seen 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, Krishnan et al. 1994). When preparations are returned to permissive conditions the membranes utilize 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 (Figure 8). Membrane retrieval of this type has been detected in PC12 cells transfected with a chimeric P-selectin (Blagoveshchenskaya, Norcott 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 out of a degradative pathway and allow them to be incorporated into the standard synaptic vesicle recycling mode.

The distribution of neuronal AP-3 in the brain shows that while there is some overlap in its expression with synaptophysin, it is not identical. A backup retrieval pathway or large dense core vesicle 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 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 μ3B knockout 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.

Materials and Methods

Reagents

[^{125}I]Na, ECL reagents, and Protein G-Sepharose were obtained from Amersham Pharmacia. ATP, creatine phosphate, and creatine kinase were obtained from Boehringer Mannheim Corp. Geneticin and IPTG were purchased from GIBCO BRL. Superfrost/Plus slides and Lab-Tek chamber slides were received from Fisher. The Vectastain ABC kit was obtained from Vector Labs. Rat and mouse brains were obtained from Pel-Freez. Female Sprague-Dawley rats were obtained from Bantin and Kingman. Cell culture media and reagents were purchased from the University of California San Francisco Cell Culture Facility. Collagen was purchased from Collaborative Biomedical Products. GTPγS, glutathione agarose, DAB tablets, H_2O_2, and other reagent grade chemicals were obtained from Sigma.

Cell Culture

Wild type and stably transfected N49A 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 to 18 hours before experiments with 6mM sodium butyrate to induce VAMP-TAg expression. Differentiated PC12 cells were grown on Lab-Tek chambers coated with Collagen (75 μg/ml) and Poly Lysine (50 μg/ml). They were grown in Low Serum Medium (DME H-21 containing with 1% horse serum, 0.5% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin, and 0.25 mg/ml geneticin for N49A cells) supplemented with Nerve Growth Factor (50ng/ml). Cells were differentiated on average between eight to eleven days.

Production of GST Fusion Proteins

To prepare a GST fusion protein containing a segment of the β3B hinge domain, complementary oligonucleotides containing sequence from the hinge domain with overhanging restriction sites were annealed with one another and ligated into the pGEX-2T vector (Amersham Pharmacia). The inserts were cloned in frame into the BamHI-EcoRI cloning sites of the vector. The DNA sequence was confirmed from sequencing by the UCSF BRC sequencing facility. The fusion protein was expressed in *Escherichia coli* cells and then purified using glutathione agarose beads following manufacturer's instructions.

Antibodies

Polyclonal antibodies to β3B were raised in rabbits by immunization with the GST-β3B hinge (Alpha Diagnostics Inc.). Polyclonal pan μ3 and pan σ3 antibodies were similarly prepared but against GST fusion proteins containing

regions residues 393-404 of rat p47a (μ3A) and residues 16-180 of σ3B, respectively. Monoclonal antibodies to synaptophysin (SY38) were purchased from Boerhinger Mannheim. The monoclonal antibody to the clathrin light chain (neuronal variant) was purchased from Synaptic Systems. Monoclonals to δ, μ3 (p47A) and σ3 were purchased from Transduction Labs. Biotinylated Goat anti Rabbit IgG (H+L) was purchased from Vector. KT3 monoclonal antibody against the T antigen (TAg) epitope tag was prepared as described. The polyclonal synaptophysin antibody is from ZYMED. The monoclonal synaptotagmin antibody is purified from hybridoma cell lines obtained from Dr. Reinhard Jahn. Affinity purified Donkey anti Rabbit IgG (H+L) HRP and affinity purified Donkey anti Mouse IgG (H+L) HRP were purchased from Jackson Labs. The secondary antibodies Texas-red conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-mouse used for immunofluorescence were purchased from Cappel.

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 previously described in (Faundez, Horng et al. 1997).

Cell-Free SV Biogenesis Assay

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

SV 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 previously described in (Faundez, Horng et al. 1998; Faundez and Kelly 2000).

Immunofluorescence

Differentiated PC12 cells were washed three times in PBS and fixed in 4% paraformaldehyde for 20 minutes. The slides were then washed in 25mM

glycine/PBS and blocked for one hour in 2% BSA, 1% fish skin gelatin, and 0.02% saponin in PBS (block solution). The slides were then incubated in their respective primary antibodies for 90 minutes at room temperature and subsequently washed three times in block solution, after which they were incubated in secondary antibody for one hour at room temperature. They were then washed three times in block solution and then two times in PBS.

Immunohistochemistry

Adult rat brain sections were generously provided by Dr. Matt Troyer (UCSF). The perfused tissue (4% paraformaldehyde) was cut into 40 μm 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 minutes at room temperature. Tissue was then washed in cmf PBS and blocked in Buffer B (0.2% Triton X-100, 10% Normal Goat Serum, cmf PBS) for one hour at room temperature. Sections were then 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 then thoroughly washed in Buffer C for 60 minutes between five and seven times, and then once for 60 minutes in Buffer B. Sections were then incubated overnight at 4°C in secondary antibody diluted in Buffer C. The following day, sections were again washed 6 X 60 minutes in Buffer C, then washed twice in cmf PBS. Sections were then incubated in the ABC Vectastain mix (according to manufacturers' instructions) for 30 minutes at room temperature. Fresh DAB was prepared and 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 following day in EtOH followed by Xylene.

Transgenic mouse

The μ3B knockout mouse used here expresses no detectable μ3B 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 (Nakatsu et al., manuscript in preparation).

Figure Legends

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 minutes. (A) *Mocha* mice brain cytosol shows a 50% reduction in the production of synaptic vesicles from the donor endosome compartment as compared to wild type brain cytosol. *Mocha* cytosol supplemented with brain-purified AP-3 rescued the defect in budding, returning vesicle production to wild type levels. Data shown represent an average +/- standard error of the mean (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, represents the newly budded pool of synaptic vesicles, while the label on the right is free antibody. (C) The *in vitro* budding assays were performed with brain cytosol depleted for the σ3 subunit. The results show a 50% reduction in synaptic vesicle biogenesis, as compared to wild type budding production. (n=3). (D) A representative assay when cytosol is depleted of σ3. The fractions collected from the gradient are shown along the X axis. Here, a no cytosol

control (□), wild type brain cytosol (◆), 4 degree rat brain cytosol (●), and brain cytosol immunodepleted using the σ3 antibody (▲) were tested. When AP-3 is removed, the height of the peak is 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 by 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 non-specifically 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 was also preincubated with either purified β3A hinge (β3Ah) or with purified β3B hinge (β3Bh) and then used for Western blots. Anti-β3B recognizes the neuronal subunit as well as antibody preincubated with β3A hinge. Antibody preadsorbed with β3B hinge can no longer bind the neuronal subunits on blots, showing its specificity. The β3B subunit often appears as a doublet in purified AP-3, perhaps due to 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 using cytosol either mock depleted (wild type budding) or cytosol immunodepleted for β3B, for σ3. The inset shows immunoblots of either mock (+) or (-) immunodepleted cytosols. The top blot was probed for β3B in either mock or immunodepleted, and the lower 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 as compared to wild type levels of synaptic vesicle production. (B) *In vitro* budding assays were also performed using cytosol from mice heterozygous for μ3B, and for mice that lacked all expression of the μ3B subunit. While the heterozygote cytosol showed robust SLMV biogenesis, the knockout mouse cytosol showed a 50% reduction in budding as compared to cytosol from its heterozygous littermate.

Figure 4

Neuronal AP-3 is not the major form of AP-3 in brain. (A) Brain cytosol from heterozygotes of μ3B (+/-) and knockouts for the neuronal μ3B subunit were run on SDS-PAGE gels. To determine if 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 unchanged in the knockout as compared to 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 no more than that in the knockout, which contains 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, were also compared in mock depleted cytosol versus cytosol immunodepleted for β3B. Equal amounts of protein were run in each lane. While β3B is removed in the depleted cytosol, levels of σ3 are unchanged from the amount in mock depleted cytosol.

Figure 5

AP-3 is necessary to coat synaptic vesicles. (A) Purified synaptic vesicles that are run over sucrose gradients sediment at approximately 22% sucrose. The same vesicles that are incubated with wild type brain cytosol, and 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 fully coat synaptic vesicles. 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, brain cytosol, did not undergo a density shift. Vesicles incubated with either mock depleted rat brain cytosol (RBC) or β3B depleted rat brain cytosol did undergo a density shift. (C) Synaptic vesicles could be fully coated 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 incubating at 37°C, also could not recruit coat.

Figure 6

Neuronal AP-3 is localized to varicosities of neuronal-like processes. (A) Differentiated PC12 cells were stained using the β3B antibody. While there was no specific staining in the cell body (inset), we observed staining in the varicosities along the processes, yet absent at the tips. (B) Differentiated cells were double stained for synaptotagmin and the staining was in contrast to that seen with the β3B antibody. Synaptotagmin staining is most intense at tips of processes. (C) Differentiated PC12 cells were also stained for the δ subunit of AP-3, a subunit present in all AP-3 complexes. δ staining is seen in varicosities, as for β3B, but in addition there is punctate staining in the cell body. (D) A representative tip of a differentiated PC12 cell stained with the 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 entiched in δ expression.

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, as well as the stratum oriens, stratum radiatum, and the molecular layer of the dentate gyrus. (C) Adjacent adult rat brain sections were stained for synaptophysin immunoreactivity. Synaptophysin is also 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, while it appears to label the mossy fiber pathway more intensely than neuronal AP-3. (E) Adult rat brain sections were stained using the β3B antiserum that had been preadsorbed with the GST fusion protein the antibody was made against. No immunoreactivity is observed. (F) A close-up view of the hippocampus also shows no staining observed in the negative control.

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 through the AP-2/clathrin pathway of endocytosis (arrow A), but escape recovery at the plasma membrane and may recycle through the AP-3 pathway. Such synaptic vesicle proteins may be retrieved into specialized axonal endosomes which use neuronal AP-3 to bud synaptic vesicles (arrow B). The endosomal pathway of synaptic vesicle production may also function to recycle components of Large Dense Core Vesicles (LDCVs). LDCV proteins recycle through 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 which 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).

Ahle, S. and E. Ungewickell (1990). "Auxilin, a newly identified clathrin-associated protein in coated vesicles from bovine brain." J Cell Biol **111**(1): 19-29.

Altschuler, Y., S. M. Barbas, et al. (1998). "Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms." J Cell Biol **143**(7): 1871-81.

Ball, C. L., S. P. Hunt, et al. (1995). "Expression and localization of alpha-adaptin isoforms." J Cell Sci **108**(Pt 8)(3): 2865-75.

Blagoveshchenskaya, A. D., J. P. Norcott, et al. (1998). "Lysosomal targeting of P-selectin is mediated by a novel sequence within its cytoplasmic tail." J Biol Chem **273**(5): 2729-37.

Clift-O'Grady, L., C. Desnos, et al. (1998). "Reconstitution of synaptic vesicle biogenesis from PC12 cell membranes." Methods **16**(2): 150-9.

Cowles, C. R., G. Odorizzi, et al. (1997). "The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole." Cell **91**(1): 109-18.

Dell'Angelica, E. C., H. Ohno, et al. (1997). "AP-3: an adaptor-like protein complex with ubiquitous expression." Embo J **16**(5): 917-28.

Dell'Angelica, E. C., C. E. Ooi, et al. (1997). "Beta3A-adaptin, a subunit of the adaptor-like complex AP-3." J Biol Chem **272**(24): 15078-84.

Desnos, C., L. Clift-O'Grady, et al. (1995). "Biogenesis of synaptic vesicles in vitro." J Cell Biol **130**(5): 1041-9.

Faire, K., F. Trent, et al. (1992). "Analysis of dynamin isoforms in mammalian brain: dynamin-1 expression is spatially and temporally regulated during postnatal development." Proc Natl Acad Sci U S A **89**(17): 8376-80.

Faundez, V., J. T. Horng, et al. (1997). "ADP ribosylation factor 1 is required for synaptic vesicle budding in PC12 cells." J Cell Biol **138**(3): 505-15.

Faundez, V., J. T. Horng, et al. (1998). "A function for the AP3 coat complex in synaptic vesicle formation from endosomes." Cell **93**(3): 423-32.

Faundez, V. V. and R. B. Kelly (2000). "The AP-3 complex required for endosomal synaptic vesicle biogenesis is associated with a casein kinase Ialpha-like isoform." Mol Biol Cell **11**(8): 2591-604.

Feng, L., B. W. Rigatti, et al. (2000). "Genomic structure of the mouse Ap3b1 gene in normal and pearl mice." Genomics **69**(3): 370-9.

Feng, L., A. B. Seymour, et al. (1999). "The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness." Hum Mol Genet **8**(2): 323-30.

Folsch, H., H. Ohno, et al. (1999). "A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells." Cell **99**(2): 189-98.

Hirst, J. and M. S. Robinson (1998). "Clathrin and adaptors." Biochim Biophys Acta **1404**(1-2): 173-93.

Hussain, N. K., M. Yamabhai, et al. (1999). "Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells." J Biol Chem **274**(22): 15671-7.

Jackson, A. P., H. F. Seow, et al. (1987). "Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments." Nature **326**(6109): 154-9.

Kantheti, P., X. Qiao, et al. (1998). "Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles." Neuron **21**(1): 111-22.

Kirchhausen, T. (1999). "Adaptors for clathrin-mediated traffic." Annu Rev Cell Dev Biol **15**: 705-32.

Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**(9): 699-727.

Kretzschmar, D., B. Poeck, et al. (2000). "Defective pigment granule biogenesis and aberrant behavior caused by mutations in the Drosophila AP-3beta adaptin gene ruby." Genetics **155**(1): 213-23.

Kuromi, H. and Y. Kidokoro (1998). "Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive Drosophila mutant, shibire." Neuron **20**(5): 917-25.

Le Borgne, R., A. Alconada, et al. (1998). "The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins." J Biol Chem **273**(45): 29451-61.

Lichtenstein, Y., C. Desnos, et al. (1998). "Vesiculation and sorting from PC12-derived endosomes in vitro." Proc Natl Acad Sci U S A **95**(19): 11223-8.

Maycox, P. R., E. Link, et al. (1992). "Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling." J Cell Biol **118**(6): 1379-88.

Meyer, C., D. Zizioli, et al. (2000). "mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors." Embo J **19**(10): 2193-203.

Miller, C. L., M. Burmeister, et al. (1999). "Hippocampal auditory gating in the hyperactive mocha mouse." Neurosci Lett **276**(1): 57-60.

Morris, S. A. and S. L. Schmid (1995). "Synaptic vesicle recycling. The Ferrari of endocytosis?" Curr Biol **5**(2): 113-5.

Morris, S. A., S. Schroder, et al. (1993). "Clathrin assembly protein AP180: primary structure, domain organization and identification of a clathrin binding site." Embo J **12**(2): 667-75.

Mullins, C., L. M. Hartnell, et al. (1999). "Defective expression of the mu3 subunit of the AP-3 adaptor complex in the Drosophila pigmentation mutant carmine." Mol Gen Genet **262**(3): 401-12.

Munoz, D. G. (1990). "The distribution of chromogranin A-like immunoreactivity in the human hippocampus coincides with the pattern of resistance to epilepsy-induced neuronal damage." Ann Neurol **27**(3): 266-75.

Murthy, V. N. and C. F. Stevens (1998). "Synaptic vesicles retain their identity through the endocytic cycle." Nature **392**(6675): 497-501.

Newman, L. S., M. O. McKeever, et al. (1995). "Beta-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein." Cell **82**(5): 773-83.

Ohno, H., T. Tomemori, et al. (1999). "Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells." FEBS Lett **449**(2-3): 215-20.

Ooi, C. E., J. E. Moreira, et al. (1997). "Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the Drosophila eye color mutant garnet." Embo J **16**(15): 4508-18.

Page, L. J. and M. S. Robinson (1995). "Targeting signals and subunit interactions in coated vesicle adaptor complexes." J Cell Biol **131**(3): 619-30.

Pearse, B. M. and M. S. Robinson (1990). "Clathrin, adaptors, and sorting." Annu Rev Cell Biol **6**(9): 151-71.

Ramaswami, M., K. S. Krishnan, et al. (1994). "Intermediates in synaptic vesicle recycling revealed by optical imaging of Drosophila neuromuscular junctions." Neuron **13**(2): 363-75.

Richards-Smith, B., E. K. Novak, et al. (1999). "Analyses of proteins involved in vesicular trafficking in platelets of mouse models of Hermansky Pudlak syndrome." Mol Genet Metab **68**(1): 14-23.

Robinson, M. S. (1993). "Assembly and targeting of adaptin chimeras in transfected cells." J Cell Biol **123**(1): 67-77.

Salem, N., V. Faundez, et al. (1998). "A v-SNARE participates in synaptic vesicle formation mediated by the AP3 adaptor complex." Nat Neurosci **1**(7): 551-6.

Seaman, M. N., C. L. Ball, et al. (1993). "Targeting and mistargeting of plasma membrane adaptors in vitro." J Cell Biol **123**(5): 1093-105.

Shi, G., V. Faundez, et al. (1998). "Neuroendocrine synaptic vesicles are formed in vitro by both clathrin-dependent and clathrin-independent pathways." J Cell Biol **143**(4): 947-55.

Stepp, J. D., K. Huang, et al. (1997). "The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole." J Cell Biol **139**(7): 1761-74.

Swank, R. T., E. K. Novak, et al. (2000). "Abnormal vesicular trafficking in mouse models of Hermansky-Pudlak syndrome." Pigment Cell Res **13 Suppl 8**(10): 59-67.

Takatsu, H., M. Sakurai, et al. (1998). "Identification and characterization of novel clathrin adaptor-related proteins." J Biol Chem **273**(38): 24693-700.

Verderio, C., S. Coco, et al. (1999). "Tetanus toxin blocks the exocytosis of synaptic vesicles clustered at synapses but not of synaptic vesicles in isolated axons." J Neurosci **19**(16): 6723-32.

Vogt, K., J. Mellor, et al. (2000). "The actions of synaptically released zinc at hippocampal mossy fiber synapses." Neuron **26**(1): 187-96.

Yang, W., C. Li, et al. (2000). "Defective organellar membrane protein trafficking in Ap3b1-deficient cells." J Cell Sci **113**(Pt 22)(11): 4077-86.

Zakharenko, S., S. Chang, et al. (1999). "Neurotransmitter secretion along growing nerve processes: comparison with synaptic vesicle exocytosis." J Cell Biol **144**(3): 507-18.

Zhen, L., S. Jiang, et al. (1999). "Abnormal expression and subcellular distribution of subunit proteins of the AP-3 adaptor complex lead to platelet storage pool deficiency in the pearl mouse." Blood **94**(1): 146-55.

Figure 1

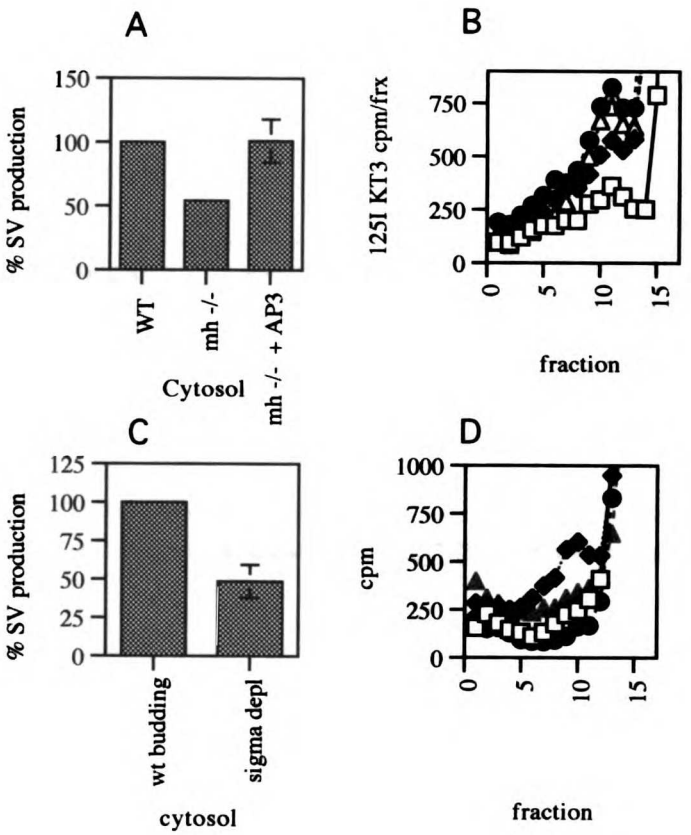
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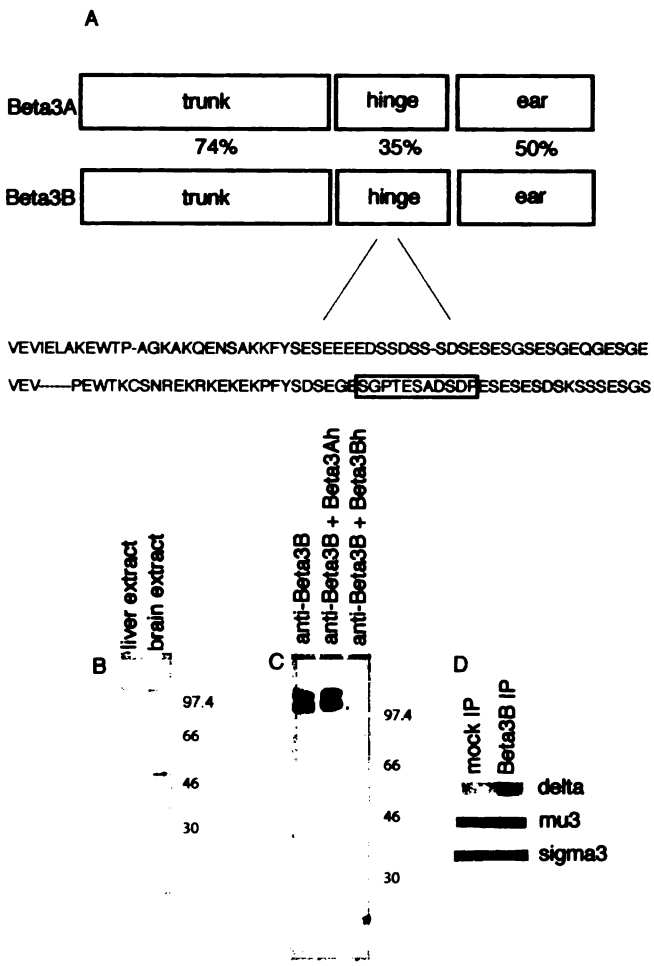
Figure 2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sigma3

Figure 3

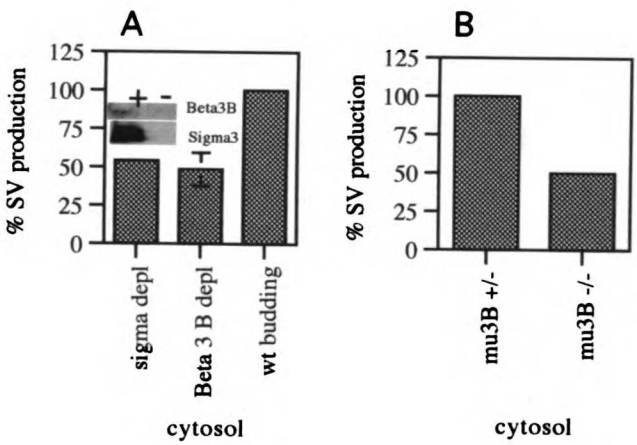

A
% SV production
125
100
75
50
25
0
+ -
Beta3B
Sigma3
+
sigma depl
Beta 3 B depl
wt budding
cytosol
B
% SV production
125
100
75
50
25
0
mu3B +/-
mu3B -/-
cytosol

Figure 4

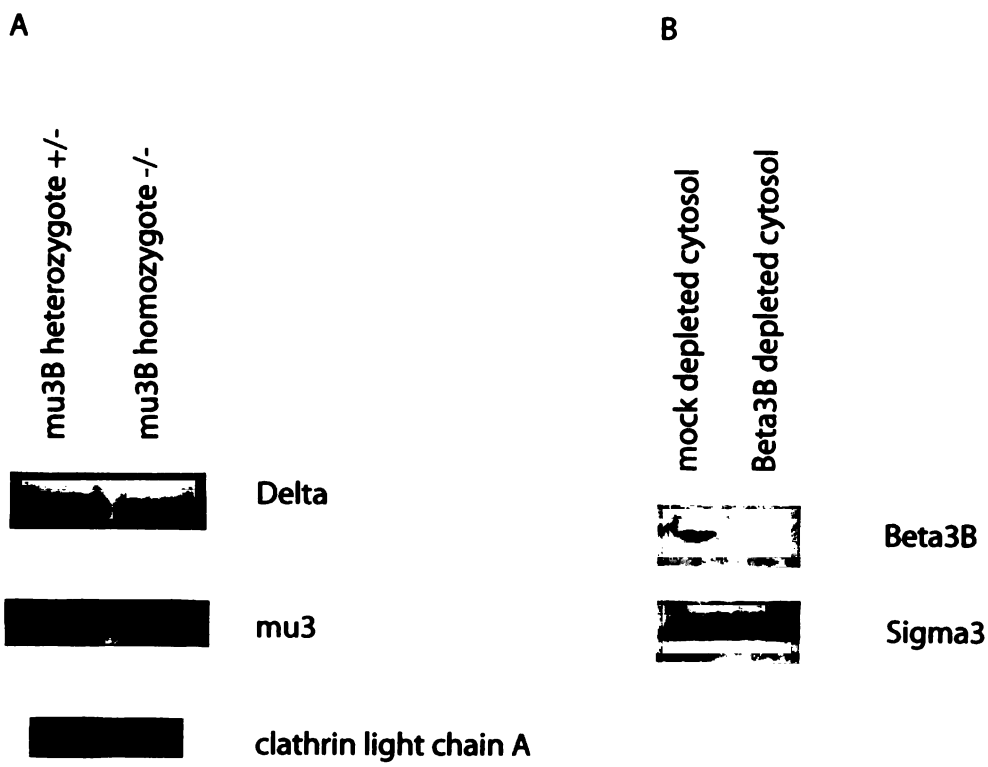

A
mu3B heterozygote +/-
mu3B homozygote -/-
Delta
mu3
clathrin light chain A
B
mock depleted cytosol
Beta3B depleted cytosol
Beta3B
Sigma3

Figure 5

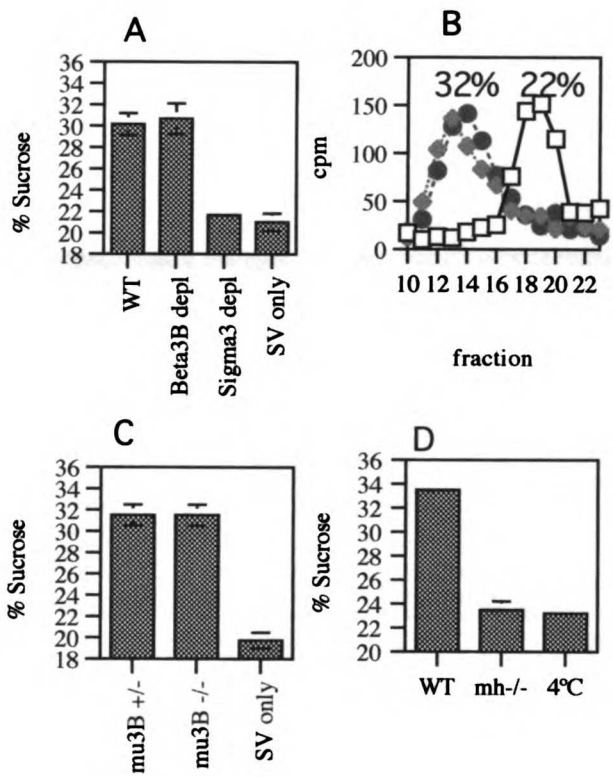

A
% Sucrose
36
34
32
30
28
26
24
22
20
18
WT
Beta3B depl
Sigma3 depl
SV only
B
cpm
200
150
100
50
0
32%
22%
10 12 14 16 18 20 22
fraction
C
% Sucrose
mu3B +/-
mu3B -/-
SV only
D
% Sucrose
WT
mh-/-
4°C

Figure 6

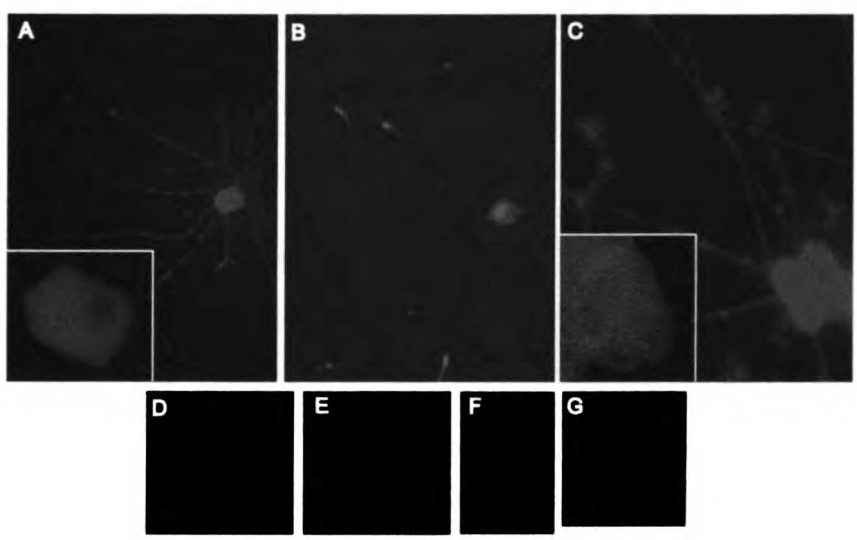


Figure 7

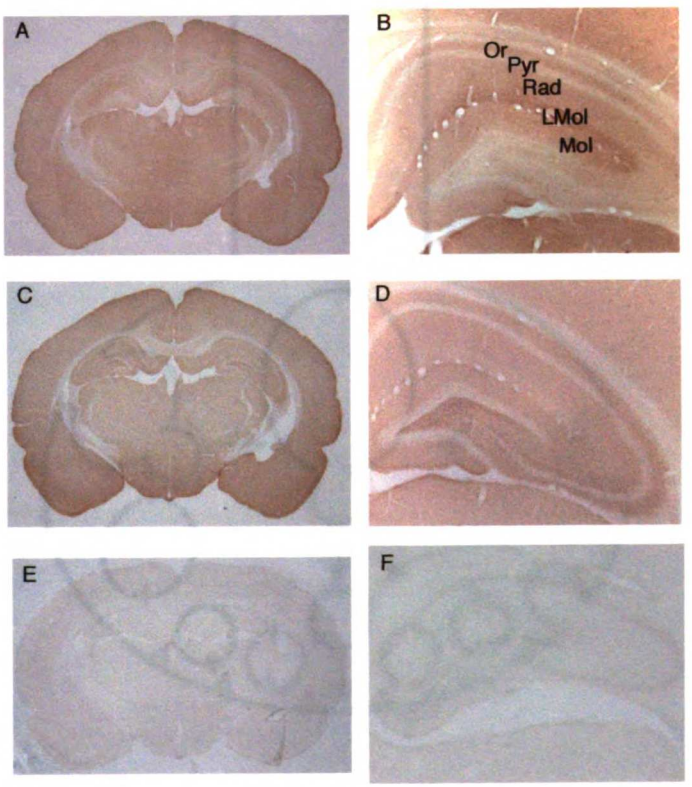
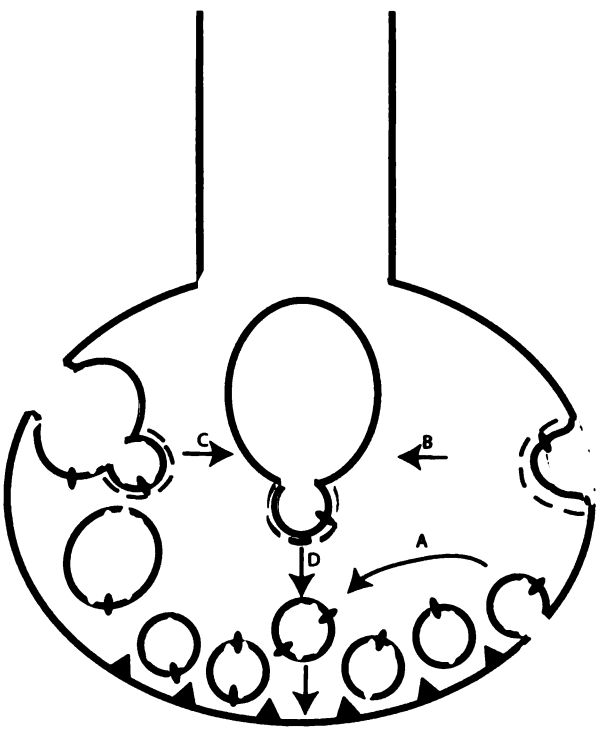


Figure 8



My work has helped us understand why there are different forms of adaptor protein complexes in the brain. I have shown that neuronal AP-3 clearly serves an important function in synaptic vesicle biogenesis, in at least a subset of neurons, and its function is quite separate than that of its ubiquitous AP-3 counterpart. There is more than one way to make a synaptic vesicle, and it also appears there is more than one type of synaptic vesicle. Questions that arise from my work are: why are there separate pools of synaptic vesicles and why are there separate pathways of synaptic vesicle biogenesis? In addition, my work on neuronal AP-3 brings up questions about the complexity of membrane traffic as well as vesicle biogenesis in the brain. The results of my studies, as well as those of others, have exposed the limitations of some in vitro assays to reveal functions of adaptor complexes. They also suggest how specificity is achieved for adaptor protein complexes, why specialized adaptor proteins, as well as brain-specific isoforms exist in neurons, why neuronal AP-3 is important, and finally the synaptic vesicle pathway that is likely to use neuronal AP-3.

There has been some speculation about why alternative or multiple isoforms of membrane trafficking components exist in the brain. In the review, "The Ferrari of Endocytosis (Morris and Schmid 1995)," the authors suggest that the demands of neurotransmission (recycling of synaptic vesicle membranes, etc.) require additional or better machinery than that found in non-neuronal cells. In addition to the numerous membrane trafficking protein isoforms, many other

proteins found in neurons are alternatively spliced or encoded for by separate genes as well. Understanding why there are neuronal isoforms of proteins may provide greater understanding about function in the brain in general beyond just membrane trafficking.

There are several major ways of generating protein diversity: alternative splicing, separate genes encoding distinct proteins, and post-translational modifications. I will now examine several examples of neuronal isoforms, seeking to illustrate how their functions may differ from their non-neuronal counterparts.

I will focus mainly on proteins that are involved in the pathways of synaptic vesicle biogenesis. Most proteins that have been examined function in the endocytotic events, but others will be mentioned as well. In neurons, once an exocytotic event takes place, the fused membrane must be recovered. There are many theories about what happens at this step, and while there is some controversy associated with it, there are some proteins known to be important for the internalization event from the plasma membrane. Many of the proteins required for internalization are known to cluster in nerve terminals, their place of action. Examples of such proteins are dynamin, clathrin, amphiphysin, synaptojanin, and intersectin. While many of these proteins, or other members of the families they belong to also act in compensatory endocytosis in non-neuronal cells, they may provide additional functions in the brain.

Protein diversity can be generated by genetic duplication followed by divergence to produce similar but not identical function. New functions would

seem to be required otherwise the duplicated gene would be lost due to redundancy.

Alternative splicing of single genes is another way to generate diversity. Exons and/or introns are either included or excluded from the RNA that gets translated into the protein product. Tissue-specific alternative splicing is known to generate great protein diversity and can have significant effects on development. In the brain, alternative splicing clearly plays a major role in generating diversity. While the recently sequenced human genome revealed only 30,000 genes, there are hundreds of thousands of proteins expressed in the human brain. The levels of alternative splicing in the brain can be enormous (Grabowski and Black 2001). Why? Perhaps because of its complex development, or brain region-specific and cell type specific functions. In general it appears that isoforms generated by alternative splicing in the brain alter the kinetics of action or affinity of that protein to bind to a partner, something that could be important in rapid neurotransmission. These changes could be in response to sudden and/or increased stimulation (proteins could be produced at the synapse in response as evidence has accumulated to support protein synthesis in axons, (Koenig and Giuditta 1999; Martin, Barad et al. 2000)) or a change in synaptic demands and neurotransmission.

Dynamin 1, synapsins, synaptotagmin 1, synaptojanin, are all examples of proteins that are encoded for by separate genes and have new functions in neurons. While the separate functions are not always clear (dynamin 1 versus dynamin 2) it is clear that some proteins serve similar roles in either neuron-

specific compartments (synaptotagmins I and II) or different roles (the synaptojanins). There are also families of proteins expressed only in the brain but are encoded by separate genes. This is very common for proteins involved in synaptic vesicle traffic, neurotransmitter uptake, or receptors at the synapse. While functional differences have not clearly been identified for most of these proteins, some studies have elucidated some differences among the family members.

Synaptic vesicle proteins, for example, can be divided up into two classes (Fernandez-Chacon and Sudhof 1999). There are those proteins that are involved in the uptake of synaptic vesicle components such as neurotransmitters, and there are those proteins that play some role in the trafficking of the vesicle. Most of the traffic or transport proteins (if not all) are members of gene families with multiple isoforms (the isoforms may be brain-specific but some may also be ubiquitous). Most synaptic vesicle proteins appear to be specific for localization on synaptic vesicles, yet some, such as SCAMP1, or cysteine-string protein (csp) are present on other organelles.

The SCAMPs (secretory carrier membrane proteins) all share a similar protein structure of a cytoplasmic amino terminus consisting of NPF repeats, four transmembrane regions, and a short cytoplasmic tail at the carboxy terminus. SCAMP1 is highly enriched in synaptic vesicles but is also expressed in other non-neuronal cells (Fernandez-Chacon, Achiriloaie et al. 2000). It was thought SCAMP1 was essential for function because it is so enriched in synaptic vesicles, yet when a knockout mouse of SCAMP1 was generated, the resulting

phenotype was weak. Another SCAMP was later identified, SCAMP5, that is expressed only in brain and is enriched in synaptic vesicles (Fernandez-Chacon and Sudhof 2000). While it is unclear the role the SCAMPs play, the essential function in brain may be encoded by SCAMP5, and the other SCAMPs may play slightly overlapping roles, yet not be required in synaptic vesicles or be essential for neuronal function. An interesting thing to note about SCAMP5 is that it, unlike SCAMPs 1-3, does not contain any NPF repeats. NPF motifs bind EH domains and this interaction has been observed for several proteins involved in endocytosis at the plasma membrane. SCAMP5 then may have no role in endocytosis. It is also expressed in the brain quite late developmentally. SCAMP1 is detectable in the earliest embryonic stages observed, while SCAMP5 expression resembles other synaptic vesicle proteins such as synaptotagmin 1 that appear only with the growth of synapses after birth, in this case two weeks. Its expression in the brain as compared to that of SCAMP1 is also distinct. While in brain they are both expressed on synaptic vesicles, their distribution in brain tissue is different. SCAMP1 strongly labels the mossy fibers while SCAMP5 expression, while present in the mossy fibers, is enriched in other layers of the hippocampus a distribution reminiscent of neuronal AP-3 expression. SCAMP5 is also not expressed in neuroendocrine cells such as PC12 cells. This data suggests that the role SCAMP5 plays is really a very specific neuronal one, not like the ubiquitous roles the other SCAMPs play. An unresolved issue is whether all synaptic vesicle proteins essential for targeting or fusion, can also be found on

other subcellular compartments. It is possible that the proteins entirely restricted to synaptic vesicles have not yet been identified.

The rab3 proteins, members of the rab family of GTPases, important in vesicle trafficking, are present in synaptic vesicles. Rab3A, Rab3B, and Rab3C are all found in synaptic vesicles and are important for synaptic vesicle fusion, but their unique functions are unknown. A knockout mouse for Rab3A has been generated and these mice lacked mossy fiber LTP, and showed increased glutamate release (Castillo, Janz et al. 1997; Geppert, Goda et al. 1997). It is possible each Rab3 is important for different fusion events or they individually help define specific populations of synaptic vesicles.

The SV2 protein is also found in synaptic vesicles yet its function remains unknown. To try and determine function, knockout mice were generated for two of the three isoforms of SV2 (SV2A and SV2B). While SV2A knockouts were eventually lethal, SV2B knockouts were not (Janz, Goda et al. 1999). This may be a case where individual SV2 proteins are characteristic of one type of synaptic vesicle and help define populations of vesicles. Perhaps SV2A is associated with the major pathway of synaptic vesicle biogenesis, like AP-2 (which is lethal when not expressed), whereas SV2B could be found in a minor population of synaptic vesicles, perhaps associated with neuronal AP-3. SV2C is a very minor form expressed in the brain and its expression is limited to "phylogenetically old" regions of the brain such as the substantia nigra, midbrain, and olfactory bulb (Janz and Sudhof 1999). When all the SV2 isoforms were compared for expression within the hippocampus, SV2A is expressed throughout the entire

region, SV2B is expressed throughout most of the region, except the mossy fibers/dentate gyrus, and SV2C is not expressed at all in the region. Once again, while the functions of the SV2 proteins remain unclear, the SV2s are important since lack of SV2A leads to seizures in those mice that survive birth. They may share some redundant function, but have some specificity in a particular region of the brain and/or population of synaptic vesicles. This seems to be a recurring theme of proteins in the brain- one of apparent redundancy in function but clear differences and specificity for certain cell types or regions of the brain.

Maybe some synaptic vesicle proteins, such as the synaptotagmins are required to be present in all types of synaptic vesicles. The synaptotagmin gene family consists of proteins with a short amino terminus, a transmembrane domain, and tandem C2 domains and are also important in endocytic events from the plasma membrane. The synaptotagmins are believed to be potential receptors for the adaptor protein AP-2. This family is made up of at least twelve different members. When knockout mice are generated that lack the synaptotagmin I gene, they die shortly after birth. Fast synchronous release of neurotransmitter was reduced in the hippocampal neurons of these mice.

Synaptotagmins I-V and IX-XI are enriched in the brain, while VI-VIII are expressed mainly in non-neuronal tissues (Marqueze, Berton et al. 2000). There is some overlap of expression of the different synaptotagmins in the same regions of the brain, but most of the synaptotagmins may actually serve different functions in neurons. An exception could be synaptotagmins I and II, which share very high homology with one another and may have similar or identical

functions. The two genes' patterns of expression are complementary to one another, supporting the belief that they may serve similar or redundant functions, just in different regions. Yet there are also cases where they are expressed in the same cell and can hetero-oligomerize with one another, which could perhaps modify the function of either of them individually.

The other brain synaptotagmins may serve quite different functions than synaptotagmins I or II. For example, synaptotagmin III, while originally thought to be a synaptic vesicle protein, is now believed not to be, although it is enriched in synapses. Synaptotagmin VI, also found in the brain is not found in synaptic vesicles. Synaptotagmin IV is found in at least two populations of synaptic vesicles, one of which lacks synaptotagmin I. Some studies suggest however that synaptotagmins I and IV are not found in the same regions of the brain, and that synaptotagmin IV is not found in synaptic vesicles of the hippocampus. Synaptotagmin IV expression has also been examined in different stages of developing rat brains.

While synaptotagmins I and II appear to be the only members of this large gene family that function in fast Ca^{2+}-triggered neurotransmitter release, the other members of this family appear to have functions in the same or similar pathways and by hetero-oligomerizing with one another, can modulate their functions. Their patterns of expression, as well as timing of expression, suggest unique and important roles in neurotransmission.

Synaptotagmin family members are also subject to alternative splicing. Exon 2 of synaptotagmin mRNAs encodes the transmembrane domain which is

often skipped or spliced out of the message and not included in the translated product. Some of the synaptotagmins that lack the transmembrane domain have been studied and it appears that they localize differently than the form that contains the transmembrane domain. The alternatively spliced isoform of synaptotagmin I could be localized perhaps to an intracellular compartment, a different synaptic vesicle population, or perhaps colocalize with synaptotagmin I that contains the transmembrane domain. It will be interesting to study such splice isoforms especially as we learn there are multiple routes of internalization from the plasma membrane, as well as multiple routes of traffic once intracellular.

Amphiphysin I and II are other proteins involved in endocytosis from the plasma membrane. They contain SH3 domains through which they interact with dynamin and synaptojanin, and independently of their SH3 domains they interact with AP-2 and clathrin. Amphiphysins I and II form heterodimers and concentrate in nerve terminals. Multiple amphiphysin II splice variants have been identified. Two of these variants contain clathrin binding sites that are not included in the non-neuronal amphiphysin proteins. (The two clathrin-binding domains are found on two separate exons.) There is also a domain within the amino terminus of amphiphysin II, the NTID, that is present in some splice variants. This region is required for targeting to clathrin-coated pits, while the clathrin-binding domains are not. Yet there are some splice variants of amphiphysin II that do not bind clathrin directly but still get targeted to the plasma membrane. This binding is unlikely to be through an AP-2 interaction because the domain of amphiphysin I that binds to AP-2 is not present in an amphiphysin II splice variant that does

target to the plasma membrane. Amphiphysin IIa is the most predominant form found in the brain, and this variant contains both the NTID as well as the clathrin binding domains. Amphiphysin II can also dimerize through its N-terminus, with or without the NTID, but binding is improved with the presence of the NTID. It is possible that while the clathrin binding domains found within some variants of amphiphysin II are not required for membrane targeting, the clathrin domains aid the process. Since amphiphysin is enriched in nerve terminals, endocytosis at the plasma membrane of neurons may require faster kinetics of vesicle recycling, fulfilled or helped by some neuronal variants of amphiphysin. Other factors that also need to be recruited to the plasma membrane may be able to bind amphiphysins that contain both domains better or faster to aid the process which is not required in non-neuronal cells.

Synaptojanin is a PIP 5-phosphatase that is important in signal transduction in both brain and non-neuronal tissues. There are two forms, synaptojanins 1 and 2. The protein consists of three protein domains, an N-terminal domain homologous to the yeast trafficking protein SAC1, a central region that contains the phosphatase activity, and a C-terminal domain enriched in prolines (Haffner, Paolo et al. 2000; Harris, Hartwieg et al. 2000). Synaptojanin 1 is alternatively spliced to produce a short protein in the brain, and a longer form in other tissues. In the brain a stop codon after the proline-rich domain is used, while in other tissues this stop codon is spliced out to produce a longer protein. In brain, synaptojanin colocalizes with dynamin in nerve terminals. Dynamin and synaptojanin interact with the SH3 domains of

amphiphysin, through their proline-rich domains. The 170 kDa protein expressed in non-neuronal cells has different membrane binding properties than that of the brain enriched 145 kDa isoform. Interestingly, the expression of the alternatively spliced isoforms also falls under developmental regulation. In rat E12 brains, only the 170 kDa isoform is detected, while in E16 and E18 brains, both 145 kDa and 170 kDa forms are present. Yet in adult brains, levels of the short form increase dramatically while the longer form is no longer detectably expressed. A similar alternative splicing occurs for synaptojanin 2, generating a short and a long form based on splicing at the C-terminus, with similar distributions of expression of the isoforms. The alternative splicing of synaptojanin 2 also appears under developmental regulation. No splicing was detected in early development, until much later than synaptogenesis (the first week of life). In mice postnatal days 9 and 13, splicing was first observed in the cortex and cerebellum, then several days later in the olfactory bulb.

Recently, a new splice variant of synaptojanin was identified (Nemoto, Wenk et al. 2001). Synaptojanin 1, as discussed above is important in synaptic vesicle recycling while synaptojanin 2 (now classified as 2A) is localized to the mitochondria. Synaptojanin 2B seems to share overlapping function with synaptojanin 1. Synaptojanin 2B also undergoes further alternative splicing to generate at least two variants, 2B1 and 2B2. Synaptojanin 2B1 binds to amphiphysin while synaptojanin 2B2 can bind both amphiphysin and endophilin. Synaptojanin 1 can also bind both amphiphysin and endophilin. This is a good example of how alternative splicing generates proteins that function differently,

here in a way that could significantly impact endocytosis of synaptic vesicle proteins in the brain.

Intersectin is another protein that is important in endocytosis at the plasma membrane (Yamabhai, Hoffman et al. 1998). Intersectin contains two EH domains, originally identified in Eps15 as the domain that allows its binding to epsin (through NPF domains), another protein involved in endocytosis. Intersectin also contains five SH3 domains, domains which have also been implicated in regulating endocytosis for other proteins, such as amphiphysins I and II. Intersectin is homologous to the Drosophila protein Dap160, which binds dynamin and may serve as a scaffold for endocytosis around sites of exocytosis (Roos and Kelly 1998). And intersectin, like so many proteins, undergoes alternative splicing (Hussain, Yamabhai et al. 1999). This produces a short form, intersectin-s, expressed ubiquitously, and a long form, intersectin-l. The long form contains other domains at the C-terminus such as a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, and C2 domains, which presumably add new functionality to the protein. Intersectin colocalizes with clathrin and may serve as a scaffold for endocytotic proteins. Work is currently being pursued on characterizing the neuronally expressed splice variant, but one could guess that the PH domain might serve as a link to the actin cytoskeleton and that the C2 domain may bind calcium, which is important in synaptic vesicle recycling (De Camilli).

VAMPs, or synaptobrevins, are type II membrane proteins found in vesicles which bind t-SNAREs and SNAP-25 on target membranes. The VAMPs

make up a large protein family that is expressed in all cell types. Different VAMPs are localized to different subcellular compartments, while VAMP was originally identified in synaptic vesicles. VAMP-2 is targeted to synaptic vesicles and synaptic-like microvesicles of the PC12 neuroendocrine cell line. While there are many genes in this family, further diversity of the synaptobrevins is achieved through alternative splicing. Alternative splicing of the VAMPs appears to affect the C-terminus of the protein, which is the found in the lumen of the vesicle/compartment. An additional form of VAMP-2 was identified, called VAMP-2B, which contains an extra intronic sequence in the RNA that gets included (Mandic and Lowe 1999). VAMP-2B and VAMP-2 are both found in PC12 cells, while only VAMP-2 is found in brain. It is possible that both forms of VAMP-2 may sort differently from one another, as do both splice isoforms of VAMP-1 (Mandic, Trimble et al. 1997; Isenmann, Khew-Goodall et al. 1998). Although the current data shows good overlap of both isoforms of VAMP-2 by subcellular fractionation of PC12 cells over equilibrium density and velocity gradients, VAMP-2B may have an altered function or targeting than that of VAMP-2. Such isoform usage could affect the way results are interpreted in results seen from PC12 neuroendocrine cells.

Dynamin, another protein involved in endocytosis from the plasma membrane, is encoded by three distinct genes; and each of these undergoes alternative splicing to produce no fewer than four protein isoforms. Dynamin I expression is limited to neuronal tissue, while dynamin II is ubiquitously expressed and dynamin III is expressed in brain, lung, and testis (Urrutia, Henley

et al. 1997). This means 12 different dynamins could be expressed in brain tissue at any one time. While it is well established that dynamin has a role in clathrin-mediated endocytosis, it is unclear and quite possible that dynamins I and III have more specialized endocytic functions. There are so many additional requirements for membrane trafficking within neurons and so much additional machinery, that Dynamin I could quite possibly function for rapid and efficient recycling of synaptic vesicle membranes after exocytosis. There is some evidence that dynamin I and dynamin II have redundant yet distinct functions in endocytosis (Altschuler, Barbas et al. 1998). Both are involved in receptor-mediated endocytosis from the plasma membrane yet they are targeted to and function within different subdomains. How they do this, what is different about the vesicles they help internalize, and how they recognize different components of the machinery will be important to know.

Two of the subunits of the adaptor protein AP-2, αA and β2 also undergo alternative splicing which produces isoforms present only in the brain that are slightly longer than the ubiquitously expressed proteins. Both of the clathrin light chains also undergo alternative splicing. No difference in function has been reported for such cases, yet it is quite possible they offer a kinetic advantage for synaptic vesicle recycling in the brain which has been yet to be elucidated.

Many other neuronal proteins are also present in multiple isoforms, either produced from large gene families or by alternative splicing. One example is with N-type calcium channels which are localized to synapses and regulate neurotransmitter release. The α_{1B} subunit couples excitation to neurotransmitter

release (Grabowski and Black 2001). These RNAs are subject to alternative splicing in the brain. When the N-type calcium channel undergoes alternative splicing, small changes are made to the two extracellular loops of the α_{1B} subunit. One type of change that can occur adds a glutamic acid and a threonine whose presence slows the activation kinetics of the channel, while other alternative splicing events do not affect the kinetics. It is thought that these additional residues in the extracellular loop region may be near the voltage-sensing center of the channel, which could potentially alter the activity of the ion channel. In addition, expression of the channel that contains the ET residues is enriched in sympathetic and sensory ganglia. It is possible that expression of the ET isoform optimizes neurotransmitter release in the central nervous system as compared to the peripheral nervous system.

The NMDA receptors known as R1, which appears important in synaptic plasticity as well as neuronal development, produce a major class of ion channels, localized on post-synaptic membranes (Grabowski and Black 2001). There are at least seven alternatively spliced isoforms of the NMDA R1 receptor. Inclusion of a specific exon allows the protein to cluster on the plasma membrane, as well as bind calcium/calmodulin, bind neurofilaments, and mediate signaling by the receptor. The $GABA_A$ receptor is also alternatively spliced which produces two forms that alter sensitivity to agonists. Dopamine receptors are also found in multiple forms due to alternative splicing. Such splicing alters binding interactions for the receptor, such as different binding of G-protein coupled receptors. Other proteins that are key in the development of neurons

also are subject to alternative splicing such as transcription factors, cell adhesion molecules, and axon guidance factors. The neuron-restrictive silencer factor, NRSF/REST, blocks transcription of neuronal genes in non-neuronal cells and in undifferentiated neurons. The function of NRSF is controlled by its own tissue specific alternative splicing.

Alternative splicing is clearly important for generating protein diversity in neurons, as well as other tissues. Specific isoforms may be expressed in only certain regions of the brain, or they may be expressed only at specific developmental time periods. What will also be interesting to figure out is how neuronal signaling/transmission causes a response in the splicing machinery, causing alternative splicing of specific transcripts.

Yet one conclusion we may be able to draw about alternative splicing in the brain is that while it generates a great deal of protein diversity in the brain, it seems to generate a certain kind of diversity. In all of the known examples of splice isoforms, the neuronal form has basically the same function as the non-neuronal form, just slightly altered. Neuronal trafficking is similar to membrane trafficking in non-neuronal cells, but there are different demands. Synaptic vesicles are a cellular component not found in other cell types, but are similar to other organelles. Neurotransmission does not take place elsewhere, but receptors are found on other cell types. It seems alternative splicing of neuronal RNAs is a way to regulate the timing and/or expression patterns of proteins that function to aid their ubiquitous counterparts. They may also have unique protein interactions that help regulate specificity of function or additional mechanisms in

neurons. This is in contrast to neuron-specific protein isoforms encoded by different genes. In most cases, it appears that these proteins have unique functions in neurons, as with neuronal AP-3 subunits, and that isoforms generated this way cannot be replaced with the non-neuronal proteins.

A review written in 1999, stated that "no two families of SV proteins exhibit the same differential distribution [in the brain]. Therefore, the biological rationale, if any, for multiple isoforms of SV proteins with distinct expression patterns is unclear (Fernandez-Chacon and Sudhof 1999)." This seems shortsighted. The rationale could be that there are many different types of synaptic vesicle proteins, expressed in a complex fashion in order to produce many types of synaptic vesicles in the brain, which is more likely than a simplistic view of one kind of synaptic vesicle biogenesis in the brain or one kind of synaptic vesicle.

There has been much work done and significant evidence accumulated to establish more than one way to generate a synaptic vesicle, as well as synaptic vesicles who have different properties from one another. For years people have tried to establish the pathway of synaptic vesicle biogenesis. Work from the Kelly lab and several others has clearly defined at least two routes of production (Shi, Faundez et al. 1998; Faundez and Kelly 2000). In the brain (although quite minor in PC12 neuroendocrine cells) the predominant pathway of synaptic vesicle biogenesis is from the plasma membrane and utilizes the adaptor protein AP-2 and the coat clathrin, as well as many other proteins involved in receptor-mediated endocytosis. This pathway, sometimes referred to as "kiss and run," is the pathway that recycles synaptic vesicle membranes after exocytosis. It is also

referred to as the direct pathway of synaptic vesicle production. The "reserve pool" of synaptic vesicles that does not fuse with the plasma membrane under stimulation may be generated in an "indirect pathway." This pathway of synaptic vesicle biogenesis goes through an endosomal intermediate (or some cisternal intermediate) before producing a new vesicle. As evidenced by the results I produced in the Kelly lab, at least one pathway of synaptic vesicle production relies upon the neuronal AP-3 adaptor protein.

Along with all of the evidence discussed above that describes the complexity and diversity in the brain, additional data exists which supports multiple types or pools of synaptic vesicles and their pathways of production. Some data also directly supports our work on neuronal AP-3. In Popov's lab, release of acetylcholine along developing axons was examined. They found that release along the developing process was Brefeldin A sensitive, while release at the growth cone/nerve terminal was Brefeldin A resistant (Zakharenko, Chang et al. 1999). This suggests that neuronal AP-3 and AP-2 function in different places, perhaps in different times (developmentally), and for synaptic vesicles that may have different properties. In PC12 cells, the AP-2 pathway of synaptic vesicle production is minor and the AP-3 pathway is predominant, while in the brain it seems the reverse is true. Perhaps this is consistent with the developing axon results from Popov's lab. PC12s may resemble immature neurons and perhaps because they do not form synapses, lack the AP-2 component. The AP-3 pathway is clearly important in the brain, but serves a minor role in actual neurotransmission, (which does not occur in PC12 cells) as compared to AP-2

complexes that are so similar, which they all are, how is specificity achieved, especially when they can traffic to other compartments where other APs are localized? AP-1, AP-2, AP-3, and AP-4 are all heterotetramers with a great deal of homology to one another (each of the subunits). They all recognize the same kinds of sorting signals found within the cytoplasmic tails of cargo they are believed to transport in intermediate vesicles (based on in vitro binding assays such as the yeast two hybrid). By further exploiting the differences between neuronal AP-3 and the ubiquitous complex, we may learn important information about adaptor specificity.

The best way to get that information however appears elusive. What one would like to do is compare the two neuronal subunits of AP-3 to the two ubiquitous ones, and what they each uniquely bind. There should, for example, be proteins on early endosomes, in addition to the synaptic vesicle cargo proteins that bind neuronal AP-3 but not the ubiquitous complex. The ubiquitous complex, however, should bind TGN/endosomal proteins that get targeted to the lysosome. Yet current methods, although providing some information, appear limited in their ability to provide physiological results. Regulation of coat/adaptor binding is often examined by binding to liposomes that do not contain protein components known to bind to adaptors specifically. In addition, binding is measured in the presence of GTPγS or in extreme excess of coat proteins and so does not reveal how the binding actually works *in vivo*. Yeast two hybrid assays have also been used to determine whether or not a subunit of an AP complex binds to a cargo protein, specifically to a tyrosine or dileucine based sorting

signal. These studies have yielded some valuable information. The medium (μ) subunits do bind to tyrosine-containing signals, and crystallographic studies reveal they do so though a binding pocket (Owen and Evans 1998). Yet, this appears to be the limits of what can be learned through the two hybrid system, at least in its current configuration. Normally, either a directed two hybrid is performed, seeing if an interaction exists between an adaptor and an expected cargo protein, or all of the medium subunits of different AP complexes have been tested in their binding to tyrosine based peptides. The problem is, every μ subunit can and does bind to every tyrosine based motif in the two hybrid assay. This is misleading. The μ1A and μ1B sorting differences, described in the Introduction, were discovered by more physiological experiments, but were missed in the yeast two hybrid experiments. Perhaps in the absence of the context of a whole complex, specificity is lost. The liposome studies from the Kornfeld lab seem to contradict with physiological results from the Kelly lab, as well as others. All *in vivo* data has shown that AP-3 does not bind clathrin and that AP-3 does not form clathrin-coated vesicles. Yet liposome binding assays can force AP-3 as well as clathrin to bind to the same vesicle (as well as two hybrid studies that show an interaction with the β3B subunit and clathrin) (Drake, Zhu et al. 2000). Although it is unclear what the best experiments are to do, there are some obvious things to pursue. Could a more physiological binding assay find the real binding differences between neuronal AP-3 and ubiquitous AP-3? Perhaps assays that examined neuronal AP-3 versus ubiquitous AP-3 binding to purified endosomes would reveal differences. Hopefully one would

see something that bound to neuronal but not ubiquitous AP-3. These types of experiments could complement a yeast two hybrid assay done with the neuronal subunits which might identify some important components of the neuronal AP-3 pathway.

Recent work from other labs has also started to explain how adaptors can act specifically, even when they bind the same cargo protein. The mannose six-phosphate receptors (MPRs) transport newly synthesized lysosomal hydrolases from the Golgi to prelysosomal compartments and then return to the Golgi to pick up more enzymes. TIP47 is a protein which binds selectively to the cytoplasmic domains of mannose 6-phosphate receptors and is required for the transport of the MPRs from endosomes back to the Golgi. Studies from the Pfeffer lab have provided evidence to suggest how adaptors can act specifically. If cargo proteins bind other components specifically, and at specific times, the binding of adaptors can be regulated. In this example, MPRs are sorted from multiple compartments in the cell. Different adaptors, specifically in this case AP-1 and AP-2 are involved in the sorting of MPRs. How is it that the correct AP binds only at the right time? A clue comes from work that looked at binding of TIP47 to cation-independent MPRs. Because binding sites for AP-2 on MPRs are in the C-terminus of CI-MPR, close to the TIP47 binding site, when AP-2 binds, TIP47 cannot, leaving only a binding site for AP-1. Thus when AP-2 does bind and TIP47 cannot, even if AP-1 does bind to MPRs, it cannot function because TIP47 is necessary for sorting from endosomes to Golgi. The reverse should hold true for AP-2 function as well if, when TIP47 binds, the AP-2 binding site is blocked

and hence only AP-1 can come on (Orsel, Sincock et al. 2000). This could be the way all adaptor protein binding is regulated; other partners are required to help regulate proper timing of binding. It will be interesting to see if the other adaptor proteins are regulated in such a way, and the examples of neuronal and ubiquitous AP-3 will be optimal ones to examine.

Other things that will be interesting to study are the regulation of AP complex binding. Work done by Faundez et al. has implicated a casein kinase in the regulation of binding of AP-3 to membranes and subsequent synaptic vesicle formation. Residues within the hinge domains of the β3 subunit get phosphorylated by the kinase (Faundez and Kelly 2000). Finding other factors that are involved will be important in understanding the function of AP-3, specifically neuronal AP-3.

There are many ways to generate protein diversity- by separate gene families, alternative splicing of RNAs, and post-translational modification. The brain, more than any other tissue appears to have higher numbers of protein isoforms expressed than anywhere else. While my focus has been on the complexity of membrane trafficking in neurons, it is clear that the requirements and demands within the brain demand a greater complexity of proteins to perform the jobs of trafficking, synaptic vesicle formation, and synaptic plasticity, etc. The example of neuronal AP-3 has been a useful one to study because it demonstrates that synaptic vesicles can be made in more than one way. This has implications in cell biology- how do adaptors function specifically- and in neurobiology- why is there more than one pathway of synaptic vesicle biogenesis

and what is different about the vesicles? Further work on neuronal AP-3 may help to answer these questions and the larger questions about complexity in the brain by tackling a small issue.

In addition, having examined the function of neuronal AP-3 in the context of isoform expression in the brain has been provoking. It seems that with so many isoforms being expressed in the brain, specifically separate regions of the brain, one can start to think about the brain as not a uniform tissue, but as many different components. Each region appears to express its own subset of protein isoforms, some due to different genes of gene families, while others are due to alternative splicing. Perhaps some of the genes that arose from duplication have not acquired new functions distinct from the original gene, yet it appears some have, as in the case of the neuronal AP-3 subunits. In addition, there is alternative splicing of genes to produce a variety of protein products. Perhaps by examining and comparing expression patterns of protein isoforms in different regions of the brain, we can learn not only about the uniqueness of each region and its function, but about the evolution of the brain as a whole. Which regions have the most protein isoform expression? By examining "old" brain regions versus "new" ones, can we learn something about such regions and how they are changing? It is possible, and likely, that some brain isoforms have not acquired any new functions as of yet, but it is also likely that some allow proteins to be regulated differently, function differently, and be expressed so that "evolution" of the tissue or region is independent of the evolution of other regions or even of the organism. It would be informative to compare such isoform

expression not only within mammalian, specifically primate, brains, but compare such expression to lesser evolved species' brains. Would we see similar isoform numbers and expression in such brains as compared to "old" regions of primate brain, and by examining the "new" regions and their newer protein isoforms, see how evolution is progressing and determine what these new isoforms add? Could we find hot spots of evolution in the brain now by identifying which regions have the most protein isoforms, and whether or not they have acquired new or unique functions? My work on neuronal AP-3 suggests that new functions are being acquired, and that they are important within the brain. It will be exciting to see what we can learn about the brain by examining it at the level of isoform expression.

Altschuler, Y., S. M. Barbas, et al. (1998). "Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms." J Cell Biol **143**(7): 1871-81.

Castillo, P. E., R. Janz, et al. (1997). "Rab3A is essential for mossy fibre long-term potentiation in the hippocampus." Nature **388**(6642): 590-3.

Drake, M. T., Y. Zhu, et al. (2000). "The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes." Mol Biol Cell **11**(11): 3723-36.

Faundez, V. V. and R. B. Kelly (2000). "The AP-3 complex required for endosomal synaptic vesicle biogenesis is associated with a casein kinase Ialpha-like isoform." Mol Biol Cell **11**(8): 2591-604.

Fernandez-Chacon, R., M. Achiriloaie, et al. (2000). "SCAMP1 function in endocytosis." J Biol Chem **275**(17): 12752-6.

Fernandez-Chacon, R. and T. C. Sudhof (1999). "Genetics of synaptic vesicle function: toward the complete functional anatomy of an organelle." Annu Rev Physiol **61**: 753-76.

Fernandez-Chacon, R. and T. C. Sudhof (2000). "Novel SCAMPs lacking NPF repeats: ubiquitous and synaptic vesicle- specific forms implicate SCAMPs in multiple membrane-trafficking functions." J Neurosci **20**(21): 7941-50.

Geppert, M., Y. Goda, et al. (1997). "The small GTP-binding protein Rab3A regulates a late step in synaptic vesicle fusion." Nature **387**(6635): 810-4.

Grabowski, P. J. and D. L. Black (2001). "Alternative RNA splicing in the nervous system." Prog Neurobiol **65**(3): 289-308.

Haffner, C., G. D. Paolo, et al. (2000). "Direct interaction of the 170 kDa isoform of synaptojanin 1 with clathrin and with the clathrin adaptor AP-2." Curr Biol **10**(8): 471-4.

Harris, T. W., E. Hartwieg, et al. (2000). "Mutations in synaptojanin disrupt synaptic vesicle recycling." J Cell Biol **150**(3): 589-600.

Hussain, N. K., M. Yamabhai, et al. (1999). "Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells." J Biol Chem **274**(22): 15671-7.

Isenmann, S., Y. Khew-Goodall, et al. (1998). "A splice-isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal." Mol Biol Cell **9**(7): 1649-60.

Janz, R., Y. Goda, et al. (1999). "SV2A and SV2B function as redundant Ca2+ regulators in neurotransmitter release." Neuron **24**(4): 1003-16.

Janz, R. and T. C. Sudhof (1999). "SV2C is a synaptic vesicle protein with an unusually restricted localization: anatomy of a synaptic vesicle protein family." Neuroscience **94**(4): 1279-90.

Koenig, E. and A. Giuditta (1999). "Protein-synthesizing machinery in the axon compartment." Neuroscience **89**(1): 5-15.

Mandic, R. and A. W. Lowe (1999). "Characterization of an alternatively spliced isoform of rat vesicle associated membrane protein-2 (VAMP-2)." FEBS Lett **451**(2): 209-13.

Mandic, R., W. S. Trimble, et al. (1997). "Tissue-specific alternative RNA splicing of rat vesicle-associated membrane protein-1 (VAMP-1)." Gene **199**(1-2): 173-9.

Marqueze, B., F. Berton, et al. (2000). "Synaptotagmins in membrane traffic: which vesicles do the tagmins tag?" Biochimie **82**(5): 409-20.

Martin, K. C., M. Barad, et al. (2000). "Local protein synthesis and its role in synapse-specific plasticity." Curr Opin Neurobiol **10**(5): 587-92.

Morris, S. A. and S. L. Schmid (1995). "Synaptic vesicle recycling. The Ferrari of endocytosis?" Curr Biol **5**(2): 113-5.

Nemoto, Y., M. R. Wenk, et al. (2001). "Identification and characterization of a synaptojanin 2 splice isoform predominantly expressed in nerve terminals." J Biol Chem **9**: 9.

Orsel, J. G., P. M. Sincock, et al. (2000). "Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein." Proc Natl Acad Sci U S A **97**(16): 9047-51.

Owen, D. J. and P. R. Evans (1998). "A structural explanation for the recognition of tyrosine-based endocytotic signals." Science **282**(5392): 1327-32.

Roos, J. and R. B. Kelly (1998). "Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with Drosophila dynamin." J Biol Chem **273**(30): 19108-19.

Shi, G., V. Faundez, et al. (1998). "Neuroendocrine synaptic vesicles are formed in vitro by both clathrin-dependent and clathrin-independent pathways." J Cell Biol **143**(4): 947-55.

Urrutia, R., J. R. Henley, et al. (1997). "The dynamins: redundant or distinct functions for an expanding family of related GTPases?" Proc Natl Acad Sci U S A **94**(2): 377-84.

Yamabhai, M., N. G. Hoffman, et al. (1998). "Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains." J Biol Chem **273**(47): 31401-7.

Zakharenko, S., S. Chang, et al. (1999). "Neurotransmitter secretion along growing nerve processes: comparison with synaptic vesicle exocytosis." J Cell Biol **144**(3): 507-18.

Appendix

After working on this project for the past several years, I have also come to appreciate the shortcomings or weaknesses of such a project. For one, working in a neuroendocrine cell line, as opposed to actual neurons, is a serious limitation. We supposedly study synaptic vesicle biogenesis, and have come to understand that there are differences in synaptic vesicles and the way they are formed, yet we are trying to draw physiological conclusions based on work with synaptic-like microvesicles. These conclusions are not necessarily going to be valid. As well, I have learned that binding assays have severe limitations. As my work has shown, under certain non-physiological conditions, one can get a binding to occur that is non-physiological. This is not only true for my work (yet we elucidated this), but for the work of others. This gives one pause when reading the results obtained with such assays, including the yeast-two hybrid assay.

Through my studies, I have also seen evidence of partial complex formation of adaptor complexes. While most people refer to the AP complexes as being able to only form with a certain set of subunit isoforms, I do not believe this is necessarily the case. While we are uncertain as to why, I have results suggesting partial complex formation- mixed of neuronal and ubiquitous subunits. While we cannot explain these results yet, and they do not fit into a story as of now, they may be important.

Inhibition of coating

A question we had in the Kelly lab was why were synaptic vesicles that were purified/prepared in Bud Buffer unable to recruit coat (AP-3) in the *in vitro* coating assay, whereas synaptic vesicles purified/prepared in Buffer A (high salt) could recruit coat. Was there potentially a protein inhibiting coat recruitment that was removed under high salt conditions?

Rat brain synaptic vesicles were purified using our standard methods. The only difference between the two preparations was the use of either Buffer A or Bud Buffer. One difference I observed was that synaptic vesicles purified in Bud Buffer did not contain synaptophysin. I could not detect synapsin in any synaptic vesicle fraction, although it was found elsewhere.

What I found in the coating assay was that the Buffer A prepared vesicles had a clear density shift, while the Bud Buffer prepared vesicles had a partial shift. About 50% or less of the vesicles recruited coat, while the rest did not. The coat that was recruitedin this case was AP-3, detected by using the sigma3 antibody produced by Victor Faundez. Perhaps the reason for this "partial" coating was due to inherent differences in the vesicles purified under different conditions. For example, the majority of vesicles purified in Bud Buffer are synaptic vesicles that cannot recruit AP-3 as coat, so perhaps these vesicles normally utilize the predominant AP-2 pathway. Maybe in Buffer A, more AP-3 vesicles are

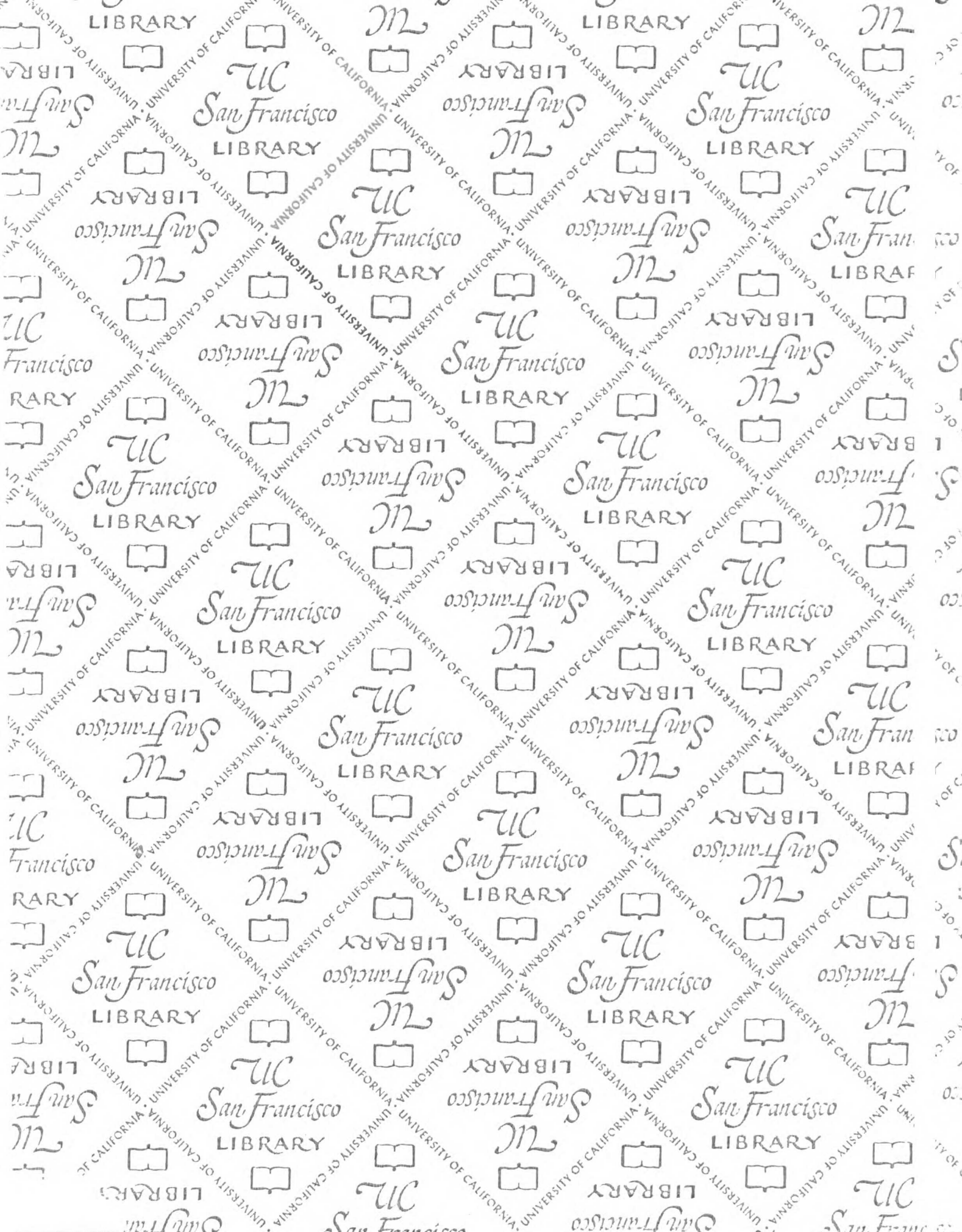
extracted from the preparation or a factor on these vesicles that is specific for AP-2 recruitment is removed or altered. I tried to use Bud Buffer P3 extracts to find the inhibitory factor by adding/incubating the extracts to Buffer A-purified synaptic vesicles, and then perform the coating assays to see if these vesicles could recruit coat. The idea was that perhaps now the inhibitory factor would be transferred to the Buffer A-purified synaptic vesicles and be no longer able to recruit coat, but they could.

Coating requirements

The standard protocol in the lab for coating of synaptic vesicles has been to use 3 mg/ml of rat brain cytosol per coating reaction. In my experiments, I noticed this was saturating and I could lower the concentration of cytosol per reaction and still achieve full coating of the synaptic vesicles. In earlier work done by Faundez et al., using 3mg/ml of cytosol per reaction, only brain cytosol could coat synaptic vesicles, while the same concentration of liver or yeast could not coat the vesicles. What was unknown in this work was how much ubiquitous AP-3 was in liver versus how much was in brain. When I reduced the levels of cytosol to 0.5 mg/ml I could recruit neuronal AP-3, or ubiquitous AP-3 (in the absence of the neuronal form) to synaptic vesicles. The majority of AP-3 in the brain is in the ubiquitous form and may be present in significantly higher levels than in liver or yeast. Knowing that ubiquitous AP-3 can bind in the absence of neuronal AP-3, this was missed, perhaps partly due to saturating the reactions with neuronal AP-3, so that ubiquitous could not compete for binding.

Role of σ 3 subunit

Another interesting thing to note briefly, is a result I observed in the coating assay when I used rat brain cytosol that had been immunodepleted of the σ3 subunit. This coating reaction always gave three peaks, one of coated synaptic vesicles, one of uncoated synaptic vesicles, and one intermediate. This is confusing because it is not clear why an intermediate would be trapped halfway-coated or only half the synaptic vesicles would be coated. It would be interesting to pursue and may provide information about the role of the σ3 subunit.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