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Neuronal Protein NP185 Is Developmentally Regulated, Initially Expressed During Synaptogenesis, and Localized in Synaptic Terminals

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Contents

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Abstract

Evidence is presented here that demonstrates the presence of NP185 (AP_3) in neuronal cells, specifically within syn-aptic terminals of the central nervous system and in the peripheral nervous system, particularly in the neuro-muscular junction of adult chicken muscle. Biochemical results obtained in our laboratories indicate that NP185 is associated with brain synaptic vesicles, with clathrin-coated vesicles, and with the synaptosomal plasma membrane. Also, NP185 binds to tubulin and clathrin light chains and the binding is regulated by phosphorylation (Su et al., 1991). Based on these properties and the data reported here, we advance the postulate that NP185 fulfills multiple functions in synaptic terminals. One function is that of a plasma membrane docking or channel protein, another of a signaling molecule for brain vesicles to reach the synaptic terminal region, and a third is that of a recycling molecule by binding to protein components on the lipid bilayer of the synaptic plasma membrane during the process of endocytosis. In support of these premises, a thorough study of NP185 using the developing chick brain, adult mouse brain, and chicken straited muscle was begun by temporally and spatially mapping the expression and localization of NP185 in evolving and mature nerve endings. To achieve these objectives, monoclonal antibodies to NP185 were used for immunocytochemistry in tissue sections of chicken and mouse cerebella. The distribution of NP185 was compared with those of other cytoskeletal and cytoplasmic proteins of axons and synapses, namely synaptophysin, vimentin, neurofilament NF68, and the intermediate filaments of glial cells (GFAP). The data indicate that expression of NP185 temporally coincides with synaptogenesis, and that the distribution of this protein is specific for synaptic terminal buttons of the CNS and the PNS.

Abbreviations: ab:antibody; AchR:acetylcholine receptor; En:embryonic day n; DHS:donor horse serum; FCS:fetal calf serum; FIF:formaldehyde-induced fluorescence; FITC:fluorescein isothiocyanate; FS:full strength; GFAP:glial fibrillary acidic protein; GLU:0.1% glutaraldehyde/PFA/PBS 7.4; mAb:monoclonal antibody; MES:2(*N*-morpholino)-ethanesulfonic acid, pH 6.5 (unless otherwise stated); NGF:neural growth factor; NGS: normal goat serum; o.n.:overnight; OCT:optimum cutting temperature compound:10% polyvinyl alcohol, 5% carbowax; PBS:phosphate buffered saline:19 m*M* NaH_2PO_4, 81 m*M* Na_2HPO_4, 150 m*M* NaCl, pH 7.4 (unless otherwise stated); PFA:4% paraformaldehyde/PBS 7.4; Pn:posthatch day n; RT:room temperature; SCa, SCb:slow component a or b of slow axonal transport; SDS-PAGE:sodium dodecyl sulfate–polyacrylamide gel electrophoresis; stg:stage; TBS/BSA:0.1% bovine serum albumin/TBS pH 7.5; TBS/Tween:0.1% Tween-20/TBS pH 7.4 TBS:Tris buffered saline:50 m*M* Tris, 150 m*M* NaCl, pH 7.4I.

Introduction

The assembly polypeptides (APs) (Zaremba and Keen, 1983), also called adaptor molecules (AMs) (Robinson, 1989; Pearse, 1989) belong to a diverse group of proteins that bind to membrane-limited organelles at specific locations in the cytoplasm and are involved in the budding off of vesicles for endocytosis. Functionally, APs represent docking sites for the assembly of protein lattices on the cytoplasmic surface of vesicles and plasma membrane. Structurally, AMs form the inner coat of a multilayer system situated directly beneath the membrane surface. On clathrin-coated pits (CCPs) and on clathrin-coated vesicles (CCVs), the protein middle layer is created by the inner projections of the 60 kD terminal domain of the clathrin heavy chains (CHCs) and probably by other associated molecules bound directly or indirectly to clathrin. The outer coat protein layer consists of the clathrin lattice *per se* formed by the polygonal arrays of CHCs with its associated clathrin light chains (LCs).

The AMs may also link coat proteins with receptor molecules embedded in the vesicle membrane. The carboxyl terminal domain of receptors protruding from beneath the membrane is the contact site for AMs. The AMs are divided in groups, based on their binding aflnity for each other after elution from hydroxylapatite columns

(Robinson and Pearse, 1986), and named HA-I and HA-II. The HA-I adaptor complex has a heterodimer of ~100 kD polypeptides, γ-adaptin and β–adaptin, and two associated polypeptides of 47 and 19 kD. The HA-II adaptor complex also has a heterodimer of ~100 kD polypeptides, α-adaptin and β-adaptin, and two other associated polypeptides of 50 and 16 kD. Other putative AMs have low binding affinity for these complexes and when purified by chromatography appear as single molecules. Some are known as AP155 (Keen, 1987), AP180 (Ungewickel et al., 1986), and NP185 (Kohtz and Puszkin, 1986, 1988). The HA-I adaptors are present in CCPs and CCVs of Golgi cisternae while HA-II adaptors are beneath the plasma membrane of CCPs and CCVs. The HA-II or AP_2 proteins are similar to the inositol polyphosphate receptor complex (Timerman et al., PNAS, in press).

The AMs putatively bind membrane receptors, and different AMs may bind the same membrane receptor (Pearse et al., 1989; Glickman et al., 1989). The mannose-6-phosphate (M6P) receptor reportedly is found on both Golgi and plasma membrane. Both HA-I and HA-II adaptors may bind to the cytoplasmic tail of M6P receptors, but at different locations, as suggested by binding competition experiments. Using site-directed mutagenesis, the cytoplasmic portion of the receptor was modified to replace two tyrosine residues with alanine and valine. This modification did not affect HA-I/M6P receptor interaction, but abolished HA-II affinity for this receptor. Thus, a "tyrosine signal" could play a part in the recognition of AMs by plasma membrane CPs, where HA-II is naturally found. This suggests an involvement of AMs in the cellular traffic of membrane receptors.

Extensive work was done on the α-adaptins of HA-II (Robinson, 1989). The α-adaptin has two bands on SDS-PAGE, A and C (band B is β-adaptin). Adaptins α_A and α_C are closely related to each other. Adaptin α_C is found in all tissues (including brain), and α_A is primarily expressed in brain (Robinson, 1987). Robinson cloned the cDNAs for these adaptins and determined that each is encoded by separate but related genes. Screening a mouse brain cDNA library, she surmised that no other related genes are present, and she also carried out *in situ* hybridization in mouse brain sections using antisense DNA to determine where α_A is expressed. Both α_A, and α_C were present in brain, as expected, but α_A was found highly concentrated in an area corresponding to the dentate gyrus of the hippocampus. In contrast, α_C was concentrated in both this structure and the habenular nucleus of the thalamus. Both areas consist of high concentrations of neuronal cell bodies. These varying levels of expression in areas of the brain suggest differences in cellular activation and different roles for the individual components of the AMs. Further resolution of the hippocampus revealed that α_A was distributed evenly between granule cell and pyramidal cell layers. The α_C was much more concentrated in the pyramidal cell layer. From these results Robinson deduced that α_A and α_C are most abundant in neuronal cell bodies, and that both adaptins are produced by the same cells but in different amounts. She speculated that the intracellular differences in α-adaptin abundance may be associated with subpopulations of coated vesicles, such as synaptic versus nonsynaptic vesicles.

In studying these molecules, it was reported that there are polypeptides in CCVs that interact with various components of the cytoskeleton (Kohtz, at al., 1990; Kohtz and Puszkin, 1989; Brady et al., 1985; Puszkin et al., 1982; Schook et al., 1979). One of these molecules, recently identified in our laboratories and named Covesin, appears to act as an organizing site for the polymerization of actin filaments (Kohtz et al., 1990). Kinesin, α-tubulin-activated vesicle-bound motor ATPase molecule, binds to CCVs as well as to other vesicular organelles (Brady et al., 1982), and may facilitate transport of these vesicles. A prospective AM, which has been extensively studied in our laboratories and named NP185 (Neuronal Protein of 185 kDa), was found to bind tubulin and the CLCs. Binding was regulated by protein kinases which phosphorylated tubulin and CLCs. A Ca^{2+} and calmodulin-dependent

protein kinase (bound to tubulin) decreased NP185 binding to tubulin, while a casein kinase II (bound to NP185) enhanced NP185-tubulin binding (Kohtz and Puszkin, 1989). Furthermore, the NP185 was found to bind to unphosphorylated CLCs, but not to CLCs phosphorylated by the CCVcasein kinase II (Schook and Puszkin, 1985; Su et al., 1991). Finally, NP185 can assemble clathrin into cages. NP185 is similar to AP_3 (Murphy et al., 1991).

It was reported that important interactions of clathrin lattices with the cytoskeleton and used in those studies highly purified clathrin and individual cytoskeletal proteins. The authors published evidence showing that α-actinin, actin, and also calmodulin could bind in vitro to clathrin (Schook et al., 1979; Bloom et al., 1981; Lisanti et al., 1982, Puszkin et al., 1981). Binding was several years later corroborated in reports from other laboratories. The binding of clathrin lattice components to elements of the cytoskeleton, including calmodulin (Lisanti et al., 1982; Salysbury et al., 1980) and protein kinases (Schook et al., 1979; Hanson et al., 1988,1990), may explain the formation of protein layers between clathrin lattices and the vesicle or the plasma membrane (Su et al., 1991). Most importantly, these layers of proteins constitute an ideal CCVs transport system to shuttle cargo molecules between the plasma membrane, the Golgi, and endosomes. These vesicular organelles are propelled to and oriented by docking molecules using the cytoskeleton as guiding tracks.

Nascent and mature synapses contain numerous CCVs (Miller and Heuser, 1984; Puszkin et al., 1983; Rees et al., 1976; Stelzner et al., 1973). Unlike any other cells, vesicles in neurons have long distances to navigate their cargo of molecules "containerized" at the endoplasmic reticulum region to their destination at the synaptic terminal. To reach this target, neurons require an active, organized, and efficient docking, loading, and unloading process at the synapse. This explains why there are abundant and diverse types of CCVs in brain cells (Puszkin et al., 1983; Bloom et al. 1980; Cheng et al., 1980; Puszkin et al., 1989). In support of this postulate it was shown that the several types of CCVs that exist in brain tissue are categorized in accordance with their polypeptide composition, which in turn determines their functions (Puszkin et al., 1989). Thus, CCVs cargo molecules, constitutive elements, and their AMs make them fulfill the unique functions of recycling neurotransmitters and receptors at synaptic terminals. Since neurons are endowed with an axon and their cytoplasm is packed with neurofilaments, it is not surprising that specific molecules are needed to sustain release and retrieval of neurotransmitters at the synapse. The NP185 may play such a role in mediating and regulating some of these functions.

Methods and Procedures

Tissue Preparation

Where appropiate all details of procedures already published are referred to their original article in the bibliography. Chick embryos were staged chronologically and developmentally. Staging time began day of egg laying (embryo day zero: E0) and continued for 21 day to hatching (E21). Hatching was day zero (P0) and subsequent days were P1, P2, and so on. Developmental staging followed a system based on precise defined morphological criteria (Hamburger and Hamilton, 1951).Terms used for each developmental stage were S1, S2, and so on, up to S46, and then hatching. Certified pathogen-free fertilized eggs were obtained from a commercial supplier (SPAFAS) and placed in a rocking egg incubator (Lyon Electric). Within 12 h, and every day thereafter, embryos were harvested, weighed, and staged by the same method (Hamburger and Hamilton, 1951). For stages E1 to E5, whole embryos at each stage were pooled to obtain sufficient amounts of protein. On E6 and E7, embryonic heads were pooled, and on E8 and E10 individual heads were used; individual brains were used thereafter. Assuming tissue density to be near to that of water, equal volumes of PBS

were added to each tissue and the contents homogenized in a small glass grinder at 4°C. The homogenate was diluted 1:1 with 50 m*M* Tris, 1% SDS, 5% 2-mercaptoethanol, 0.02% phenol red, and 30% glycerin ("sample buffer").

Immunoblot Technique

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed generally following one method (Laemmli, 1970). Sample preparations were loaded onto one-dimensional gels consisting of 10% acrylamide as described elsewhere (Puszkin et al., 1989).

Tissue Preparation for Immunolocalization

Chicken embryos were harvested and staged, fixed in 0.2% picric acid, 4% formaldehyde, and phosphate-buffered saline (PBS), for 30 min (Zamboni and de Martino, 1967). Details on the use of Healthy adult Balb/c mice, white leghorn chickens, including labeling with monoclonal antibodies and preparation of all controls were described previously (Perry et al., 1991). Primary monoclonal antibodies (mAbs) consisted of 8G8, raised against NP185 in this laboratory (Kohtz and Puszkin, 1988); obtained from Boehringer Mannheim were SY38, NR4, G-A-5, and 3B4, recognizing synaptophysin, neurofilament NF68, glial fibrillary acidic protein (GFAP), and vimentin, respectively. Control tissue sections were incubated with either the second antibody only, or with mouse control ascites and second antibody. False positive and false negative reactions were possible, and rigorous controls were applied in all the experiments (Perry et al., 1991). When examining embryonic tissues, which in the earliest developmental stages were not expected to express NP185, the problem of false negative reactions became important. To make sure that the incubation conditions were favorable for desired labeling, sections of adult chicken cerebella were always included with the embryonic sections, as positive controls.

Results

Developmental Expression of NP185

To establish crossreactivity of mAb 8G8 in avian brain tissue, and to determine the onset of expression of NP185 in chick embryo, embryo samples were prepared for Western blot analysis. Figures 1A and 1B show immunoblots of the prepared samples. Lanes 2 of Figs. 1A and 1B present a sample of bovine brain coated vesicles purified by specific method (Schook and Puszkin, 1985). These are included as positive controls for immunoreactivity. The antibody to NP185 crossreacted with avian brain tissue, demonstrated by the bands that first appear at E10 (lane 4). Expression levels increased with each embryonic day thereafter, based on the qualitative increase in band density. The total protein content in each sample lane was controlled by proportional pooling of tissue samples, and by equal dilution based on sample weight.

The results shown above establish the presence of NP185 in avian brain tissue, and that the onset of expression occurs no later than E10. However, earlier expression of NP185 cannot be ruled out. The sensitivity of Western blot analysis is dependent upon adequate levels of protein in the migrating sample. It is likely that at earlier embryonic days NP185 is expressed, but at levels below immunoblot detection. Therefore, immunohistochemistry was performed and the results obtained were confirmatory.

Immunolocalization of NP185

Protocols were designed to establish the ontogenic distribution of NP185 and it was found that the embryonic day of NP185 expression detected immunologically was E10. A control with chick embryo at E6 displayed no NP185 fluorescence. Within the one-day window that occurs during embryo sampling, the onset of NP185 expression was rapid, and in samples from four similar experiments, E9 tissue sections were nega-

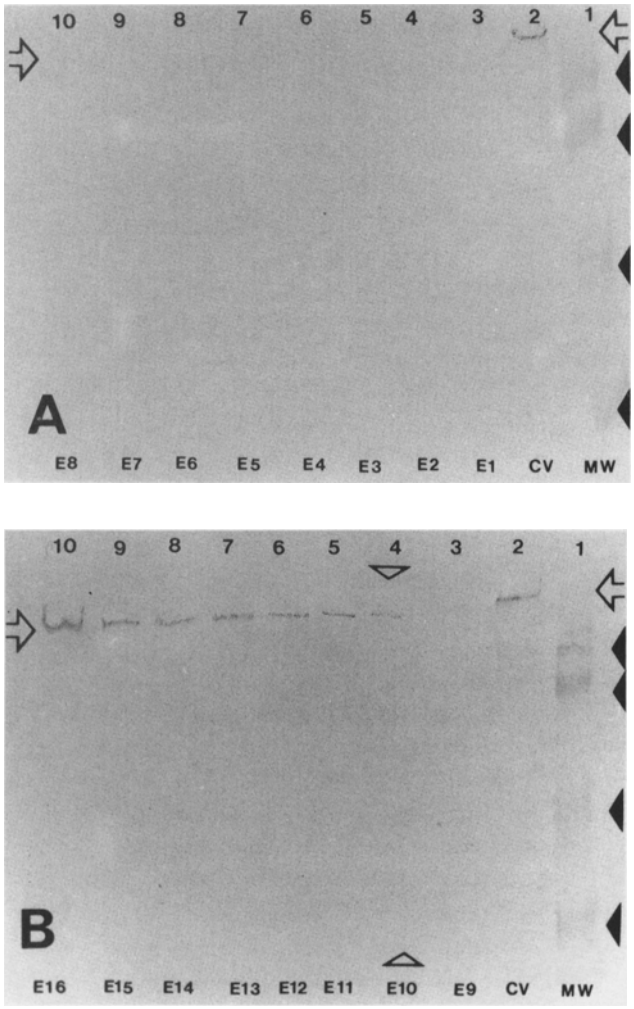

Fig. 1 A. Western blots for NP185. Lane 1, MW standards; lane 2, brain CCVs (positive control for mAb to NP185); lanes 3–10, purified chick embryo tissue, embryonic days E1 through E8. NP185 is not expressed. B. Lanes 1–2, as before; lanes 3–10, purified embryo tissue, embryonic days E9 through E16. NP185 appears at day E10 and continues thereafter.

tive for NP185 while E10 sections were clearly positive. The labeling pattern obtained is shown in Fig. 2. Histologically, at E10 the cerebellum consists of the external granular layer (E) and the mantle (M) layer (Nelsen, 1953). The external granular layer is the proliferative zone, which produces uncommitted neuroblasts. These cells are preneuronal, thus, the external granular layer has no synapses. The mantle layer is the migratory zone, where neuroblasts migrate and differentiate into neurons. Migration and cell differentiation is influenced by early, indiscriminate synaptic formation (Jacobson, 1978), and the mantle accommodates synapses from its onset. Immunolabeling of NP185 was strong and completely restricted to the mantle. Labeling patterns showed reticular fluorescent networks around dark cell bodies, a pattern that resembled developing rat cerebellum for p65, a synaptic vesicle-associated protein with exclusive synaptic localization (Cambray-Deakin et al., 1987).

The reticular nature of the labeling pattern is better seen at higher magnification in Fig. 3, where the division between the synapse-rich mantle (M) and the nonsynaptic external granular layer (E) is clear and where neuroblasts begin their medial migration (arrow). The mantle's matrix was clearly labeled for NP185 while the external granular layer was not. Neuroblasts adjacent to the mantle surrounded the labeled matrix. An identical distribution pattern was reported with synaptic vesicle-associated p65 (Cambray-Deakin et al., 1987). Significantly, the committed neuroblasts, which in the external granular layer have no protosynaptic neurites, are negative for NP185, and the same was also found for p65. As the cerebellum matures, synaptic distribution becomes more organized and regionally distinct and Fig. 4 gives a panoramic view of a single folium at E14, labeled for NP185. The labeling pattern manifestly follows synaptic distribution. The early mantle, which arises at E4 (Fujita, 1969), divides into the superficial mantle and deep mantle zones as early as E6 (Hanaway, 1967). By E12, these zones are more distinct, and have been referred to as the intermediate and ventricular layers (Chuong et al., 1987). The superficial mantle zone, nearest to the external granular layer, becomes enriched with abundant synaptic connections, as the parallel fibers begin to synapse with the dendritic spines of Purkinje cells (Fults et al., 1985; Gray, 1961). As synaptic density and organization continue to develop, NP185 labeling in this region also becomes more dense.

In contrast, the deep mantle zone is rich in neuronal cell bodies, caused by the migration of immature neurons from the external granular layer (Fujita, 1969) and from the ependyma (Miale and Sidman, 1961). Synapses, which are not as extensive as in the superficial mantle zone, are displaced by nerve soma. This results in a synaptic distribution pattern that is characteristically reticular—a net with holes, giving the appearance of a sectioned sponge (Fig. 5). The major contributing factor to this synaptic distribution is the strong adhesion of the granule cells (Mugnaini, 1969). The clumping of the granule cell bodies encourages focal organization of the synapses around protoglomeruli. The synaptic terminals of the mossy fibers, which are so large that they have been referred to as "synaptic bags" (Gray, 1961) and "synaptic vesicle-filled varicosities" (Burgoyne and Cambray-Deakin, 1988), surround the clusters of granule cells. The mossy fiber terminals synapse with granule cell dendrites, which are close to their originating somas, but searated by astrocyte processes (Mugnaini and Forstrønen, 1967). This establishes a synapse-dense neuropil with a nonsynaptic core—the glomerulus of the granular layer. Labeling of any synapse-specific protein in this structure will yield halos of fluorescence surrounding dark cores, exactly the pattern seen in labeling of NP185 (Fig. 5). This pattern is carried into maturity. The internal granular layer, which arises from the mantle (Fults et al., 1985), maintains this reticular synaptic architecture.

Throughout cerebellar development, the external granular layer remains negative for NP185. At E13, the granule cells of the extrnal granular layer begin migrating down Bergmann glial fibers to form the internal granular (I) layer (Chuong et al., 1987; Palacios-Prü et al., 1981; Rakic, 1971). As these cells become situated, they are enveloped by mossy fiber rosettes to form glomeruli. The granule cells are surrounded by intense fluorescent labeling of NP185, while the premigratory granule cells remain negative (Fig. 6). The focal synaptic organization in the internal granular layer is now well established (I). Climbing and mossy fibers, granule cell dendrites, and early Golgi cells contribute to the synaptic organization in this region (Fults et al., 1985; Altman, 1972c).

In Fig. 7, E15 marks the appearance of a monolayer of Purkinje cells (p), which constitutes the nascent Purkinje layer (P) in Fig. 6 (Foelix and Oppenheim, 1974). The Purkinje cells have intense areolate labeling of NP185. At E15, Purkinje cell bodies are studded with spiny processes, or pseudopodia. These serve as anchoring sites for climbing fibers and basket cell axons, with which they form extensive axosomatic synapses (Altman, 1972b; Mugnaini, 1969). As the axons form baskets around the Purkinje cells (hence the name) the pseudopodia retract. Significantly, NP185 is localized to the perimembranous space corresponding to the site of extensive, transient axosomatic synapses, creating intensely fluorescent halos around the cells (Fig. 7). By E15, many of the Purkinje cells have developed apical cones and early dendritic projections (Palacios-Prü, 1981). These make direct contact with parallel fibers to form shaft junctions (Landis, 1987). Shaft junctions are specialized, transient synapses present only during neuronal development. They serve as precursors of mature synaptic organization, perhaps through target recognition. By electron microscopy, Landis observed coated vesicles associated with these structures. In Fig. 8, an apical cone was visualized. It is strongly labeled for NP185. Figure 9 provides a side-by-side example of the different patterns of NP185 distribution in the developing chick cerebellar cortex. NP185 is not found in the external granular layer, where synaptic connections are absent.

The early molecular layer is labeled for NP185 in a grainy pattern. In the molecular layer, synaptogenesis and cellular maturation begin at the Purkinje layer, and progress outwardly toward the pia mater (Altman, 1972b). Inwardly migrating granule cells lay down trailing axons that lengthen and align in parallel arrays, as their name implies. As younger granule cells migrate, their axons are deposited on top of the existing parallel fibers. While this is taking place, imma-

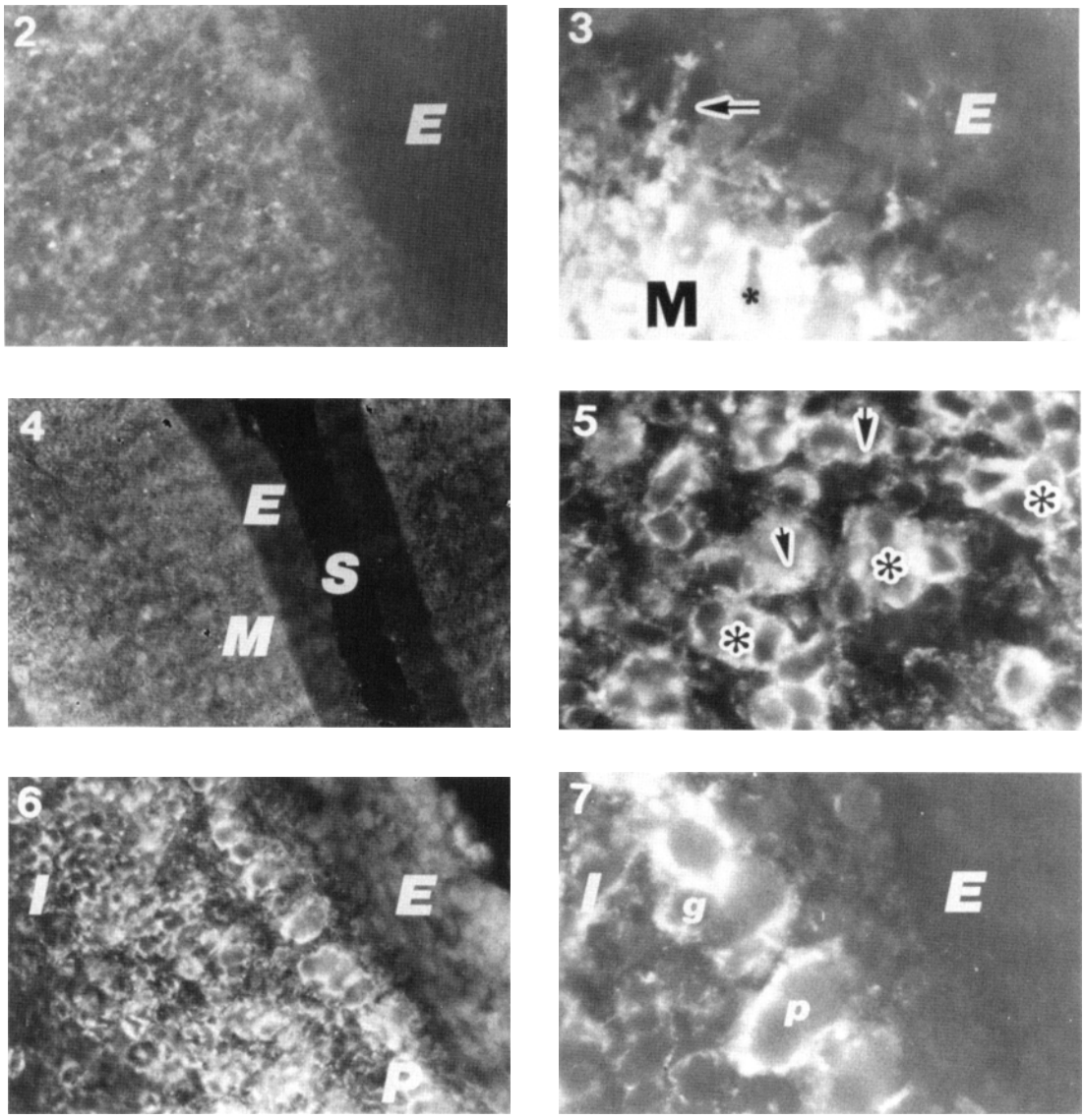

Fig. 2. Typical NP185 label in early embryo. NP185 distribution in chick embryo cerebellum, 128×. E: external granular layer; fluorescence is seen in the mantle. Reticular labeling surrounds the cell bodies. Fig. 3. Reticular immunoreactivity surrounding the cell bodies. NP185 distribution at E10 in embryonic chick cerebellum, 320×. E: external granular layer; M: mantle. Border between external granular layer and mantle shows cells with leading points into the mantle and with intercalated premigratory neuroblasts. Fig. 4. Distribution of NP185 at E14 in chick embryo cerebellum. Panoramic view of folium, 320×. S: interfolial space; E: external granular layer; M: mantle is positive for NP185. There is a denser, stronger labeling at the edge of the mantle layer. Fig. 5. Early neuropil labeling for NP185 in E15 chick embryo cerebellum, mantle, 320×. Clusters of immature granule cells (asterisks) are surrounded by synapse-rich neuropils (arrows). Fig. 6. NP185 distribution at E15 in chick cerebellum, 128×. E: external granular layer; P: Purkinje layer; I: internal granular layer. There is intense perimembranous labeling of Purkinje cells and labeled proglomerulus in the internal granular layer. Fig. 7. Labeling of young Purkinje cells for NP185 in E15 chick embryo cerebellum, 320×. E: external granular layer; I: internal granular layer; p: labeled Purkinje cell for NP185; g: migrating granule cell.

ture Purkinje cells produce apical cones that wend into the molecular layer. The apical cones evolve into dendrites, and establish synapses with parallel fibers (Landis, 1987). Dendritic arborization progresses outwardly as the parallel fibers continue to stratify. This results in directional synaptogenesis, beginning at the Purkinje layer and moving externally, toward the pia mater. The early molecular cell layer, adjacent to the Purkinje layer, is labeled for NP185 in a grainy manner (Fig. 9). This labeling pattern is consistent with the progression of synaptogenesis (Woodward et al., 1969). The label is always strongest in the deep molecular layer (nearest the Purkinje layer), and progressively diminishes towards the superficial molecular layer (nearest the pia mater).

The Purkinje layer in Fig. 9 is labeled for NP185 in the same manner as in Fig. 7. The areolate labeling of the Purkinje cells is apparent in the cell marked P. This is characteristic of early Purkinje cell labeling for NP185. The staining is clearly not confined to the cell body. At E15 (the developmental stage of the tissue in this figure), Purkinje cell somata are studded with spiny processes (Foelix and Oppenheim, 1974). These processes serve as synaptic anchors for the climbing fibers (Altman, 1972b) and basket cell axons (Mugnaini, 1969). The fuzzy, intense staining seen here conforms to the synaptic arrangement on the perisomatic processes. Notably, these transient spiny processes begin to retract at E16 and disappear by E20. The mature Purkinje cell soma is covered with a glial sheath, and possesses significantly fewer axosomatic synapses. In like manner, the Purkinje cell label for NP185 diminishes from a bright, fuzzy halo at E15 to a well-defined perimembranous ring at maturity. (Compare Fig. 9 with Fig. 12.) As in the developing molecular layer, NP185 distribution appears to follow synaptic distribution.

Finally, Fig. 9 shows the lattice structure of the early internal granular layer. A protoglomerulus is well visualized (g). Individual unlabeled granule cell bodies can be seen clumped together to form the glomerular core, which is covered by a glial sheath. Mossy fiber synaptic bags and granule cell dendrites contribute to the synapse-dense neuropil. Corsistent with the labeling patterns of the molecular (M) and Purkinje cell (arrow) layers (p), NP185 distribution closely adheres to synaptic distribution in the internal granular layer. These findings all suggest a synaptic distribution of NP185, even in the immature and transient synapses of developing neural tissue.

Granule cells arise from the external granular layer and migrate through the mantle to reside in what will become the internal granular layer (Fults et al., 1985). Figure 10 captures an immature granule cell (arrow) during this migration. As granule cells migrate, they assume a spindle shape, and leave trailing axons that eventually become the parallel fibers of the molecular layer (Quesada and Genis-Galvez, 1983; Altman, 1972a). In Fig. 10, both of these characteristics are well visualized by the NP185 label. The migrating granule cell (arrow) is followed by its axon, which is decorated with punctate fluorescence (arrowhead), imparting a beads-on-a-string appearance. The fusiform granule cell body is wider at the axonal base, which is characteristic of this cell (Mugnaini and Forstrønen, 1967). As soon as granule cells enter the matrix of the mantle layer, they initiate dendritic development (Quesada and Genis-Galvez, 1983), and establish axodendritic synapses with immature Purkinje cells (Landis, 1987; Kim, 1975; Miale and Sidman, 1961). With this in mind, the appearance of NP185 around the migrating granule cell body and immature parallel fiber in Fig. 10 is significant, and again suggests a synaptic distribution of this protein.

NP185 Distribution in Mature Cerebellum

A low magnification photomicrograph gives a multilayered view of NP185 distribution in the cerebellar cortex at maturity (Fig. 11). In keeping with its high synaptic density, the molecular layer is densely labeled for NP185. The labeling in this layer is uniform, with the exception of dark holes that represent basket cell somata (Obata and

Fujita, 1984), and perhaps a fortuitous cross-section of a dendritic trunk traversing out of plane. Turning back to Fig. 8 it can be seen that the NP185 label in the nascent molecular layer is composed of innumerable fluorescent grains. In the mature, synapse-dense molecular layer (Fig. 11), this grainy labeling is so extensive that it almost gives a pointillistic illusion of a solid, continuous stain.

In order to map the synaptic distribution in chick cerebellum, antisera was generated against subcellular fractions enriched in synaptic vesicles and synaptic plasma membrane (Howe et al., 1977). Their immunofluorescent data are in excellent agreement with the distribution of NP185 reported here. In their study, the molecular layer is intensely labeled with dense, grainy fluorescence. This pattern is characteristic of synaptic labeling, as reported in other investigations of synapse-specific proteins (Leclerc et al., 1989; Cambray-Deakin et al., 1987; Wiedenmann and Franke, 1985; de Camilli et al., 1983).

The Purkinje layer has discrete, perimembranous staining around the Purkinje cell bodies (P) and dendritic trunks (D). Gone is the areolate pattern seen at E15 (Fig. 9). This is because the transient perisomatic processes, which are so abundant in synaptogenesis, have all retracted. The mature Purkinje cell body is left with axo-somatic synapses provided by basket cell axons (Uchizono, 1969). These connections are less numerous and more spatially confined. This transformation is demonstrated in two Purkinje cells shown in closeup in Fig. 12, where the discrete localization of NP185 at the perimembranous region of the Purkinje cell body is well displayed. The Purkinje cell cytoplasm (p) is entirely unlabeled. Punctate concentrations (arrowheads) of fluorescent label may signify sites of axo-somatic synapses that happen to be precisely in the focal plane. An identical label pattern for the Purkinje cell was observed (Howe et al., 1977) in synaptic mapping study of the cerebellum. Further corroboration of the synaptic nature of this labeling pattern is provided by others; the distribution of neuronal membrane protein p29 was mapped and compared its distribution to that of the synapse-specific, integral protein synaptophysin (Baumert et al., 1990). The somata of brain stem neurons, known to have well established axo-somatic synapses, were labeled in a pattern identical to that seen in Fig. 12. In a study of cerebellar pathology, the distribution of synaptophysin in the normal cerebellum, including Purkinje cells was observed (Goto et al.,1989). Their findings on synaptophysin labeling of the Purkinje cell body are identical to the above findings. An examination of the general distribution of synapsin I in rat cerebellum produced identical findings to those above (De Camilli et al., 1983).

Figure 13 also provides a good labeling representation of the granular layer. This layer is referred to *in ovo* as the internal granular layer, to distinguish it from its germinal namesake, the external granular layer, which disappears shortly after hatching. Once established by the proto-glomeruli, the overall synaptic architecture (at the tissue level) in the granular layer remains in a reticular, or net-like, formation. Dark, unlabeled glomeruli (G) reside next to bright, intensely labeled mossy fiber synaptic bags (S). The glomerulus (G) is outlined with immunofluorescent label. This is the vicinity of synapsis between Golgi cell axons, mossy fibers, and granule cell dendrites (Jacobson, 1978). As in the molecular and Purkinje layers, this label pattern is identical to another report (Howe et al., 1977).

In summary, NP185 expression *in embryo* conforms with synaptogenesis, including the appearance of transient protosynapses. The localization of NP185 waxes with early synaptic proliferation, and wanes with synaptic retrenchment, as seen in developing Purkinje cells. In mature cerebellum, NP185 distributes according to established synaptic organization. Conversely, NP185 is not found in those places where synapses do not exist. For example, NP185 is not present in neuronal somata, even those of Purkinje cells, in which the cytoplasm is easily visualized. These findings strongly suggest that NP185 is synapse-specific.

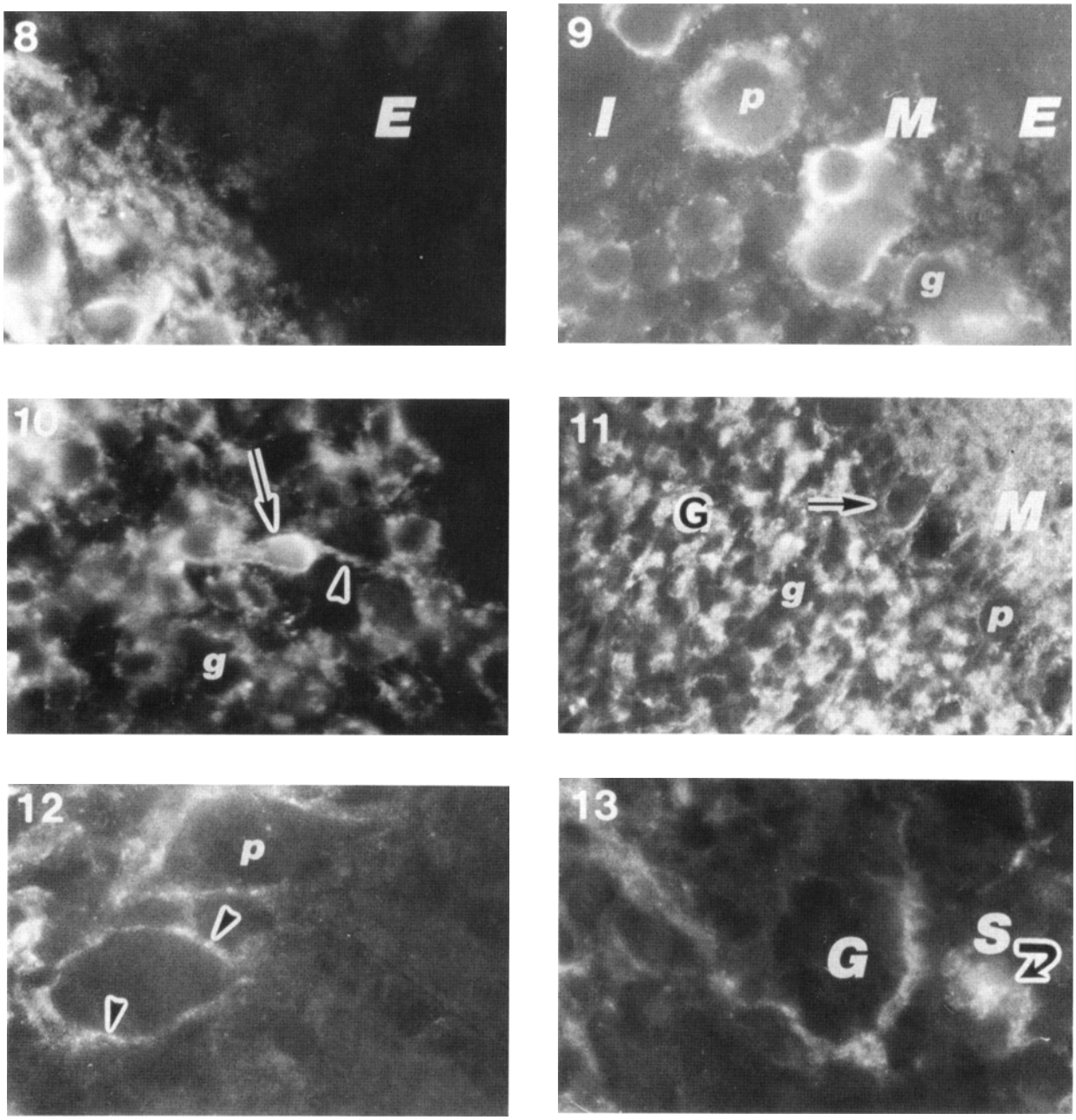

Fig. 8. Apical cones and dendritic processes are labeled for NP185 at the mantle/external granular layer border. E: external granular layer. Fig. 9. NP185 labeling in the chick embryonic cerebellar cortex at E15. E: external granular layer; M: molecular layer; I: internal granular layer; g: migrating granule cell; p: Purkinje cell. Fig. 10. Migrating granule cell labeled for NP185 at E15 in chick embryo cerebellum, 320×. Arrow: migrating granule cell, medially directed with a trailing axon. Arrowhead: trailing axon with studded labeling; g: glomerulus. Fig. 11. Immunofluorescent labeling of NP185 in mature cerebellar cortex, 128×. M: molecular layer; G: granular layer; g: glomerulus; p: Purkinje cell; Arrow: mossy fiber synaptic bag reaching the Purkinje cell layer. Fig. 12. Perimembranous NP185 labeling of Purkinje cells in adult mouse cerebellum, 320×. p: Purkinje cell soma; Arrowhead: punctate perimembranous labeling of Purkinje cells. Fig. 13. Glomerular NP185 labeling in adult mouse cerebellum, 320×. G: glomerulus; S: synaptic bag of mossy fiber.

Comparative Distribution of NP185 with Other Neuronal Proteins

Synaptophysin

To confirm that NP185 is confined to the synapse, its distribution was compared with that of two neuronal proteins, synaptophysin and NF68. In neurons, synaptophysin is synapse-specific (Wiedenmann and Franke, 1985) and NF68 is predominantly axonal (Liem et al., 1978). Synaptophysin is an integral protein (Jahn et al., 1985) found in presynaptic membrane (Wiedenmann and Franke, 1985). It is also found in the neuromuscular junction (Torri-Tarelli et al., 1990). It is a hexamer that is very similar to other channel-forming proteins (Thomas et al., 1988). It has four transmembrane domains (Südhof et al., 1987), and binds to calcium (Burgoyne, 1990). It probably plays a central role in membrane retrieval (Valtorta et al., 1989).

Synaptophysin has been used by others as a comparative synaptic marker when mapping the distribution of other proteins (Cabalka et al., 1990; Goto et al., 1989; Reynolds and Wilkin, 1988). Synaptophysin expression correlates well with synaptogenesis (Knaus et al., 1986). All of these features makes synaptophysin a useful comparative marker in the present study. The monoclonal antibody SY38 does not crossreact with chicken brain or chick embryo. Therefore, comparison of synaptophysin distribution with NP185 distribution was limited to adult murine brain. The cerebella of the chicken and mouse agree very well in cytoarchitecture and neurogenesis (Fujita, 1969), although much of the maturation of the murine cerebellum occurs postnatally.

Figure 14 shows the distribution of immunoreactivity for synaptophysin in adult mouse cerebellum. The distribution pattern seen here is identical with that reported by others (Leclerc et al., 1989; Goto et al., 1989; Reynolds and Wilkin, 1988). The molecular layer is intensely labeled in a dense, continuous arrangement, consistent with the high density of parallel fiber-Purkinje cell dendrite synapses. The granular layer has a flocculent pattern of immunoreactivity, that reflects the tufted formation of the synaptic glomeruli. The white matter, which essentially is a large bundle of axons, is completely negative for stain. Figure 15 provides a more magnified view of the labeling patterns in the different cerebellar layers. At the higher magnification seen in this figure, the reticular labeling pattern in the granular layer is more apparent. The label in the molecular layer is dense and grainy. The distribution of NP185 is virtually the same, as can be seen by comparing this figure with Fig. 11. In Fig. 16, the distribution of synaptophysin (arrows) labeling around a Purkinje cell (P), known to be synapse-specific in the cerebellum, agrees with the distribution of NP185. This supports the argument that NP185 is localized to synapses.

Neurofilament NF68

Neurofilaments are a class of intermediate filaments that are neuron-specific (Romand et al., 1988). They comprise a triplet of proteins with molecular weights of 68, 160, and 200 kD (Liem et al., 1978). They are distributed in most neurons of the central nervous system (Trojanowski et al., 1986), primarily in axons (Hoffman and Lasek, 1975). As such, they are useful neuronal markers (Luthman et al., 1988; Osborn, 1983), especially for axonal histochemistry (Hoffman and Cleveland, 1988). The 68 kD neurofilament (NF68) undergoes self-assembly and participates in the organization of the axonal cytoskeleton (Geisler and Weber, 1981). The authors of this review chose the protein as a nonsynaptic neuronal marker.

Chick embryos beginning with embryonic day E2 were examined. Although it was reported that neurofilaments are expressed in the chick embryo as early as E3 (Tapscott et al., 1981), no expression of NF68 in the cerebellar plate was detected until E6. This is the period during which the external granular layer and mantle are forming (Fujita, 1969). This is also when nascent Purkinje cells

arise from neuroblasts in the ventricular (ependymal, E) layer (Miale and Sidman, 1961). In the E6 embryo, labeling for NF68 appears as a ring surrounding the outer border of the ependyma around the fourth ventricle (V). This is shown in Fig. 17. By E7, the distribution is more extensive, covering more than half of the ependymal layer (Fig. 18). The organization has increased, and the labeled area is comprised of an array of parallel bands, which are not to be confused with the thin, distinct radial fibers discussed in the section of comparison for neurofilaments.

In contrast to these findings, NP185 does not appear until E10. The ependyma, like the external granular layer, remains negative for NP185. By E18, NF68 is concentrated in the cytoplasm and axons of Purkinje cells (Fig. 19, p). Here NF68 is found exclusively in the Purkinje cells. The cytoplasm is strongly labeled, allowing visualization of the dendritic hillock up to the first bifurcation. The nucleus is unlabeled, and stands out in strong contrast to the perikaryal stain. The axon (arrowhead) of these cells are well labeled. The axons can be easily seen projecting all the way to the white matter.

At higher magnification, the perikaryal stain is seen to be punctate and concentrated in a region corresponding to the endoplasmic reticulum (Fig. 20). This finding exactly agrees with a study that confirmed this subcellular distribution by electron microscopy (Langley et al., 1988). They noted that in the cytoplasm, the neurolaments remain in a granular, nonfilamentous form. The perikaryal concentration of granular neurofilaments is transient, and disappears by maturity (Bignami et al., 1985). It probably reflects intense production for axonal expansion (Sinicropi and McIlwain, 1983).

At this stage of cerebellar development, the differences in distribution between NF68 and NP185 are marked. NP185 is extensively distributed throughout the cerebellar cortex in a grainy or flocculent pattern. It never is seen in any filamentous type of distribution. In contrast, at E18–E19, NF68 is exclusively confined to the cytoplasm and axons of Purkinje cells, as well as axons of other neurons. This is the expected distribution of an axonal, nonsynaptic neuronal protein.

In the immature Purkinje cell, such as the E15 Purkinje cell seen in Fig. 9, some cytoplasmic staining for NP185 is apparent. This can signify the buildup phase of NP185 production, analogous to that speculated for NF68. Yet more significant is the much more intense areolate staining *outside* of the Purkinje cell body, where early synaptogenesis is taking place. This pattern is never seen with NF68.

A remarkable shift in NF68 distribution occurs by the time Purkinje cells reach maturity. The Purkinje cells become entirely negative for NF68; concomitantly, basket cell axons become intensely labeled for NF68, and remain so throughout maturity. Basket cell axons are readily identified by their distribution. They run along the Purkinje/molecular layer border, perpendicular to the longitudinal axis of the Purkinje cells (Altman, 1972a). These axons then descend into the Purkinje layer to embrace the Purkinje cell body by forming their characteristic basket of pinceaux (Langley et al., 1988; Uchizono, 1969). An example of this basket arrangement is given in Fig. 21.

Here the Purkinje cells are completely unlabeled. Their presence is only indicated by the highly stained baskets which surround them (p, arrows). This result is identical to other published studies (Sternberger et al., 1982; McKay and Hockfield, 1982). These baskets are of course formed by the axons of their namesakes. The filamentous labeling seen in the granular layer probably represents axons of mossy fibers and Purkinje cells (Langley et al., 1988). Figure 22 gives a closeup of the Purkinje layer (one Purkinje cell is delineated). The labeling seen here is so different from that seen in Fig. 19 that at first it is hard to imagine that the same monoclonal antibody has been used. In superficial appearance, NF68 seems to redistribute from the cytoplasm and axons of the Purkinje cells to the axons of basket cells. This startling change is best appreciated by comparing Fig. 20 with Fig. 23. In Fig. 23, an individual Purkinje cell is

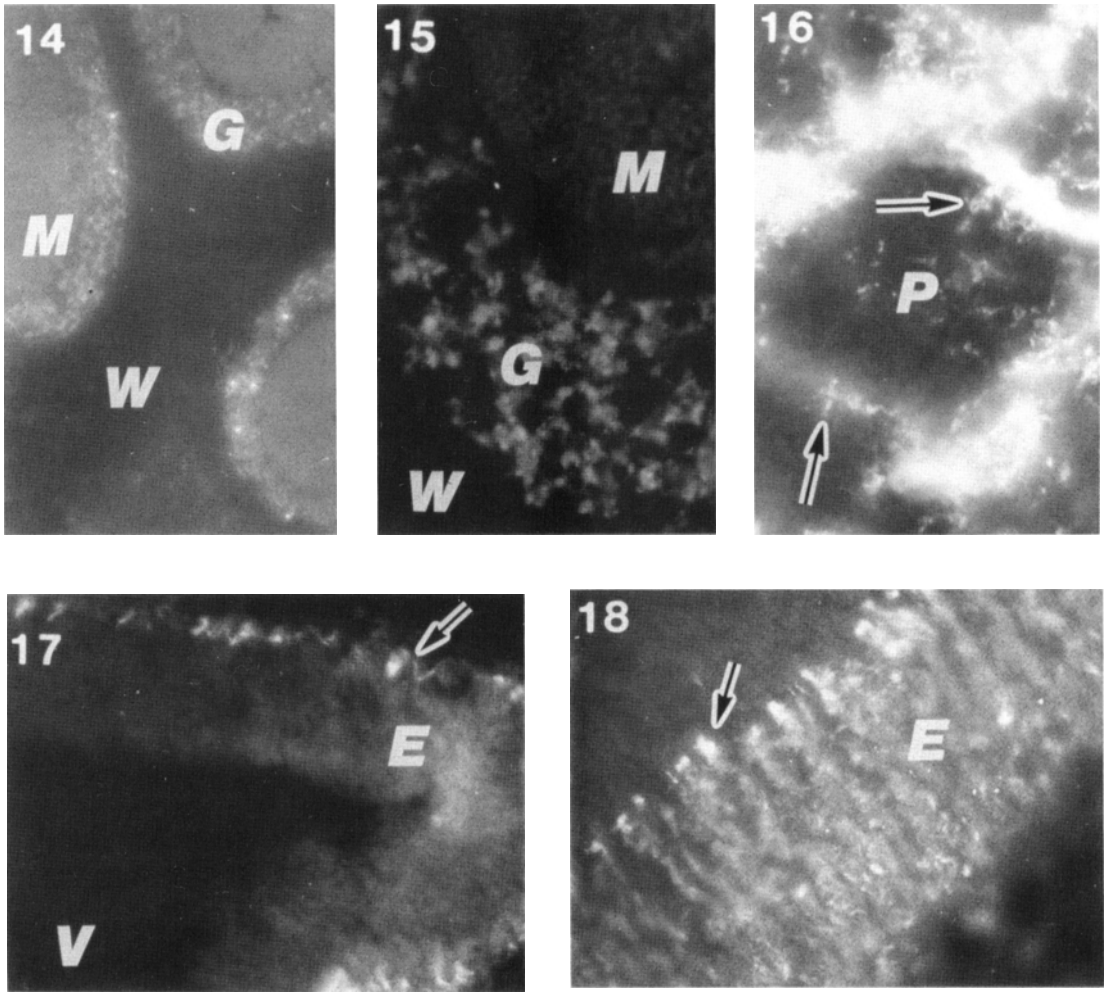

Fig. 14. Synaptophysin distribution in adult mouse cerebellum, 32×. W: white matter, M: molecular layer; G: granular layer. Fig. 15. Differential distribution of synaptophysin in adult mouse cerebellum, 80×. W, M, G: same as Fig. 14. Fig. 16. Perimembranous synaptophysin labeling of Purkinje cells in adult mouse cerebellum, 360×. Arrows: punctate labeling resembling P185 labeling. Fig. 17. Earliest expression of NF68 at day E6 in chick embryo, 80×. V: fourth ventricle; E: ependyma outer perimeter labeled for NF68. Arrow: cytoplasmic foot-pads with intense NF68 fluorescence. Fig. 18. Expression of NF68 at day E7 in chick embryo, 128×. Label covers outer half of ependyma and it is organized into parallel bands.

outlined by hand drawn dots and by an immunofluorescent pinceaux of basket cells. The Purkinje cell body, which is so clearly labeled in Fig. 20, appears absent.

This seemingly paradoxical result was first noticed by Bignami et al. (1985), who obtained exactly the same results presented here. They noted that the cytoplasm of the immature Purkinje cells, while immunochemically positive for neurofilament proteins, was devoid of actual filaments. From this they surmised that in the cytoplasm of these immature cells, the neurofilaments must reside in a nonpolymerized form. This "granular" form of neurofilament proteins probably represents the early synthesis of the nonpolymerized subunits, which are being produced in abundance to meet the needs of the growing cell. The subcellular localization of NF68 in the endoplasmic reticulum (Langley et al., 1988) supports this hypothesis.

The apparent redistribution of NF68 from the immature Purkinje cell to the mature basket cell axon is caused by the conversion of NF68 from its early, nonpolymerized form to its mature, polymerized state. In the immature neuron, the neurofilaments are rapidly expressed and processed within the cell body; as the cell matures, the proteins are transported to their natural destination away from the cell body, where they are concentrated. This redistribution is probably regulated by phosphorylation (Langley, 1988). Viewed in this light, it is not surprising to observe NF68 leaving the cytoplasm of one cell even as it collects in the axon of a different cell, if both cells are undergoing maturation. This is the case for the basket and Purkinje cells.

As a further comparison of the distributions of NP185 and NF68 in the mature Purkinje layer, Fig. 12 may be contrasted with Fig. 22. The basket cell axons make synaptic connections to the Purkinje cell somata (Landis and Reese, 1974), although not as extensively as in immaturity. The axonal pattern of distribution in Fig. 22 is entirely different than that seen in Fig. 12, which has no filamentous label. NP185 stain is limited to the membrane region of the Purkinje cell; NF68 stain loosely surrounds the cell. The punctate labeling around the Purkinje cell, seen with NP185 stain, is absent with NF68 stain. These results further confirm the synaptic—as opposed to axonal—distribution of NP185.

Comparative Distribution of Nonneuronal Proteins

To determine whether NP185 is truly neuron-specific, as opposed to nervous tissue-specific, the distribution of two intermediate filaments, glial fibrillary acidic protein (GFAP) and vimentin was mapped. In the cerebellum, both proteins are exclusively located in glia cells (Graeber et al., 1988; Schiffer et al., 1986). The distribution of these proteins was compared with that of NP185.

Glial Fibrillary Acidic Protein

GFAP is an intermediate filament found in astrocytes (Pelc et al., 1986; Bovolenta et al., 1984). It is distributed in the granular, Purkinje, and molecular layers of the cerebellum (Ghandour et al., 1980). As such, this protein is a good marker of cerebellar glial cells. Chick embryo cerebella were examined from E8, which is the day of onset of glial differentiation in the avian cerebellum (Fujita, 1969). Expression of GFAP was first detected at E16, long after initial glial differentiation. The late onset of GFAP expression relative to glial development in the mouse was reported (Schnitzer et al. in 1981). The distribution of immunofluorescent labeling shown in Fig. 24 gives an example of the labeling pattern in the molecular layer. The Bergmann glia fibers are strongly and exclusively labeled (arrows). This finding is identical to other published results (Wilkin and Levi, 1986; Sommer et al., 1981; Bignami and Dahl, 1973). Bergmann glia are readily identified by their linear, parallel organization in the molecular layer, sometimes referred to as palisade architecture (de Blas, 1984). The fibers of the Bergmann glia radiate—hence their common name, the radial fibers—out toward the pial surface of the cerebellar cortex (bf). During migration, the granule cell is in direct contact with these fibers (Burgoyne and Cambray-Deakin, 1988). These glial processes serve as guiding pathways for medial migration of the nascent granule cell from the external granular layer to the internal granular layer (Grovas and O'Shea, 1984; Rakic, 1971). In Fig. 11 there is a complete absence of neuronal staining. The distribution of the immunofluorescent label in the molecular layer is entirely unlike that seen for MP185 (Fig 11).

Vimentin

This molecule forms an intermediate filament in cells of mesenchymal origin (Franke et al., 1978), including microglia (Graeber et al., 1988). It is also found in astrocytes and ependymal cells (Schnitzer et al., 1981). Like GFAP, it is a useful marker of nonneuronal cells in the cerebellum. Chick embryo brains were examined from E2 (the cerebellar plate from E4) up through hatching. Vimentin expression is first detected in the cerebellum at E7. The expression of this glial protein precedes that of GFAP by 9 embryonic days, a

considerable span in the 21-d developmental period of the chick embryo. This marked difference in onset between vimentin and GFAP was noted (Schnitzer et al., 1981). In the cerebellar cortex, labeling occurred exclusively in the radial fibers of the Bergmann glia in the molecular layer. At earliest expression, E7, these fibers were short, sparse, unaligned, and deep within the mantle. By E10, these fibers had established their terminal pods at the pial surface. The fibers were still sparse, but more aligned. By E16, the day of onset of GFAP expression, the radial fibers were well aligned in an orderly array—the palisade arrangement (de Blas, 1984). An example is given in Fig. 25. Although the expression of GFAP lagged behind that of vimentin by nine embryonic days, once they both are expressed, the distribution of these proteins is identical in Bergmann fibers (bf, arrows). Others noted this in previous comparative studies (Bovolenta et al., 1984; Schnitzer et al., 1981). As was seen with GFAP distribution of the labeling was nonneuronal, and followed a distribution entirely different than that of NP185.

To summarize, the labeling patterns of the nonneuronal proteins GFAP and vimentin are entirely different from those of NP185. This is true throughout all developmental stages after the proteins first appear. Vimentin is earliest in expression, commencing at E7; NP185 follows next at E10; GFAP is last at E16. NP185 does not codistribute with either glial protein, which are reliable markers for nonneuronal cerebellar cells (Schnitzer, 1981). This evidence strongly suggests that NP185 is neuron-specific.

Localization of NP185 in the Neuromuscular Junction

Based on the above findings, which strongly suggest that NP185 is synapse-specific, it seemed reasonable to look for NP185 in a peripheral synapse. If NP185 is synapse-specific, one could predict that it is also localized in the neuromuscular junction. From the earliest days of coated vesicle research, CCVs were observed in the neuromuscular junction (Heuser and Reese, 1973). Clathrin-coated plaques (0.5–1 μm), pits (0.1–0.15 μm), and vesicles are all found in neuromuscular junctions, even embryonic ones (Bloch and Pumplin, 1988). Coated pits and vesicles participate in membrane insertion and removal of acetylcholine receptors. Bursztajn demonstrated that coated vesicles internalize acetylcholine receptors (Bursztajn and Fischbach, 1984), and are involved in AchR transport (Bursztajn et al., 1987). These observations make it appropriate to look for NP185 in the neuromuscular junction.

Establishing NP185 Localization

The immunolabeling shown in Fig. 26 establishes the presence of NP185 in chicken skeletal muscle. The distribution of NP185 is confined to areas, some as large as 120 μm diameter, with distinct concentrated points. This distribution pattern is consistent with the morphology of the neuromuscular junction (Purves and Lichtman, 1985). The motor nerve terminal is often referred to as a single synapse, but in fact it is a collection of many synaptic boutons. The boutons are polymorphic sacs that extend into the complementary troughs of the postsynaptic endplate (Carry and Morita, 1984). The general arrangement of a neuromuscular junction is illustrated in Fig. 26, which is a high-resolution scanning electron micrograph of a striated muscle fiber. The saccular structure of the terminal bouton constitutes a pocket (curved large black arrows); the presynaptic material localized in these pockets has been mostly removed during the treatment of the fiber for scanning electron microscopy (small black arrows) and are unevenly distributed over the general area associated with the neuromuscular junction (Scott et al., 1988). However, for immunohistochemical tagging of a presynaptic protein, this limitation is not found, since in the frozen muscle the presynaptic structure remains on the muscle fiber, while the axon shaft ia removed. Immunofluorescence is manifested as an archipelago of irregular bright dots when the neuromuscular junction is viewed from above. This is exactly what is seen in Figs. 26 and 28 (arrows). In Fig. 28, the labeled area follows the sagital axis of the underlying muscle fiber, which is shown by phase contrast microscopy in

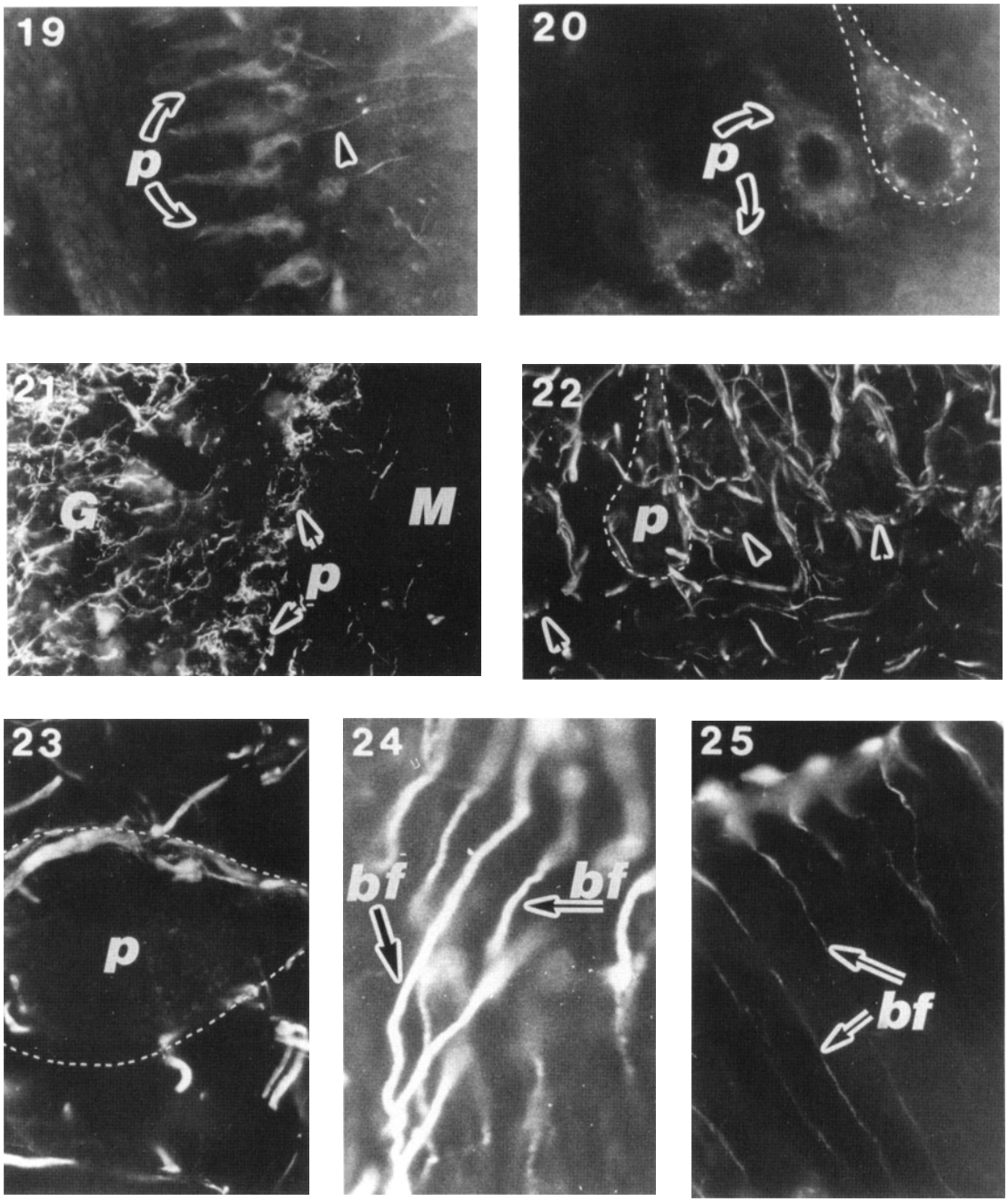

Fig. 19. Purkinje cells labeled for NF68 at E18 chick embryo. p: Purkinje cells; Arrowhead: axon with NF68 labeling. Fig. 20. Perikaryal labeling of NF68 at E19 chick embryo cerebellum, 320×. p: Purkinje cells with punctate labeling round nucleus. Dashed lines show contour of a Purkinje cell. Fig. 21. Immunolabeling for NF68 in adult chicken cerebellar cortex, 80×. G: granular layer; M: molecular layer; p: Purkinje cells are unlabeled but are surrounded by immunolabeled basket cell axons. Fig. 22. Same as before showing NF 68 in basket cell axons at 128× p: contour of Purkinje cells also shown by arrowheads. Fig. 23. Same as before showing NF68 labeled basket cell axons around a Purkinje cell in adult chicken cerebellum, 320×. Fig. 24. Immunolabeling for GFAP in the molecular layer of E17 chick embryo cerebellum. The Bergmann fibers are exclusively labeled. Fig. 25. Vimentin in Bergmann glial fibers of E16 chick embryo cerebellum. bf: parallel lines are individual fibers of Bergmann glia.

Fig. 29 (white asterisks and curved arrows). The speckled labeling in Fig. 28 is very loosely organized, following short lines of fluorescent dots giving an overall parallel pattern (curved arrows).

The organization of the fluorescent points varied. Often the speckles were seemingly randomly distributed within the labeled area, as seen in Fig. 30. The speckles were sometimes queued. This figure gives a more magnified view of NP185 localization in a neuromuscular junction of skeletal muscle. The punctate labeling is distributed over a small region. The points seen in Fig. 26 occasionally seem to form small constellations of curves or loops. This looping is reminiscent of the endplate visualized by Gunther and Letinsky using zinc-iodide/osmium tetroxide staining (1982), and Fig. 27 shows evidence that this pattern really reflects an underlying structure. When the skeletal muscle was viewed in crosssection, labeling of NP185 occurred along the muscle fiber perimeter. An example is shown in Fig. 31. In this instance the label is continuous, not punctate. The absence of punctation is likely when the neuromuscular junction is viewed laterally, rather than from above as shown in tubular-like structure at the bottom of Fig. 27. The labeling configuration seen here is identical to published results of neuromuscular junction labeling taken in cross-section (Fontaine et al, 1988; Bender et al., 1976). Occasionally the NP185 label was laid out in linear fashion, which seemed to follow the linear arrangement of the neuromuscular junction more commonly seen in Amphibia (Silver, 1963). The incidence of positive labeling was high and the dimensions of the labeled areas agree with that of others who visualized the avian neuromuscular junction by histochemical methods (Scott et al., 1988; Silver, 1963; Cole, 1955).

Double Labeling of NP185 and AchR

The above results confirm the presence of NP185 in skeletal muscle tissue, and suggest that NP185 is sequestered in a particular region of the muscle fiber. However, these results cannot establish the localization of NP185 at the neuromuscular junction. For this reason, double-labeling experiments were performed. To label the neuromuscular junction, fluorescein-conjugated α-bungarotoxin (αBT-F) was used. This snake venom derivative is known to bind selectively and irreversibly to acetylcholine receptors (AchR) in the postsynaptic endplate of the neuromuscular junction (Chang and Lee, 1963). It can bind to the receptors both intracellularly and on the cell surface (Bursztajn et al., 1987). Rhodamine-conjugated IgG (IgG-R) was used as the probe for mAb 8G8. Figures 32A and B give an example of the double labeling of AchR and NP185. The fluorescent label for AchR is continuous; in contrast, the label for NP185 is punctate. These patterns are especially apparent in the areas accented by arrows. They appear as loops that do not correspond to the AchR label. Except for these regions, the labeling is generally coextensive, with the NP185 label tending to be more discrete than continuous. In Figs. 33C and D, another example of AchR and NP185 codistribution is seen. Once again, the label for NP185 is speckled (arrow). There is also a speckled label for AchR, but not to the same degree as for NP185. In chick myotubes, AchRs are commonly aggregated in patches in the sarcolemma (Cohen and Pumplin, 1979). This pattern is readily visible under epifluorescent light microscopy. Clustering of AchR is induced by innervation (Bloch and Pumplin, 1988; Anderson et al., 1977). This distribution is caused by cytoskeletal anchoring of the receptors (Pumplin and Bloch, 1987). The AchR clusters seen in Figs. 32 A and C are highly reactive with the bungarotoxin reagent and yield high fluorescein labeling at this focal level, which is the same used for visualization of rhodamine fluorescent clusters of NP185 labeling in Figss. 32 B and D.

However, as was always the case, NP185 has a much more punctate distribution, with many points that have overall correspondence, but not strict overlapping with the AchR clusters. is is important to note since the labeling of NP185 is coextensive with the labeling of AchR, covering the same tissue area.

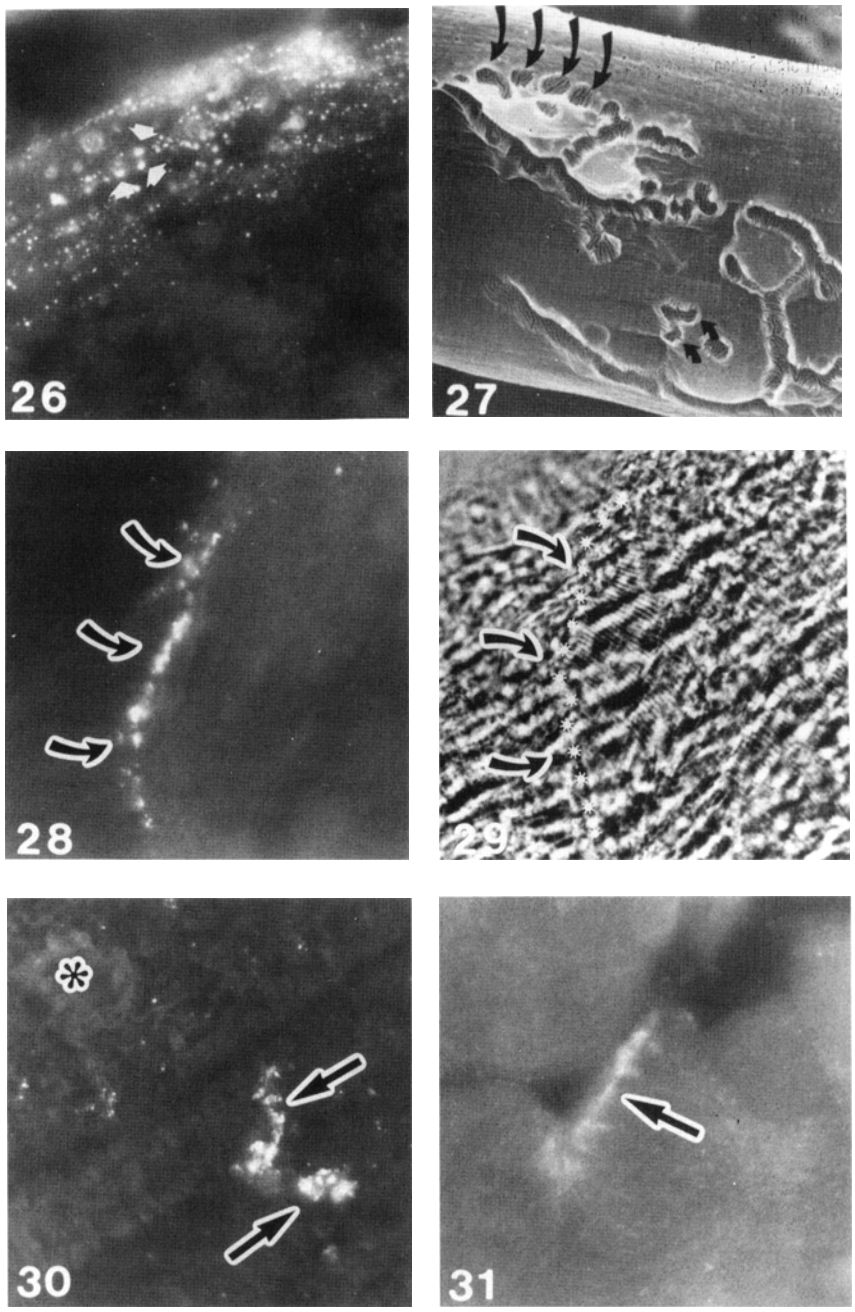

Fig. 26. Immunolocalization of NP185 in chick skeletal muscle. Longitudinal section of p34 chicken, 320×, showing fluorescent points creating a limited region. Arrows: short queue of speckles. Fig. 27. Scanning electron micrograph showing the surface of a striated muscle fiber with presynaptic material remaining from the treatment procedures. Fig. 28. Linear labeling for NP185 in a neuromuscular junction of pectoral muscle at p34 of chicken, 320×. Fig. 29. Phase contrast microscopy of muscle fiber shown in Fig. 28, delineating with asterisks and arrows the neuromuscular junction labeled for NP185 of the prior figure. Fig. 30. NP185 immunolabeled muscle synaptic terminal of shorter length than that shown before. Asterisk: blood vessel. Fig. 31. NP185 immunolabeled muscle cross-section at 320×. Arrow: synaptic junction runs along the perimeter of fiber.

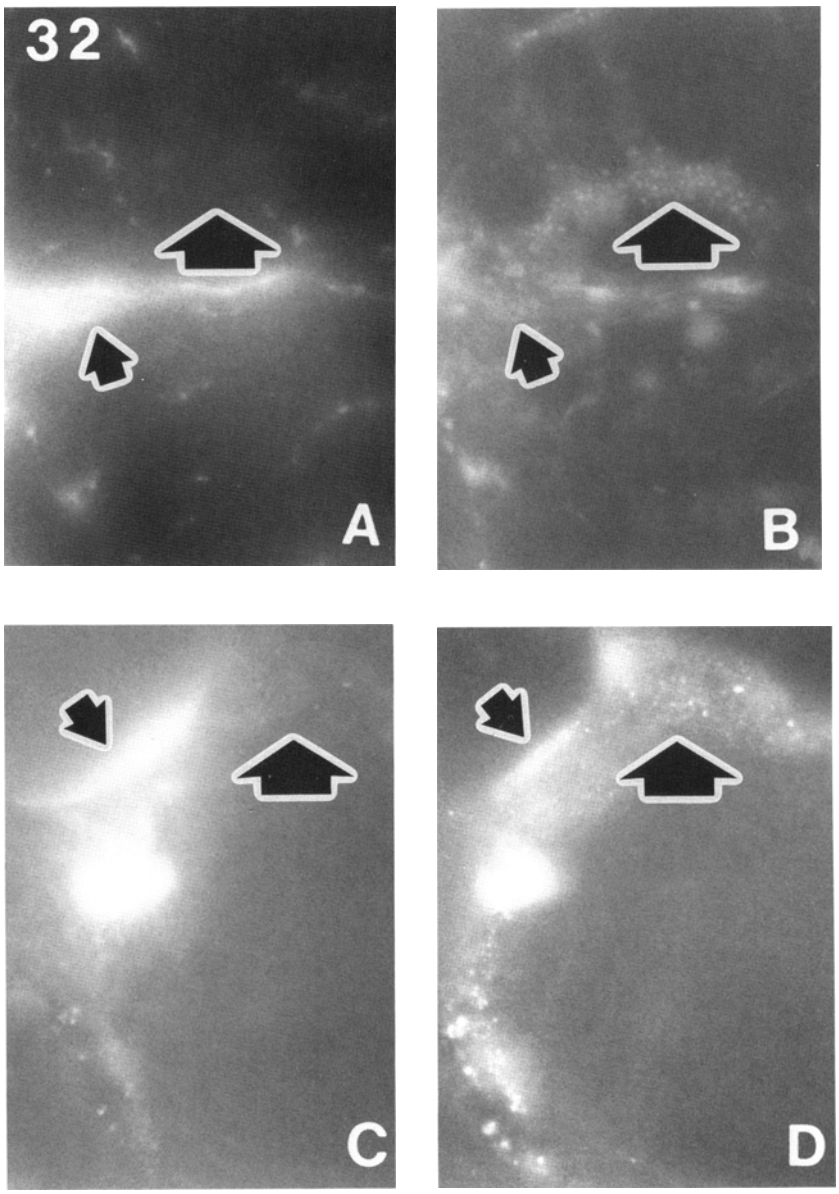

Fig. 32. Codistribution of AchR and NP185. A and C: alpha-bungarotoxin labeled neuromuscular junction showing intense green fluorescence for AchR. B and D: NP185 label of same field and depth of focus with speckling of rhodamine label at thick arrows (*see* text for further details).

Discussion

NP185 first becomes detectable in the avian cerebellum at E10, which corresponds to important neurogenic events. Although protosynapses are formed between E7 and E9, the first appearance of morphologically mature synapses in the cerebellar cortex occurs at E10 (Foelix and Oppenheim, 1974). These synapses are characterized by increased membrane thickening and a marked increase in synaptic vesicles. Within the mantle, the nascent molecular and Purkinje

layers begin to develop at this stage (Foelix and Oppenheim, 1974). There is abundant synaptogenesis in this region as the Purkinje cells migrate to their final position and climbing fibers establish synaptic contact with Purkinje cell bodies (Shimono et al., 1976).

Purkinje cell development offers an example of the close association of NP185 expression with synaptic distribution. In maturity, only one climbing fiber contacts each Purkinje cell (Mariani and Changeux, 1981), but during early development of these neurons, each Purkinje cell body is in synaptic connection with many climbing fibers. These synapses subsequently undergo retrenchment. By maturity, one climbing fiber forms synapses with the dendrites of one Purkinje cell. The Purkinje cell body itself now only has minor axo-somatic synapses with basket cell axons. The distribution of NP185 in the Purkinje layer reflects these developmental changes: The early Purkinje cell soma, covered with terminal boutons of several climbing fibers, has an intense areolate label; the mature Purkinje cell soma has only a perimembranous label with a few punctate concentrations.

Clathrin molecules appear early in neuroblasts and thereafter clathrin-coated vesicles play a pivotal role in synaptogenesis (Altman, 1971). They are concentrated at incipient synaptic sites, especially at future postsynaptic membranes. They may provide prospective synapses with the material necessary to form early adhesion plaques, through direct insertion of electron-dense membrane. It was found that coated vesicles congregated at newly formed synaptic junctions (Stelzner et al., 1973). This group postulated that clathrin-coated vesicles could contribute to synapse formation by releasing adhesion mediators into the extracellular space (exocytosis), or alternatively, by incorporating recognition factors into the cell (endocytosis). Eckenhoff and Pysh (1979) found that coated vesicles internalize apposed membrane in neurons undergoing synaptogenesis. This internalization probably allows the transfer of morphogenetic material essential for early neurite growth and for later synaptic remodeling. Coated vesicles are also common in the developing neuromuscular junction (Bloch and Pumplin, 1988).

Coated vesicles are implicated in more than early synaptogenesis. They are a general feature of the postsynaptic membrane of mature synapses (Waxman and Pappas, 1969). Based on their ubiquity, they are undoubtedly vital in maintaining mature synaptic function as well as forming new synaptic sites. Given the close connection of coated vesicles and synapses, and the association of NP185 with CCVs, it is not surprising that NP185 has a synapse-specific distribution. A key question on the role of NP185 in synaptogenesis is that while clathrin molecules are expressed early, before differentiation, why is NP185 expressed just when active synapses are established? This leads to the postulate that NP185 is needed for synapses to function or rather to maintain their function.

The synaptic association of NP185 and CCVs is supported (Cheng et al., 1980). They raised a polyclonal antibody against clathrin HC, and studied its distribution in rodent cerebellum by immunohistochemistry. The distribution pattern is identical to that seen for NP185: dense, grainy labeling in the molecular layer; reticulate labeling in the granular layer; distinct, perimembranous labeling of Purkinje cells, with punctate concentrations. From this distribution, the authors inferred that clathrin HC is highly concentrated in synaptic terminals. The work by the authors of this review adds a temporal relationship between these two proteins.

By themselves, the results in chick embryo and adult chicken strongly suggest that NP185 is localized in the synapse. The further work in this study corroborates this finding. Using antibodies to other proteins for comparative immunohistochemistry is useful in tracking a protein at the light microscopic level. This technique has been successfully used by others (Baumert et al., 1990; Goto et al., 1989; Reynolds and Wilkin, 1988; Eriksdotter-Nilsson et al., 1987; Pelc et al., 1986; Schiffer et al., 1986). NP185 distribution is identical to that of synaptophysin, which in the central

nervous system is neuronal and synapse-specific. In contrast, NF68, which is neuronal and axon-specific (nonsynaptic), has an entirely different distribution. Both of the nonneuronal proteins, GFAP and vimentin, have distributions completely unlike that of NP185. Therefore, NP185 is both neuron-specific and synapse-specific.

The distribution of NP185 in the neuromuscular junction is the final evidence of synapse-specificity. Coated vesicles are on the postsynaptic side of the neuromuscular junction, distributed in AchR clusters in domains nearby, but separate from the AchR domains (Pumplin, 1989). Coated vesicles are present from the very beginning of contact formation between the neurites of ciliary neurons and chick embryo myotubes (Bursztajn, 1984). This contact is regarded as a good in vitro model of a developing neuromuscular junction. As the growth cone comes in contact with the myotube, it extends numerous filopodia along the myotube surface. This multiple contact may be necessary for proper neuromuscular development, perhaps by preparing the myotube for mature association with a functional motor neuron. Eventually a single neuronal process gains dominance, and the developing myotube loses most of the other neurite contacts. This loss is analogous to synaptic retrenchment after initial abundant proliferation, the final step in synaptogenesis. Throughout the course of neurite-myotube association, coated vesicles are observed to be in close apposition to contact sites. As the dominant neuronal process matures, this association remains. Coated vesicles internalize acetylcholine receptors was demonstrated (Bursztajn et al.,1987). Chloroquine, which blocks lysosomal fusion, produces accumulation of acetylcholine receptors in postsynaptic coated vesicles (Bursztajn and Libby, 1981).

The contact and adhesion model (Bloch and Pumplin, 1988), assigns to clathrin-coated plaques and vesicles a role in neuromuscular synaptogenesis. AchR clustering in the postsynaptic membrane of the neuromuscular junction occurs in response to innervation of young muscle fibers. Three membrane domains are constructed in the process of receptor clustering. First, nerve and muscle membranes contact each other. Adhesion molecules, perhaps such as neural cell adhesion molecules (N-CAM), then strengthen this contact. Microfilaments attach to the acytoplasmic side of the membrane at points of innervation, where nerve and muscle membranes adhere. It is likely that talin and vinculin, interacting with the membrane-associated receptors of the adhesion molecules, establish an anchor on the cytoplasmic surface that allows further cytoskeletal organization. Clathrin-coated plaques form next to the contact domains. Clathrin-coated vesicles insert acetylcholine receptors in the postsynaptic membrane. The insertion of cetylcholine receptors triggers the formation of a spectrin–actin network. This network grows to create domains rich in acetylcholine receptors. This model probably applies to synaptogenesis in the central nervous system.

NP185 Is Synapse-Specific

In brain, NP185 is closely associated with clathrin-coated vesicles (Kohtz and Puszkin, 1988). Since clathrin-coated plaques and vesicles are crucial in synaptogenesis, NP185 undoubtedly plays an important part in synaptic function. NP185 has phosphorylation-modulated binding affinity for clathrin LCs (Su et al., 1991), and also binds to tubulin in a casein kinase II-regulated manner (Kohtz and Puszkin, 1989). These characteristics are reminiscent of synapsin I, a neuronal protein associated with synaptic vesicles on their cytoplasmic surface (Ueda and Greengard, 1977). Its binding affinity to SVs is decreased five-fold when it is phosphorylated (Schiebler et al., 1986). Phosphorylation is catalyzed by Ca^{2+}/calmodulin-dependent protein kinase II (Huttner et al., 1981). Synapsin I binds with neurofilaments (Goldenring et al., 1986) and microtubules (Baines and Bennett, 1986). When in the unphosphorylated state, it promotes crosslinking of synaptic vesicles at the presynaptic membrane (Steiner et al., 1987). Like other neuronal proteins, it is found in the neuromus-

cular junction (Valtorta et al., 1988). Lastly, it shares the same cerebellar distribution as NP185 (de Camilli et al., 1983). Considering these properties, it is tempting to speculate that NP185 may serve as a cytoskeletal docking protein for CCVs, in a manner analogous to that of synapsin I for SVs (Valtorta et al., 1989). Alternatively, NP185 may be part of or regulate an ion channel or receptor molecule for endocytosis.

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References

Ahle S., Mann A., Eichelsbacher U., and Ungewickell E. (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.* **7**, 919–929.

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

Altman J. (1971) Coated vesicles and synaptogenesis. A developmental study in the cerebellar cortex of the rat. *Brain Res.* **30**, 311–322.

Altman J. (1972a) Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J. Compar. Neurol.* **145**, 353–398.

Altman J. (1972b) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. *J. Compar. Neurol.* **145**, 399–464.

Altman J. (1972c) Postnatal development of the cerebellar cortex in the rat. III. Maturation of the components of the granular layer. *J. Compar. Neurol.* **145**, 465–514.

Anderson M. J. and Cohen M. W. (1977) Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 757–773.

Anderson M. J., Cohen M. W., and Zorychta E. (1977) Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 731–756.

Atsumi S. (1971) The histogenesis of motor neurons with special reference to the correlation of their endplate formation. *Acta Anatt.* **80**, 161–182.

Baines A. J. and Bennett V. (1986) Synapsin I is a microtubule-bundling protein. *Nature* **319**, 145–147.

Bannerman P. G. C., Mirsky H., Jessen K. R., Timpl R., and Duance V. C. (1986) Light microscopic immunolocalization of laminin type IV collagen, nidogen, heparan sulphate proteoglycan and fibronectin in the enteric nervous system of rat and guinea pig. *J. Neurocytol.* **15**, 733–743.

Bar-Zvi D. and Branton D. (1985) Casein kinase II like protein kinase in calf brain coated vesicles phosphorylates clathrin β light chain. *J. Cell Biol.* **101**, 48a. (abstract).

Baumert M., Takei K., Hartinger J., Burger P. M., von Mollard G. F., Maycox P. R., de Camilli P., and Jahn H. (1990) P29: a novel tyrosine-phosphorylated membrane protein present in small clear vesicles of neurons and endocrine cells. *J. Cell Biol.* **110**, 1285–1294.

Bender A. N., Ringel S. P., and Engel W. K. (1976) The acetylcholine receptor in normal and pathologic states. *Neurology* **26**, 477–483.

Benson R. J. J., Porter-Jordan K., Buoniconti P., and Fine R. E. (1985) Biochemical and cytochemical evidence indicates that coated vesicles in chick embryo myotubes contain newly synthesized acetylcholinesterase. *J. Cell Biol.* **101**, 1930–1940.

Bignami A. and Dahl D. (1973) Differentiation of astrocytes in the cerebellar cortex and the pyramidal tracts of the newborn rat, an immunofluorescence study with antibodies to a protein specific to astrocytes. *Brain Res.* **49**, 393–402.

Bignami A., Grossi M., and Dahl D. (1985) Transient expression of neurofilament protein without filament formation in Purkinje cell development. *Int. J. Devl. Neurosci.* **3**, 365–377.

Birkett C. R., Foster K. E., Johnson L., and Gull K. (1985) Use of monoclonal antibodies to analyse the expression of a multi-tubulin family. *FEBS Lett.* **187,** 211–218.

de Blas A. L. (1984) Monoclonal antibodies to specific astroglial and neuronal antigens reveal the cyto-architecture of the Bergmann glia fibers in the cerebellum. *J. Neurosci.* **4,** 265–273.

Bleil J. D. and Bretscher M. S. (1982) Transferrin receptor and its recycling in HeLa cells. *EMBO J.* **1,** 351–355.

Blitz A. L., Fine R. E., and Toselli P. A. (1977);Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* **75,** 135–147.

Bloch R. J. and Pumplin D. W. (1988) Molecular events in synaptogenesis: nerve-muscle adhesion and postsynaptic differentiation. *Am. J. Physiol.* **254,** C345–C364.

Bloom W. S., Fields K. L., Yen S. H., Haver K., Schook W., and Puszkin S. (1980a) Brain clathrin Immunofluorescent patterns in cultured cells and tissues. *Proc. Natl. Acad. Sci. USA* **77,** 5520–5524.

Bloom W. S., Schook W., Feageson E., Ores C., and Puszkin S. (1980b) Brain clathrin: Viscometric and turbidimetric properties of its ultrastructural assemblies. *Biochim Biophys. Acta* **598,** 447–455.

Bovolenta P., Liem R. K. H., and Mason C. A. (1984) Development of cerebellar astroglia: transitions in form and cytoskeletal content. *Devel. Biol.* **102,** 248–259.

Brady S. T., Lasek R. J., and Allen R. D. (1982) Fast axonal transport in extruded axoplasm from squid giant axon. *Science* **218,** 1129–1131.

Brady S. T. (1985) A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* **317,** 73–75.

Bretscher M. S., Thomson J. N., and Pearse B. M. F. (1980) Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA* **77,** 4156–4159.

Brodsky F. I., Galloway C. J., Blank G. S., Jackson A. P., Seow Heng-F., Drickamer K., and Parham P. (1987) Localization of clathrin light-chain sequences mediating heavy-chain binding and coated vesicle diversity. *Nature* **326,** 203–205.

Brown M. S. and Goldstein J. L. (1986) A. receptor-mediated pathway for cholesterol homeostasis. *Science* **23,** 34–47.

Buc-Caron Marie-H., Nystrom P., and Fischbach G. D. (1983) Induction of acetylcholine receptor synthesis and aggregation: partial purification of low-molecular-weight activity. *Devl. Biol.* **95,** 378–386.

Burgoyne R. D. (1990) Secretory vesicle-associated proteins and their role in exocytosis. *Ann. Rev. Physiol.* **52,** 647–659.

Burgoyne R. D. and Cambray-Deakin M. A. (1988) The cellular neurobiology of neuronal development: the cerebellar granule cell. *Brain Res. Rev.* **13,** 77–101.

Bursztajn S. (1984) Coated vesicles are associated with acetylcholine receptors at nerve-muscle contacts. *J. Neurocytol.* **13,** 503–518.

Bursztajn S. and Fischbach G. D. (1984) Evidence that coated vesicles transport acetylcholine receptors to the surface membrane of chick myotubes. *J. Cell Biol.* **98,** 498–506.

Bursztajn S. and Libby P. (1981) Morphological changes in cultured myotubes treated with agents that interfere with lysosomal function. *Cell Tissue Res.* **220,** 573–588.

Bursztajn S., Nudleman H. B., and Berman S. A. (1987) Coated and smooth vesicles participate in acetylcholine receptor transport. *Cell Tissue Res.* **248,** 535–540.

Cabalka L. M., Ritchie T. C., and Coulter J. D. (1990) Immunolocalization and quantitation of a novel nerve terminal protein in spinal cord development. *J. Compar. Neurol.* **295,** 83.

Cambray-Deakin M. A., Norman Kathryn -M., and Burgoyne R. D. (1987) Differentiation of the cerebellar granule cell: expression of a synaptic vesicle protein and the microtubuleassociated protein MA P1A. *Develop. Brain Res.* **34,** 1–7.

de Camilli P., Cameron H., and Greengard P. (1983) Synapsin I (protein I), a nerve terminal specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* **96,** 1337–1354.

Carry M. R. and Morita M. (1984) Structure and morphogenesis of the neuromuscular junction, in *The Neuromuscular Junction,* Brumback R. A. and Gerst J. W., eds., Futura, Mt. Kisco, NY, pp. 25–64.

Chang C. C. and Lee C. Y. (1963) Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch. Int. Pharmaocdyn.* **144,** 241–257.

Chappell T. G., Welch W. J., Shlossman D. M., Palter K. B., Schlesinger M. J., and Rothman J. E. (1986) Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* **45,** 3–13.

Cheng T. Po-O., Byrd F. I., Whitaker J. N., and Wood J. G. (1986) Immunocytochemical localization of coated vesicle protein in rodent nervous system. *J. Cell Biol.* **86,** 624–633.

Chuong C. -M., Crossin K. L., and Edelman G. M. (1987) Sequential expression and differential function of multiple adhesion molecules during the formation cerebellar cortical layers. *J. Cell Biol.* **104,** 331–342.

Cohen S. A. and Pumplin D. W. (1979) Clusters of intramembrane particles associated with binding sites for α-bungarotoxin in cultured chick myotubes. *J. Cell Biol.* **82,** 494–516.

Cole W. V. (1955) Motor endings in the striated muscle of vertebrates. *J. Compar. Neurol.* **102,** 671–715.

Crick F. H. C. (1983) The packing of α-helices: simple coiled-coils. *Acta Cryst.* **6,** 689–697.

Crowther R. A. and Pearse B. M. F. (1981) Assembly and packing of clathrin into coats. *J. Cell Biol.* **91,** 790–797.

Duband J. L. and Thiery J. P. (1982) Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. *Devel. Biol.* **93,** 308–323.

Eckenhoff M. F. and Pysh J. J. (1979) Double-walled coated vesicle formation: evidence for massive and transient conjugate internalization of plasma membranes during cerebellar development. *J. Neurocytol.* **8,** 623–638.

Eränkö O. (1967) The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. Roy. Micr. Soc.* **87,** 259–276.

Eriksdotter-Nilsson M., Björklund H., Dahl D., Olson L., and Ingvar M. (1987) Sustained seizures cause circumscribed cerebral changes in glial fibrillary acidic protein neurofilament and la minin immunofluorescence. *Exp. Brain Res.* **69,** 155–166.

Fine R. E. (1989) Vesicles without clathrin: intermediates in bulk flow exocytosis. *Cell* **58,** 609–610.

Foelix R. F. and Oppenheim H. (1974) The development of synapses in the cerebellar cortex of the chick embryo. *J. Neurocytol.* **3,** 277–294.

Fontaine B., Sassoon D., Buckingham M., and Changeux Jean-P. (1988) Detection of the nicotinic acetylcholine receptor α-subunit mRNA by *in situ* hybridization at neuromuscular junctions of 15-day-old chick striated muscles. *EMBO J.* **7,** 603–609.

Franke W. W., Schmid E., Osborn M., and Weber K. (1978) Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA* **75,** 5034–5038.

Friend D. S. and Farquhar M. G. (1967) Functions of coated vesicles during protein absorption in the rat vas deferens. *J. Cell Biol.* **35,** 357–376.

Fujita S. (1969) Autoradiographic studies on histogenesis of the cerebellar cortex, in *Neurobiology of Cerebellar Evolution and Development,* Llinás R., ed., American Medical Association, Chicago, pp. 743–747.

Fults D. W., Towle A. C., Lauder J. M., and Maness P. F. (1985) $pp60^{c\text{-}src}$ in the developing cerebellum. *Mol. Cell Biol.* **5,** 27–32.

Geisler N. and Weber K. (1981) Self-assembly *in vitro* of the 68,000 molecular component of the mammalian neurofilament triplet proteins into intermediate-sized filaments. *J. Mol. Biol.* **151,** 565–571.

Ghandour M. S., Vincendon G., and Gombos G. (1980) Astrocyte and oligodendrocyte distribution in adult rat cerebellum: an immunohistological study. *J. Neurocytol.* **9,** 637–646.

Glickman J. N., Conibear E., and Pearse B. M. F. (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO J.* **8,** 1041–1047.

Goldenring J. R., Lasher R. S., Vallano M. L., Ueda T., Naito S., Sternberger N. H., Sternberger I.A., and DeLorenzo R. J. (1986) Association of synapsin I with neuronal cytoskeleton. *J. Biol. Chem.* **261,** 8495–8504.

Gorden P., Carpentier Jean-L., Cohen S., and Orci L. (1978) Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **75,** 5025–5029.

Goto S., Hirano A., and Rojas-Corona R. R. (1989) A comparative immunocytochemical study of human cerebellar cortex in X-chromosome-linked copper malabsorption. (Menkes' kinky hair disease) and granule cell type cerebellar degeneration. *Neuropath. App. Neurobiol.* **15,** 419–431.

Gower D. J. and Tytell M. (1987) Axonal transport of clathrin-associated proteins. *Brain Res.* **407,** 1–8.

Gozes I. and Richter-Landsberg C. (1978) Identification of tubulin associated with rat brain myelin. *FEBS Lett.* **95,** 169–172.

Graeber M. B., Streit W. J., and Kreutzberg G. W. (1988) The microglial cytoskeleton: vimentin is localized within activated cells *in situ*. *J. Neurocytol.* **17,** 573–580.

Gray E. G. (1961) The granule cells mossy synapses and Purkinje spine synapses of the cerebellum: light and electron microscope observations. *J. Anat.* **95,** 345–363.

Griffiths G., Pfeiffer S., Simons K., and Matlin K. (1985) Exit of newly synthesized membrane proteins from the *trans* cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* **101**, 949–964.

Grovas A. C. and O'Shea K. S. (1984) An SEM examination of granule cell migration in the mouse cerebellum. *J. Neurosci. Res.* **12**, 1–14.

Gunther J. S. and Letinsky M. S. (1982) A preparation for studying dystrophic arian muscle and neuromuscular junctions. *Mus. Nerve* **5**, 7–13.

Haigler H. T., McKanna J. A., and Cohen S. (1979) Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol.* **81**, 382–395.

Hamburger V. and Hamilton H. L. (1951) A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

Hanaway J. (1967) Formation and differentiation of the external granular layer of the chick cerebellum. *J. Compar. Neurol.* **131**, 1–14.

Hanson V. G., Schook W. J., and Puszkin S. (1988) Brain coated vesicle destabilization and phosphorylation of coat proteins. *J. Neurochem.* **50**, 307–315.

Hanson V. G., Schook W. J., and Puszkin S. (1990) Novel regulatory role of phosphorylated clathrin light chain β in bovine brain coated vesicles. *J. Neurochem.* **54**, 46–50.

Hantaï D., Gautron J., and Labat-Robert J. (1983) Immunolocalization of fibronectin and other macromolecules of the intercellular matrix in the striated muscle fiber of the adult rat. *Collagen Rel. Res.* **3**, 381–391.

Helenius A., Kartenbeck J., Simons K., and Fries E. (1980) On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* **84**, 404–420.

Heuser J. E. and Reese T. S. (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **57**, 313–344.

Heuser J. (1980) Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* **84**, 560–583.

Hill B. L., Drickamer K., Brodsky F. M., and Parham P. (1988) Identification of the phosphorylation sites of clathrin light chain LCb. *J. Biol. Chem.* **263**, 5499–5501.

Hoffman P. N. and Cleveland D. W. (1988) Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific β-tubulin isotype. *Proc. Natl. Acad. Sci. USA* **85**, 4530–4533.

Hofman P. N. and Lasek R. J. (1975) The slow component of axonal transport. *J. Cell Biol.* **66**, 351–366.

Holmes N., Biermann J. S., Brodsky F. M., Bharucha D., and Parham P. (1984) Comparison of the primary structures of clathrin light chains from bovine brain and adrenal gland by peptide mapping. *EMBO J.* **3**, 1621–1627.

Howe P. R. C., Fenwick E. M., Rostas J. A. P., and Livett B. G. (1977) Immunochemical comparison o synaptic plasma and synaptic vesicle membrane antigens. *J. Neurocytol.* **6**, 339–352.

Huttner W. B., DeGennaro L. J., and Greengard P. (1981) Differential phosphorylation of multiple sites in purified protein I by cyclic AMP-dependent and calcium-dependent protein kinases. *J. Biol. Chem.* **256**, 1482–1488.

Hynes R. O. (1985) Molecular biology of fibronectin. *Ann. Rev. Cell Biol.* **1**, 67–90.

Hynes R. O. and Yamada K. M. (1982) Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* **95**, 369–377.

Imhof B. A., Marti U., Boller K., Frank H., and Birchmeier W. (1983) Association between coated vesicles and microtubules. *Exper. Cell. Res.* **145**, 199–207.

Jackson A. P. and Parham P. (1988) Structure of human clathrin light chains. *J. Biol. Chem.* **263**, 16,688–16,695.

Jackson A. P., Seow Heng-F., Holmes N., Drickamer K., and Parham P. (1987) Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments. *Nature* **326**, 154–159.

Jacobson M. (1978) *Developmental Neurobiology*, 2nd ed. Plenum, New York, p. 78.

Jahn R., Schiebler W., Ouimet C., and Greengard P. (1985) A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **82**, 4137–4141.

Johnson G. D. and de C. Nogueira Araujo G. M. (1981) A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Meth.* **43**, 349–350.

Jongsma A. P. M., Hijmans W., and Ploem J. S. (1971) Quantitative immunofluorescence. *Histochemie* **25**, 329–343.

Kanaseki T. and Kadota K. (1969) The "vesicle in a basket." *J. Cell Biol.* **42**, 202–220.

Keen J. H., Willingham M. C., and Pastan I. H. (1979) Clathrin-coated vesicles: isolation dissociation and factor-dependent reassociation of clathrin baskets. *Cell* **16,** 303–312.

Keen J. H. (1987) Clathrin assembly proteins: Affinity purification and a model for coat assembly. *J. Cell Biol.* **105,** 1989.

Kim S. U. (197) Formation of unattached spines of Purkinje cell dendrite in organotypic cultures of mouse cerebellum. *Brain Res.* **88,** 52–58.

Kirchhausen T. and Harrison S. C. (1981) Protein organization in clathrin trimers. *Cell* **23,** 755–761.

Kirchhausen T., Scarmato P., Harrison S. C., Monroe J. J., Chow E. P., Mattaliano R. J., Ramachandran K. L., Smart J. E., Ahn A. H., and Brosius J. (1987) Clathrin light chains LCA and LCB are similar, polymorphic and share repeated heptad motifs. *Science* **236,** 320–324.

Knaus P., Betz H., and Rehm H. (1986) Expression of synaptophysin during postnatal development of the mouse brain. *J. Neurochem.* **47,** 1302–1304.

Kohtz D. S. and Puszkin S. (1988) A neuronal protein (NP185) associated with clathrin-coated vesicles. *J. Biol. Chem.* **263,** 7418–7425.

Kohtz D. S. and Puszkin S. (1989) Phosphorylation of tubulin by casein kinase II regulates its binding to a neuronal protein (NP185) associated with brain coated vesicles. *J. Neurochem.* **52,** 285–295.

Kohtz D. S., Hanson V. G., Kohtz J. D., Schook W. J., and Puszkin S. (1987) Mapping two functional domains of clathrin light chains with monoclonal antibodies. *J. Cell Biol.* **104,** 897–903.

Kohtz D. S., Hanson V. G., and Puszkin S. (1990) Novel proteins mediate an interaction between clathrin-coated vesicles and polymerizing actin filaments *Eur. J. Biochem.* **192,** 291–298.

Kohtz D. S. and Puszkin S. (1990) Novel NGF-induced protein in PC 12 cells: Immunological evidence for their presence in brain nerve endings using a single monoclonal antibody. *J. Neurosci. Res.* **27,** 307–313.

Krebs E. G. and Beavo J. A. (1979) Phosphorylation-dephosphorylation of enzymes. *Ann. Rev. Biochem.* **48,** 923–959.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Landis D. M. D. (1987) Initial junctions between developing parallel fibers and Purkinje cells are different from mature synaptic junctions. *J. Compar. Neurol.* **260,** 513–525.

Landis D. M. D. and Reese T. S. (1974) Differences in membrane structure between excitatory and inhibitory synapses in the cerebellar cortex. *J. Compar. Neurol.* **155,** 93–126.

Langley O. K., Sternberger N. H., and Sternberger L. A. (1988) Expression of neurofilament proteins by Purkinje cells: ultrastructural immunolocalization with monoclonal antibodies. *Brain Res.* **457,** 12–20.

Larkin J. M., Donzell W. C., and Anderson R. G. W. (1986) Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices. *J. Cell Biol.* **103,** 2619–2627.

Larsell O. (1967) *The Comparative Anatomy and Histology of the Cerebellum from Myxinoids through Birds.* The University of Minnesota Press, Minneapolis, MN, pp. 226–240, 243–245, 262–273.

Lasek R. J., Garner J. A., and Brady S. T. (1984) Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* **99,** 212s-221s.

Leclerc N., Beesley P. W., Brown I., Colonnier M., Gurd J. W., Paladino T., and Hawkes R. (1989) Synaptophysin expression during synaptogenesis in the rat cerebellar cortex. *J. Compar. Neurol.* **280,** 197–212 .

Liem R. K. H., Yen Shu-H., Salomon G. D., and Shelanski M. L. (1978) Intermediate filaments in nervous tissues. *J. Cell Biol.* **79,** 637–645.

Lisanti M. P., Shapiro L. S., Moskowitz N., Hua E. L., Puszkin S., and Schook W. (1982) Isolation and preliminary characterization of clathrin-associated proteins. *Eur. J. Biochem.* **125,** 463–470.

Lisanti M. P., Flanagan M., and Puszkin S. (1984) Clathrin lattice reorganization: theoretical considerations. *J. Theor. Biol.* **108,** 143–157.

Lisanti M. P. *Tissue and Cell Research.*

Lowe J., Maclennan K. A., Powe D. G., Pound J. D., and Palmer J. B. (1989) Microglial cells in human brain have phenotypic characteristics related to possible function as dendritic antigen presenting cells. *J. Pathol.* **159,** 143–149.

Luthman J., Johansson O., Ahlström U., and Kvint S. (1988) Immunohistochemical studies of the neurochemical markers CGRP, enkephalin, galanin, gamma-MSH, NPY, PHI, proctolin, PTH, somatostatin SP, VIP, tyrosine hydroxylase and neurofilament in nerves and cells of the human attached gingiva. *Arch. Oral Biol.* **33,** 149–158.

Mares V., Viklicky V., Gerstein L. M., Dráber P., and Ciesielski-Treska J. (1988) Immunocytochemistry and heterogeneity of rat brain vimentin. *Histochem.* **88,** 576–581.

Mariani J. and Changeux Jean-P. (1981) Ontogenesis of olivocerebellar relationships. I. Studies by intracellular recordings of the multiple innervation of Purkinje cells by climbing fibers in the developing rat cerebellum. *J. Neurosci.* **1**, 697–702.

Martin J. H. (1989) *Neuroanatomy*, Text and Atlas. Elsevier, New York, pp. 45–46, 239–266.

McComb R. D. and Bigner D. D. (1985) Immunolocalization of laminin in neoplasms of the central and peripheral nervous systems. *J. Neuropath. Exp. Neurol.* **44**, 242–253.

McKay R. D. G. and Hockfield S. J. (1982) Monoclonal antibodies distinguish antigenically discrete neuronal types in the vertebrate central nervous system. *Proc. Natl. Acad. Sci. USA* **79**, 6747–6751.

McQuarrie I. G., Brady S. T., and Lasek R. J. (1986) Diversity in the axonal transport of structural proteins: major differences between optic and spinal axons in the rat. *J. Neurosci.* **6**, 1593–1605.

Mesland D. A. M., Spiele H., and Ross E. (1981) Membrane-associated cytoskeleton and coated vesicles in cultures hepatocytes visualized by dry-cleaving. *Exp. Cell Res.* **132**, 169–184.

Miale I. L. and Sidman R. L. (1961) An autoradiographic analysis of histogenesis in the mouse cerebellum. *Exper. Neurol.* **4**, 277–296.

Moskowitz N., Schook W., and Puszkin S. (1982) Comparison of calmodulin binding to brain synaptic and coated vesicles. *Biochim Biophys. Acta* **689**, 523–530.

Mugnaini E. (1969) Ultrastructural studies on the cerebellar histogenesis. II. Maturation of nerve cell populations and establishment of synaptic connections in the cerebellar cortex of the chick, in *Neurobiology of Cerebellar Evolution and Develpment*, Llinás R., ed., American Medical Association, Chicago, pp. 749–782.

Mugnaini E. (1970) The relation between cytogenesis and the formation of different types of synaptic contact. *Brain Res.* **17**, 169–179.

Mugnaini E. and Forstrønen P. F. (1967) Ultrastructural studies on the cerebellar histogenesis. I. Differentiation of granule cells and development of *glomeruli* in the chick embryo. *Z. Zellforsch* **77**, 115–143.

Murphy J., Pleasure I. T., Puszkin S., Prasad K., and Keen J. (1991) Clathrin assembly protein AP-3: The identity of the 155 kD protein AP-180 and NP185 and analysis of domain structure. *J. Biol. Chem.* **266**, 4401.

Nelsen O. E. (1953) *Comparative Embryology of the Vertebrates*. McGraw-Hill, New York, pp. 805–835.

Obata K. and Fujita S. C. (1984) Developmental changes of chick cerebellar cortex revealed by monoclonal antibodies. *Neurosci. Res.* **1**, 117–129.

Oblinger M. M. (1988) Biochemical composition and dynamics of the axonal cytoskeleton in the corticospinal system of the adult hamster. *Metab. Brain Dis.* **3**, 49–65.

Ockleford C. D. (1976) A three-dimensional reconstruction of the polygonal pattern on placental coated-vesicle membranes. *J. Cell Sci.* **21**, 83–91.

Orci L., Glick B. S., and Rothman J. E. (1986) A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* **46**, 171–184.

Osborn M. (1983) Intermediate filaments as histologic markers: an overview. *J. Invest. Dermatol.* **81**, 104s–109s.

Osborn M. and Weber K. (1982) Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures, in *Methods in Cell Biology*, vol. 24 Wilson L., ed., Academic, New York, pp. 97–132.

Partanen S. (1975) Simultaneous fluorescnce histochemical demonstration of catecholamines and tryptophyl-peptides in endocrine cells. *Histochem.* **43**, 295–303.

Patten B. M. (1951) *Early Embryology of the Chick*, 4th ed., The Blakiston, New York, p. 101.

Pauloin A., Bernier I., and Jollès P. (1982) Presence of cyclic nucleotide-Ca^{2+} independent protein kinase in bovine brain coated vesicles. *Nature* **298**, 574–576.

Pauloin A. and Jollès P. (1986) Presence of a MgATP/ADP-dependent pp50 phosphatase in bovine brain coated vesicles. *J. Biol. Chem.* **261**, 12,568–12,573.

Pauloin A., Thurieau C., and Jolles P. (1988) Cyclic phosphorylation/dephosphorylation cascade in bovine brain coated vesicles. *Biochim Biophys. Acta* **968**, 91–95.

Payne G. S. and Schekman R. (1985) A test of clathrin function in protein secretion and cell growth. *Science* **230**, 1009–1014.

Pearse B. M. F. (1975) Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* **97**, 93–98.

Pearse B. M. F. (1976) Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. USA* **73**, 1255–1259.

Pearse B. M. F. (1978) On the structural and functional components of coated vesicles. *J. Cell Biol.* **126,** 803–812.

Pearse B. M. F. (1985) Assembly of the mannose-6-phosphate receptor into reconstituted clathrin-coats. *EMBO J.* **4,** 2457–2460.

Pearse B. M. F. (1987) Clathrin and coated vesicles. *EMBO J.* **6,** 2507–2512.

Pearse B. M. F. (1989) Characterization of coated-vesicle adaptors: their reassembly with clathrin and with recycling receptors. *Meth. Cell Biol.* **31,** 229–243.

Pearse B. M. F. and Bretscher M. S. (1981) Membrane recycling by coated vesicles. *Ann. Rev. Biochem.* **50,** 85–101.

Pearse B. M. F. and Crowther R. A. (1981) Packing of clathrin into coats. *Cold Spring Harbor Symp. Quant. Biol.* **46,** 703–706.

Pearse B. M. F. and Crowther R. A. (1987) Structure and assembly of coated vesicles. *Ann. Rev. Biophys. Chem.* **16,** 49–68.

Pearse B. M. F. and Robinson M. S. (1984) Purification and properties of 100-kd proteins from coated vesicles and their reconstitution with clathrin. *EMBO J.* **3,** 1951–1957.

Pelc S., Fondu P., and Gompel C. (1986) Immunohistochemical distribution of glial fibrillary acidic protein, neurofilament polypeptides and neuronal specific enolase in the human cerebellum. *J. Neurosci.* **73,** 289–297.

Perry D. G., Hanson V., Benuck M. L., and Puszkin S. (1991) Neuronal protein NP185 in Avian and Murine Cerebellum: Expression during development and evidence for its presence in nerve endings. *J. Histochem. Cytochem.* **39,**1461–1470.

Pfeffer S. R. and Kelly R. B. (1985) The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. *Cell* **40,** 949–957.

Polak M. (1965) Morphological and functional characteristics of the central and peripheral neuroglia, in *Biology of Neuroglia,* 1st ed., vol. 15, de Robertis E. D. P. and Carrea H., eds., Elsevier, New York, pp. 12–34.

Pollard T. D. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Ann. Rev. Biochem.* **55,** 987–1035.

Porter M. E. and Johnson K. A. (1989) Dynein structure and function. *Ann. Rev. Cell Biol.* **5,** 119–151.

Pratt M. M. (1980) The identification of a dynein ATPase in unfertilized sea urchin eggs. *Devel. Biol.* **74,** 364–378.

Pretorius H. T., Nandi P. K., Lippoldt R. E., Johnson M. L., Keen J. H., Pastan I., and Edelhoch H. (1981) Molecular characterization of human clathrin. *Biochem.* **20,** 2777–2782.

Pumplin D. W. (1989) Acetylcholine receptor clusters of rat myotubes have at least three domains with distinctive cytoskeletal-membranous components. *J. Cell Biol.* **109,** 739–753.

Pumplin D. W. and Bloch R. J. (1987) Disruption and reformation of the acetylcholine receptor clusters of cultured rat myotubes occur in two distinct stages. *J. Cell Biol.* **104,** 97–108.

Purves D. and Lichtman J. W. (1985) Rearrangement of developing neuronal connections, in *Principles of Neural Development.* Sinauer Associates, Sunderland MA, pp. 271–300.

Puszkin S., Haver K., and Schook W. (1981) Brain Clathrin: a Study of its properties, in *Regulatory Mechanisms of Synaptic Transmission,* Tapia H. and Cotman C. W., eds., Plenum, New York, pp. 241–260.

Puszkin S., Lisanti M., Haver K., Hua E. L., Moskowitz N., Bloom W. S., and Schook W. J. (1982) Brain clathrin complex: II. Immunofluorescent correlation and biochemical affinity for actin. *J. Histochem. Cytochem.* **30,** 497–503.

Puszkin S., Andres A., Ores C., Lisanti M. P., and Schook W. J. (1983) Immunocytochemical characterization of clathrin-associated proteins. (CAPs). I. Neuronal distribution of CAPs, a component of clathrin-coated vesicles. *Cell Tissue Res.* **231,** 495–505.

Puszkin S., Kohtz J. D., Schook W. J., and Kohtz D. S. (1989) Clathrin-coated vesicle subtypes in mammalian brain tissue: Detection of polypeptide heterogeneity by immunoprecipitation with monoclonal antibodies. *J. Neurochem.* **53,** 51–63.

Quesada A. and Genis-Galvez J. M. (1983) Early development of the granule cell in the cerebellum of the chick embryo. *J. Morph.* **178,** 323–334.

Rakic P. (1971) Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and EM study *Macacus rhesus. J. Compar. Neurol.* **141,** 283–312.

Reynolds R. and Wilkin G. P. (1988) Expression of G_{D3} ganglioside by developing rat cerebellar Purkinje cells in situ. *J. Neurosci. Res.* **20,** 311–319.

Robinson M. S. (1987) 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* **104,** 887–895.

Robinson M. S. (1989) Cloning of cDNAs encoding two related 100-kD coated vesicle proteins. (α-adaptins). *J. Cell Biol.* **108**, 833–842.

Robinson M. S. and Pearse B. M. F. (1986) Immunofluorescent localization of 100-kD coated vesicle proteins. *J. Cell Biol.* **102**, 48–54.

Robinson M. S. (1990) Cloning and expression of gamma-adaptin a component of clathrin-coated vesicles associated with the Golgi Apparatus. *J. Cell Biol.* **111**, 2319–2326.

Romand H., Hafidi A., and Despres G. (1988) Immunolocalization of neurofilament protein subunits in the spiral ganglion of the adult rat. *Brain Res.* **462**, 167–173.

Roth T. F. and Porter K. R. (1964) Yolk protein uptake in the oocyte of the mosquito *Aaedes aegypti* L. *J. Cell Biol.* **20**, 313–332.

Rothman J. E. (1986) Life without clathrin. *Nature* **319**, 96–97.

Rothman J. E. and Fine R. E. (1980) Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. *Proc. Natl. Acad. Sci. USA* **77**, 780–784.

Salisbury J. L., Condeelis J. S., and Safir P. (1980) Coated vesicles and actin *J. Cell Biol.* **87**, 132.

Schiebler W., Jahn H., Doucet Jean-P., Rothlein J., and Greengard P. (1986) Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* **261**, 8383–8390.

Schiffer D., Giordana M. T., Migheli A., Giaccone G., Pezzotta S., and Mauro A. (1986) Glial fibrillary acidic protein and vimentin in the experimental glial reaction of the rat brain. *Brain Res.* **374**, 110–118.

Schlessinger J., Shechter Y., Willingham M. C., and Pastan I. (1978) Direct visualized binding, aggregation, and internalization of isulin and epidermal growth factor on living fibroblastic cells. *Proc. Natl. Acad. Sci. USA* **75**, 2659–2663.

Schlossman D. M., Schmid S. L., Braell W. A., and Rothman J. E. (1984) An enzyme that removes clathrin-coats: purification of an uncoating ATPase. *J. Cell Biol.* **99**, 723–733.

Schnitzer J., Franke W. W., and Schachner M. (1981) Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system. *J. Cell Biol.* **90**, 435–447.

Schook W. J. and Puszkin S. (1985) Expression of a brain coated vesicle kinase activity which has clathrin light chain 2 as its principal substrate. *J. Cell Biol.* **101**, 49a (abstract).

Schook W., Puszkin S., Bloom W., Ores C., and Kochwa S. (1979) Mechanochemical properties of brain clathrin: Interactions with actin and α-actinin and polymerization into basketlike structures or filaments. *Proc. Natl. Acad. Sci. USA* **76**, 116–120.

Schook W. J. and Puszkin S. (1985) Brain clathrin light chain 2 can be phospborylated by a coated vesicle kinase. *Proc. Natl. Acad. Sci. USA* **82**, 8039–8043.

Scott L. J. C., Bacou F., and Sanes J. R. (1988) A synapse-specific carbohydrate at neuromuscular junction: association with both acetylcholinesterase and a glycolipid. *J. Neurosci.* **8**, 932–944.

Shimono T., Nosaka S., and Sasaki K. (1976) Electrophysiological study on the postnatal development of neuronal mechanisms in the rat cerebellar cortex. *Brain Res.* **108**, 279–294.

Silver A. (1963) A histochemical investigation of cholinesterases at neuromusular junctions in mammalian and avian muscle. *J. Physiol.* **169**, 386–393.

Sinicropi D. V. and McIlwain D. L. (1983) Changes in the amounts of cytoskeletal proteins within the perikarya and axons of regenerating frog motoneurons. *J. Cell Biol.* **96**, 240–247.

Sommer I., Lagenaur C., and Schachner M. (1981) Recognition of Bergmann glial and ependymal cells in the mouse nervous system by monoclonal antibody. *J. Cell Biol.* **90**, 448–458.

Sotelo C. (1967) Cerebellar Neuroglia: Mophological and Histochemical Aspects, in *The Cerebellum*, 1st ed., vol. 25. Fox C. A. and Snider R. S., eds.. Elsevier, New York, pp. 226–250.

Steiner J. P., Ling E., and Bennett V. (1987) Nearest neighbor analysis for brain synapsin I. *J. Biol. Chem.* **262**, 905–914.

Stelzner D. J., Martin A. H., and Scott G. L. (1973) Early stages of synaptogenesis in the cervical spinal cord of the chick embryo. *Z. Zellforsch* **138**, 475–488.

Stephens R. E. (1986) Membrane tubulin. *Biol. Cell* **57**, 95–110.

Sternberger L. A., Harwell L. W., and Sternberger N. H. (1982) Neurotypy: regional individuality in rat brain detected by immunocytochemistry with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **79**, 1326–1330.

Su B., Hanson V., Perry D., and Puszkin S. (1991) Biochemical and immunological properties of the neuronal protein NP185. *J. Neurosci. Res.* **29**, 461–473.

Sôdhof T. C., Lottspeich F., Greengard P., Mehl E., and Jahn R. (1987) A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. *Science* **238**, 1142–1144.

Tapscott S. J., Bennett G. S., and Holtzer H. (1981) Neuronal precursor cells in the chick neural tube express neurofilament proteins. *Nature* **292**, 836–838.

Thomas L., Kartung K., Langosch D., Rehm H., Bamberg E., Franke W. W., and Betz H. (1988) Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science* **242**, 1050–1053.

Torri-Tarelli F., Villa A., Valtorta F., de Camilli P., Greengard P., and Ceccarelli B. (1990) Redistribution of synaptophysin and synapsin I during α-latrotoxin-induced release of neurotransmitter at the neuromuscular junction. *J. Cell Biol.* **110**, 449–459.

Towbin H., Staehelin T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Trojanowski J. Q., Walkenstein N., and Lee V. M-Y. (1986) Expression of neurofilament subunits in neurons of the central and peripheral nervous system: an immunohistochemical study with monoclonal antibodies. *J. Neurosci.* **6**, 650–660.

Uchizono K. (1969) Synaptic organization of the mammalian cerebellum, in *Neurobiology of Cerebellar Evolution and Development*, 1st ed., Llinás R., ed., American Medical Association, Chicago, pp. 549–583.

Ueda T. and Greengard P. (1977) Adenosine 3':5'-monophosphate-regulated phosphoprotein system of neuronal membranes. *J. Biol. Chem.* **252**, 5155–5163.

Unanue E. R., Ungewickell E., and Branton D. (1981) The binding of clathrin triskelions to membranes from coated vesicles. *Cell* **26**, 439–446.

Ungewickell E. and Branton D. (1981) Assembly units of clathrin-coats. *Nature* **289**, 420–422.

Ungewickell E. and Branton D. (1982) Triskelions: the building blocks of clathrin-coats. *TIBS* **7**, 358–360.

Ungewickell E., Unanue E. R., and Branton D. (1981) Functional and structural studies on clathrin triskelions and baskets. *Cold Spring Harbor Symp. Quant. Biol.* **46**, 723–732.

Usami M., Takahashi A., Kadota T., and Katoda K. (1985) Phosphorylation of a clathrin light chain of coated vesicles in the presence of histones. *J. Biochem.* **97**, 1819–1822.

Vale R. D. (1987) Intracellular transport using microtubule-based motors. *Ann. Rev. Cell Biol.* **3**, 347–378.

Valtorta F., Torri-Tarelli F., Campanati L., Villa A., and Greengard P. (1989) Synaptophysin and synapsin I as tools for the study of the exo-endocytotic cycle. *Cell Biol. Int. Rep.* **13**, 1023–1038.

Vigers G. P. A., Crowther R. A., and Pearse B. M. F. (1986) Three-dimensional structure of clathrin cages in ice. *EMBO J.* **5**, 529–534.

de Waegh S. and Brady S. T. (1989) Axonal transport of a clathrin uncoating ATPase. (HSC70): a role for HSC70 in the modulation of coat vesicle assembly in vivo. *J. Neurosci. Res.* **23**, 433–440.

Wall D. A., Wilson G., and Hubbard A. L. (1980) The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepato<ytes. *Cell* **21**, 79–93.

Waxman S. G. and Pappas G. D. (1969) Pinocytosis at postsynaptic membranes: E M evidence. *Brain Res.* **14**, 240–244.

Wenger E. and Wenger B. S. (1978) A histochemical method for gross demonstration of motor end plates. *Stain Technol.* **53**, 279–282.

Wiedemnann B. and Franke W. W. (1985) Identity and location of synaptophysin, an integral membrane glycoprotein of 38 kD characteristic of presynaptic vesicles. *Cell* **41**, 1017–1028.

Wilkin G. P. and Levi G. (1986) Cerebellar Astrocytes, in *Astrocytes* 1st ed., vol. 1. Fedoroff S. and Vernadakis A., eds., Academic, New York, pp. 245–268.

Willingham M. C., Maxfield F. R., and Pastan I. H. (1979) α_2Macroglobulin binding to the plasma membrane of cultured fibroblasts. *J. Cell Biol.* **82**, 614–625.

Woodward D. J., Hoffer B. J., and Lapham L. W. (1969) Correlative survey of electrophysiological neuropharmacological and histochemical aspects of cerebellar maturation in rat, *Neurobiology of Cerebellar Evolution and Development*, Llinás R., ed., American Medical Association, Chicago, pp. 725–741.

Zamboni L. and de Martino C. (1967) Buffered picric acid-formaldehyde:A new, rapid fixative for electron microscopy. *J. Cell Biol.* **35**, 148A (Abstract).

Zaremba S. and Keen J. H. (1983) Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin-coats. (arrow). *J. Cell Biol.* **97**, 1339–1347.