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Neuron-Specific Expression of High-Molecular-Weight Clathrin Light Chain

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High-molecular-weight forms of clathrin light chains LC_a and LC_b contain inserted sequences and are expressed in brain tissue but have not been observed in peripheral tissues. Monoclonal antibodies specific for the high-molecular-weight form of LC_b and all forms of LC_a were used to analyze their expression in different species and different neuronal cell types. High-molecular-weight light chains were found in bovine, rat, mouse, chicken, and human brain, indicating a conserved pattern of expression. Neuron-specific expression of the high-molecular-weight light chains was suggested by analysis of human brain gray matter and white matter. The former contained a higher proportion of light chains with insertion sequences. Immunohistochemical analysis localized the high-molecular-weight form of LC_b to synapses and neuronal perikarya, but not to glial cells. Immunofluorescent labeling of cultured chicken dorsal root ganglia confirmed expression in neurons but not Schwann cells. These results indicate that the high-molecular-weight forms of clathrin light chains are restricted in expression and found in neuronal cells.

Clathrin-coated vesicles mediate intracellular trafficking of membrane-bound and secreted proteins to and from the plasma membrane (Brodsky, 1988). In neurons, coated vesicles have been implicated in membrane recycling following synaptic vesicle fusion (Heuser and Reese, 1973; Miller and Heuser, 1984) and in targeting proteins to the fast axonal transport pathway (Stone et al., 1984). The major coated vesicle protein, clathrin, has a 3-legged triskelion shape (Ungewickell and Branton, 1981). It is composed of 3 heavy chains (180 kDa) and 3 light chains of 2 types, LC_a and LC_b (30–40 kDa) (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). The clathrin heavy chain provides the structural backbone for the triskelion and the protein lattice comprising the assembled clathrin coat. Examination of the light chain protein sequences suggests they contribute both structural and regulatory elements to coated vesicle formation and function (Brodsky, 1988). However, their exact roles are not yet understood.

The clathrin light chains LC_a and LC_b are encoded by 2 distinct genes and have similar features, although their sequences are only 60% homologous (Jackson et al., 1987). Both light chains undergo differential RNA splicing in the brain, generating higher-molecular-weight forms. There are 2 higher-molecular-weight forms of LC_a, with 30 and 18 amino acid inserts, respectively, and 1 of LC_b with a different but homologous 18 amino acid insert (Jackson et al., 1987; Kirchhausen et al., 1987). These inserted sequences are found at corresponding positions in both light chains and are exposed to the cytoplasm in assembled coated vesicles (Brodsky et al., 1987).

Since the higher-molecular-weight clathrin light chains are found almost exclusively in brain tissue (Brodsky and Parham, 1983; Brodsky, 1985a), they have often been referred to as "brain-specific." To gain insight into the possible function of the inserted sequences and the clathrin light chains themselves, we have analyzed the cellular distribution of the high-molecular-weight light chains in brain and neuronal tissue. These forms are predominant in brain gray matter and are expressed in cultured sensory neurons. Glial cells express only the "non-brain" or "peripheral" light-chain forms. These data suggest the high-molecular-weight clathrin light chains are restricted to neurons and may contribute to a neuron-specific function of clathrin-coated vesicles.

Materials and Methods

Preparation of tissue homogenates and immunoblotting. Tissue samples were weighed and homogenized in 2 volumes (for human samples) or 4 volumes (for nonhuman samples) of cold homogenizing buffer (10 mM Tris, pH 7.3, 1 mM $MgCl_2$, 0.5% NP-40, 0.005% phenylmethylsulfonyl fluoride) using a glass–Teflon homogenizer. Homogenates were incubated on ice for 30 min followed by centrifugation, 30 min at 12,000 × *g* at 4°C. Supernatants were collected and frozen at −80°C for more than 2 hr, thawed, and boiled for 20 min to separate heat-labile proteins from clathrin light chain. Samples were then centrifuged for 15 min, 12,000 × *g* at 4°C, to pellet denatured proteins. Resulting supernatant was diluted in 2× DTT sample buffer (1.5% SDS, 20 mM dithiothreitol (DTT), 12.5 mM Tris, pH 6.8, 0.004% bromphenol blue, 10% glycerol) and then boiled for 20 min. Reduced proteins were alkylated with 25 mM iodoacetamide for 30 min at room temperature. Reduced and alkylated samples were applied to SDS-polyacrylamide (10%) gels in the

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following amounts: 10 μl (gray matter), 20 μl (white matter), 50 μl (spinal cord and nonhuman samples). Proteins were then transferred from the gel to 0.45 μm nitrocellulose (BioRad) and incubated with either X16 or LCB.2 (Brodsky, 1985a) followed by ^{125}I-rabbit anti-mouse immunoglobulin.

Immunohistochemistry. Postmortem samples of human spinal cord and cerebellum were fixed in Bouin's solution, 70% ethanol, or microwave denatured and embedded in paraffin (Trojanowski et al., 1989). Six micron sections were cut. Antibody labeling was carried out using the peroxidase–antiperoxidase (PAP) technique (Sternberger et al., 1970). Sections were incubated in 1% fetal calf serum (FCS) in 0.5 M Tris, pH 7.6 (blocking solution) for 10 min at room temperature and then overlayed with monoclonal antibody (mAb) solutions (1–4 μg/ml in blocking solution), 4°C overnight. The mAbs used were X16 (Brodsky, 1985a), LCB.2 (Brodsky, 1985a), SY38 (anti-synaptophysin; Boehringer Manheim), and RMd0 20 (Lee et al., 1987). Linking antibody (goat anti-mouse; Cooper Biomedical) diluted 1:200 in blocking solution was layered onto washed sections and left 1 hr at room temperature. Then specimens were incubated at room temperature for 1 hr in PAP complex (mouse origin), diluted 1:40,000 in blocking solution.

After washing, immunoperoxidase staining was developed for 10 min with 0.05% 3′,3′ diaminobenzidine (DAB), 0.03% H_2O_2 in 0.5 M Tris buffer with 0.01 M imidazole. The reaction was stopped by removal of the DAB solution, and sections were counterstained with hematoxylin.

Primary cell cultures. All solutions and media were purchased from the UCSF Tissue Culture Facility except where noted. Astrocyte cultures were obtained from D. Spencer and M. Berens (Brain Tumor Research Center, UCSF). Cultures were prepared using a modification of the protocol of Pulliam et al. (1988). Brain tissue was suspended in PBS, pH 7.4 and dissociated into single cells by passage through 2 nylon screens of 200 and 100 μm mesh. Cells were washed 3 times with PBS and resuspended in DME-H21 supplemented with 15% FCS, 20 μg/ml insulin, 50 μg/ml gentamicin, and nonessential amino acids. After 48 hr, cells were trypsinized and seeded into Lab-tek slides (Nunc Intermed). Cells were grown in MEM-EBSS supplemented with 10%FCS.

Dorsal root ganglia (DRG) were dissected from four 8-d-old chick embryos and washed twice in cold Ca^{2+}/Mg^{2+}-free PBS, pH 7.4 (CMF-PBS). DRG pellet was resuspended in 5 ml CMF-PBS + 250 μl of 0.25% bactotrypsin + 0.02% EDTA and incubated at 37°C for 15–25 min. Ganglia were then triturated through a fire-polished (to one-half diameter) Pasteur pipet. Hams F-12, 5 ml, media supplemented with 5% FCS (F-12/FCS) was added to stop trypsinization. Neurons were washed 3 times with F-12/FCS and resuspended in 2.5 ml of F-12/FCS and preplated on a 60 mm culture dish for 1 hr at 37°C/5% CO_2 to remove fibroblasts. The plate was then swirled gently to collect both free-floating and partially attached neurons. Neurons were centrifuged and then plated on poly-D-lysine-coated coverslips in a 24-well culture plate and grown 48 hr in F-12/FCS + 100 ng/ml β-subunit of nerve growth factor (β-NGF). Each well was supplemented with more β-NGF (100 ng/ml) after the first 24 hr. β-NGF was prepared as described by Suda et al. (1978).

Indirect immunofluorescence. Cells were fixed for 10 min at room temperature with 3.7% paraformaldehyde in PBS and permeabilized with 0.04% saponin for 10 min at room temperature. Each well was rinsed with PBS and blocked with blocking solution (5% normal goat serum, 4% FCS, 0.1% Triton-X 100, 0.02% SDS in PBS) for 1 hr at room temperature. After blocking, antibodies (20 μg/ml in blocking solution) or antibody cocktail (undiluted) was added to each well and incubated for 1 hr at room temperature followed by four 5 min washes with PBS. The mAbs used were X22 (anti-heavy chain; Brodsky, 1985a) LCB.2 (Brodsky et al., 1987), 29B5 (anti-dinitrophenol), and GFAP-mAb cocktail (glial fibrillary acidic protein; Biomedical Technologies). Rhodamine-conjugated goat anti-mouse immunoglobulin (1:600) or fluorescein-conjugated goat anti-mouse immunoglobulin (1:250) (Organon Technika-Cappel) diluted in blocking solution was added to each well for 1 hr at 4°C, followed by four 5 min washes and 1 wash in dH_2O. Samples were then mounted in 0.1% *o*-phenylenediamine (Sigma) in Fluoromount-G (Fisher) and visualized under a microscope using a 60× oil-immersion objective.

Results and Discussion

The protein sequences determined for human, bovine, and rat LC_a and LC_b show that for each light chain the inserted sequences are completely conserved except for a single amino acid substitution in rat LC_b at position 170 (Jackson and Parham, 1988; Kirchhausen et al., 1987). To explore the extent of conservation of the inserted sequences, mAb LCB.2, which recognizes the inserted sequence of bovine LC_b, was used (Brodsky et al., 1987). Antibody reactivity with tissue from chicken, mouse, and rat was tested by immunoblotting (Fig. 1) after tissue homogenates were separated by gel electrophoresis and transferred to nitrocellulose. LCB.2 stained a polypeptide of about 37 kDa in brain tissue homogenates from all these species. This corresponds to the previously observed gel migration pattern for LC_b, which runs slower than its actual molecular weight (25.1 kDa) would predict (Jackson et al., 1987). LCB.2 did not react with liver homogenates from any of these species. In comparison, mAb X16, which reacts with all 3 forms of LC_a (Brodsky et al., 1987), stained all tissue samples. The higher-molecular-weight forms of LC_a were detected only in brain homogenates. X16 bound less avidly to LC_a in chicken samples compared to its binding to LC_a from the other species tested. These results show that for chicken, mouse, and rat clathrin, the high-molecular-weight forms of LC_a and LC_b are restricted in expression and found in brain.

To localize the higher-molecular-weight forms of clathrin light chain within neuronal tissue, homogenates of gray and white matter from human brain and human spinal cord were prepared. These samples were tested for X16 and LCB.2 binding by immunoblotting. Since the weight/volume ratio of tissue to homogenizing buffer was maintained for each human tissue sample prepared (see Materials and Methods), comparison of the relative amounts of clathrin light chain in each brain region was possible. Binding of mAb X16 produced a pattern of 3 bands: 2 higher-molecular-weight bands corresponding to LC_a with the 30 and 18 amino acid insertion sequence and a lower band representing the peripheral form of LC_a without an insertion sequence (Fig. 2). Binding of mAb LCB.2 revealed a single band corresponding to LC_b with its insertion sequence. A clear difference in light chain expression in gray and white matter was observed. Both X16 and LCB.2 showed greater binding to gray matter homogenates than to white matter, even though twice as much white matter sample was applied to the gel. This indicates that gray matter contains more clathrin light chains per gram of tissue than white matter. In X16 blots, the band corresponding to the peripheral form of LC_a was very faint in gray matter (Fig. 2). In white matter, however, this band was much more pronounced relative to the other LC_a bands. This demonstrates that the ratio of the high-molecular-weight forms of clathrin light chain to peripheral form is greater in gray matter compared to white matter. The above observations were true of gray and white matter from both occipital and frontal cortex. In addition, occipital white matter appeared to have more LC_a with the 18 amino acid insert than with the 30 amino acid insert. Although this result was consistent between different preparations of the same sample, its significance is unclear since only one tissue sample was available. Spinal cord samples also showed expression of the high-molecular-weight forms of clathrin light chain as well as the peripheral forms. Thus, the high-molecular-weight clathrin light chains are generally characteristic of CNS tissue.

The presence of glial cells correlated with the increased expression of the peripheral form of clathrin light chains in white matter. This suggested that the light chains with insertion sequences might be neuron-specific and not expressed by glial cells. Furthermore, the high concentration of light chains in gray

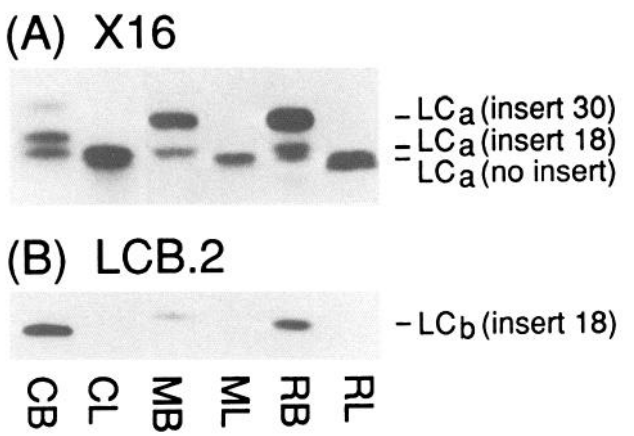

Figure 1. Expression of high-molecular-weight clathrin light chains in avian and mammalian tissues. Immunoblots of chicken brain (*CB*), chicken liver (*CL*), mouse brain (*MB*), mouse liver (*ML*), rat brain (*RB*), and rat liver (*RL*) with mAbs recognizing clathrin light chains: (*A*) X16, (*B*) LCB.2

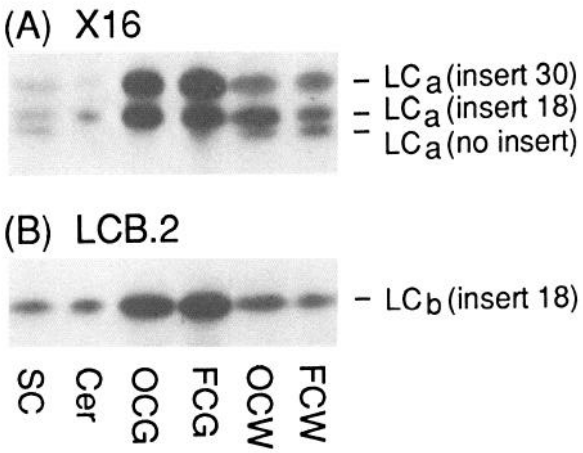

Figure 2. Clathrin light distribution in human neuronal tissue homogenates. Immunoblot of spinal cord (*SC*), cerebellum (*Cer*), occipital cortex gray matter (*OCG*), frontal cortex gray matter (*FCG*), occipital cortex white matter (*OCW*), and frontal cortex white matter (*FCW*), with mAbs recognizing clathrin light chains: (*A*) X16, (*B*) LCB.2.

matter suggested that the expression of the high-molecular-weight light chains might be increased at synaptic junctions. To look at the distribution of the clathrin light chains at the cellular level, histological sections of human brain and spinal cord were stained with X16 and LCB.2. X16 does not react with LC_a when it is bound to clathrin heavy chain. However, the X16 determinant becomes exposed after microwave treatment of tissue sections, allowing X16 to bind LC_a (see Materials and Methods; see also Trojanowski et al., 1989). Both LCB.2 and X16 reacted with brain tissue, LCB.2 reacting more intensely than X16. Figure 3, *A, B* illustrates binding in cerebellum. Both mAbs stained neuropil (neuronal processes) in the molecular layer as well as Purkinje cell perikarya and dendrites. In the granular cell layer, the most intensely stained structures were the glomeruli, sites at which mossy fibers synapse on the dendrites of granule and Golgi cells. Neuronal perikarya of granule cells appeared negative. However, occasional perikarya of Golgi neurons were labeled by LCB.2, but not by X16. In contrast, there was only weak X16 staining and almost no LCB.2 staining in the white matter (data not shown). The LCB.2 staining pattern is qualitatively similar to a combination of the staining patterns obtained with mAb SY-38 against synaptophysin and mAb RMd0 20, which recognizes nonphosphorylated high- and middle-molecular-weight neurofilament proteins (Lee et al., 1987). Synaptophysin has been shown to localize specifically to axon terminals (Wiedenmann and Franke, 1985), while the nonphosphorylated high- and middle-molecular-weight neurofilament proteins localize to nerve cell perikarya (Lee et al., 1987) (Fig. 3, *C, D*).

Further evidence for concentration of the high-molecular-weight light chains in nerve endings was seen in the staining of spinal cord sections (Fig. 3, *E, F*). Here, the larger motor neurons of the anterior horn stained weakly with LCB.2 and X16 despite their large size. In contrast, axon terminals which delineate the perimeter of some of these large neurons and their processes stained intensely. From the immunohistochemical data, it can be inferred that the high-molecular-weight clathrin light chains are concentrated in axon terminals and in the cell body of some, but not all, neurons. However, this analysis does not indicate whether the peripheral form of the light chains is also present in neuronal cells (see Discussion below). These staining patterns do suggest that in white matter axons, clathrin light chains are not abundant or not in a form detectable with LCB.2 and X16. This result is consistent with electron microscopy data indicating a paucity of clathrin-coated vesicles in the axon (Morré, 1982).

Immunohistochemical analysis showed consistently that X16 weakly stained glial cells in white matter (data not shown), but LCB.2 did not (Fig. 3*A*). This was confirmed by immunofluorescence analysis of cultured astrocytes using LCB.2 and the anti-clathrin heavy chain mAb X22 (Brodsky, 1985a) (Fig. 4). No reactivity with LCB.2 was observed, although the cells clearly contained clathrin, as indicated by X22 reactivity. These results indicated that the high-molecular-weight LC_b recognized by LCB.2 is expressed in a neuron-specific fashion. To determine whether this was also characteristic for neurons and glial cells of the PNS, cultures of chicken DRG, which contain both cell types, were examined by immunofluorescence. mAb X22 produced a punctate staining pattern with more intense staining of the Golgi and nuclear regions in both Schwann cells and neurons (Fig. 5). LCB.2 staining of neurons was identical to that observed with X22. However, LCB.2 did not react with Schwann cells; staining was similar to background staining with control mAb 29B5 (anti-dinitrophenol).

These immunofluorescence studies confirm the neuron-specific expression of the high-molecular-weight forms of clathrin light chains. While it cannot be ascertained from these experiments if the peripheral forms of clathrin light chain are also expressed in neurons, it can be inferred from the results presented in Figure 2 that neurons express none or, at best, small amounts of the peripheral form. Human gray matter, which is comprised of large numbers of neurons as well as glial cells, has a small amount of the peripheral light chain. In contrast, white matter, consisting of axons and large numbers of glial cells, has much more peripheral light chain. Thus, the small amount of peripheral light chain observed in gray matter probably derives from glial cells rather than from neurons.

Previous studies have analyzed the general distribution of clathrin in neuronal tissue and cells (Bloom et al., 1980; Cheng et al., 1980) but did not specifically characterize the location of the high-molecular-weight light chains. One of these immuno-

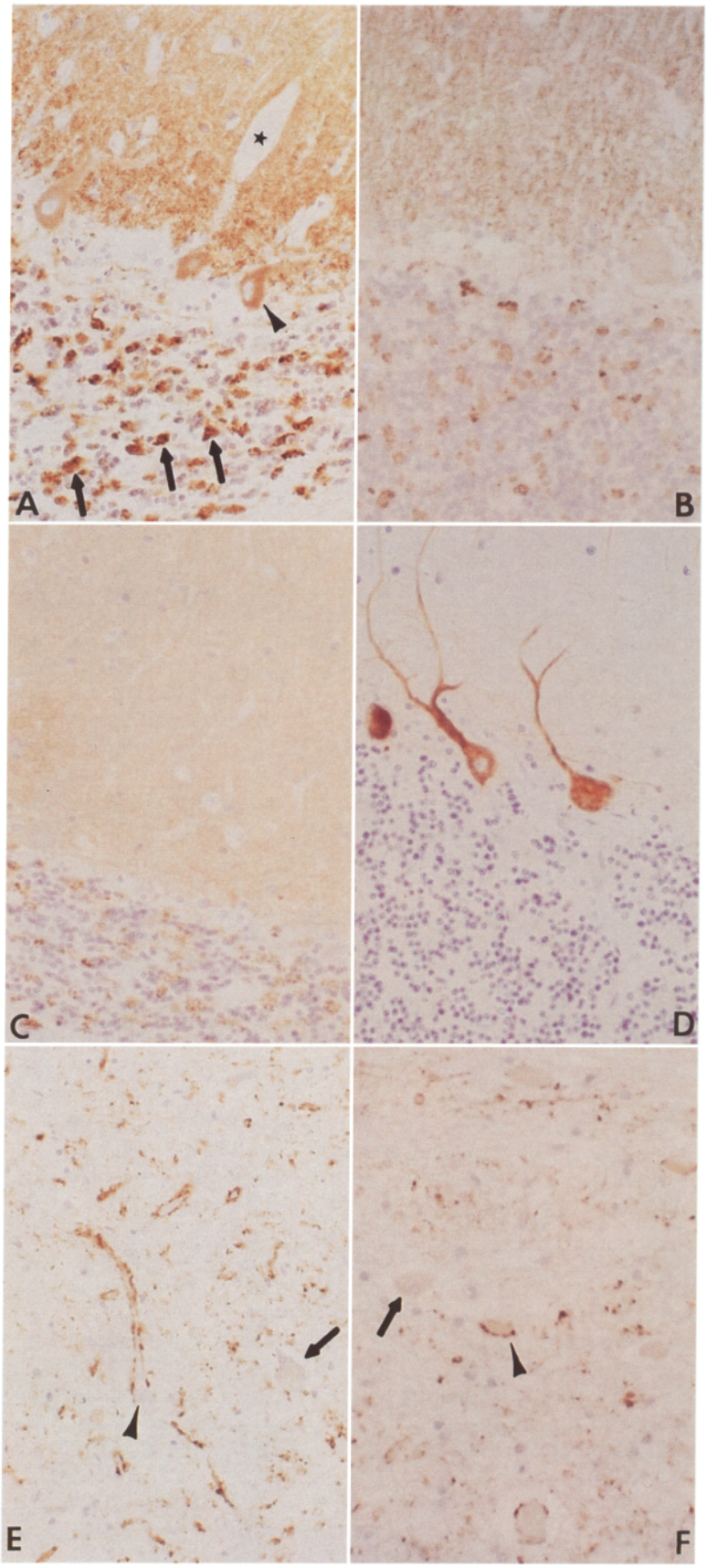

Figure 3. Binding of mAbs to neuronal tissue sections. *A,* LCB.2 binding to cerebellum: Purkinje cell perikarya (*arrowhead*), glomeruli (*arrows*) in the granular layer, nonreactive blood vessel (*) in the molecular layer, white matter (*lower right-hand corner*). Counterstained with hematoxylin. ×300. *B,* X16 binding to cerebellum. Counterstained with hematoxylin. ×300. *C,* Synaptophysin distribution in cerebellum (SY38 binding). Note that Purkinje cell perikarya do not stain. Counterstained with hematoxylin. ×300. *D,* Nonphosphorylated high- and middle-molecular-weight neurofilament protein distribution in cerebellum (RMd0 20 binding). Purkinje cell perikarya and nerve termini stain with RMD0 20 (Lee et al., 1987). Axons only stain weakly because the neurofilament proteins become phosphorylated upon export into axons which alters their ability to be recognized by this mAb. This accounts for lack of staining of the molecular layer. Counterstained with hematoxylin. ×300. *E,* LCB.2 binding to spinal cord gray matter. LCB.2 binding is punctate or distributed in stippled linear arrays that outline the perimeter of a weakly staining anterior horn cell (*arrowhead*). Not all anterior horn cell perikarya exhibit intense and/or continuous circumfirential LCB.2 staining (*arrow*). Counterstained with hematoxylin. ×300. *F,* X16 binding to spinal cord gray matter. Some anterior horn cells are outlined by X16 staining (*arrowhead*), while others are not (*arrow*). Counterstained with hematoxylin. ×300. mAb binding was detected with PAP-anti-immunoglobulin.

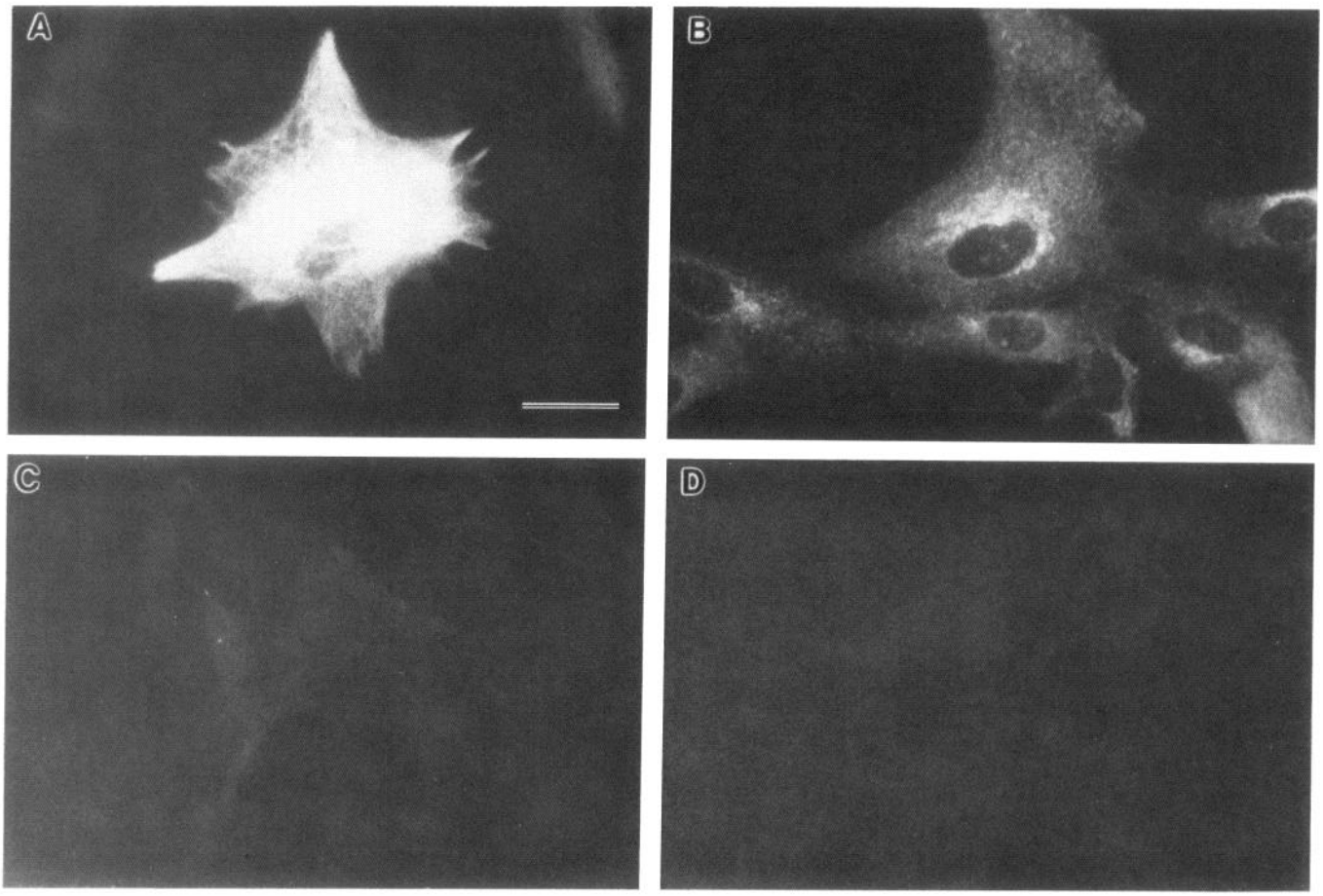

Figure 4. High-molecular-weight clathrin light chain LC_b is not expressed in astrocytes. Scale bar, 5 μm. Cultured astrocytes stained with (*A*) mAb cocktail to glial fibrillary acidic protein (*GFAP*), a glial cell marker; (*B*) mAb X22, (*C*) mAb LCB.2, (*D*) Control mAb 29B5. mAb binding was detected with rhodamine-conjugated anti-immunoglobulin.

fluorescence studies of clathrin distribution suggested that in neurons, clathrin was detectable in both soluble (depolymerized) and assembled (coated vesicle form), and it appeared concentrated at the presynaptic terminal (Cheng et al., 1980). Subsequent analysis of axonal transport of clathrin light chains and heavy chains indicated that they are transported together in slow component b (SCb) (Garner and Lasek, 1981; Gower and Tytell, 1987), correlating with previous findings that the clathrin triskelion is the functional molecular unit of clathrin *in situ* (Brodsky, 1985b). SCb also transports proteins which affect clathrin assembly including the 50 kDa assembly polypeptide (Chestnut et al., 1986) and the 70 kDa heat-shock cognate protein (HSC70) (de Waegh and Brady, 1989) that depolymerizes assembled clathrin *in vitro* (Rothman and Schmid, 1986). Other proteins transported by SCb include actin (Black and Lasek, 1979), nerve-specific enolase, creatinine phosphokinase (Brady and Lasek, 1981), and calmodulin (Brady et al., 1981). Interestingly, clathrin is not associated with the fast component (FC) of axonal transport which transports membranous organelles and transmitter vesicles (Garner and Lasek, 1981). This implies that the clathrin in anterograde transport is not in the form of coated vesicles but in the unassembled form. It has therefore been suggested that the few coated vesicles observed in the axon represent retrograde transport or local endocytic activity and that clathrin transported to the synaptic terminal is in the soluble form (Gower and Tytell, 1987). De Waegh and Brady (1989) have proposed that HSC70 protein in SCb could contribute to maintenance of the depolymerized state of clathrin during axonal transport.

The concentration of neuron-specific clathrin light chains at the synaptic terminal suggests that they play a role in clathrin-mediated membrane retrieval. It is possible that the inserted sequence could be part of a mechanism for coordinating clathrin-mediated endocytosis following synaptic vesicle fusion. The short hydrophobic stretch introduced by the insertion sequence has been proposed to be a binding site for a cytoplasmic protein that might control mobilization of clathrin for this specialized function (Jackson et al., 1987). Here we suggest a new perspective on this idea implicating axonal transport in the recruitment of clathrin for membrane recycling. In this scheme, a neuron-specific protein might bind to the inserted sequences to promote clathrin light chain association with cytoskeletal elements and prevent triskelia from assembling into cages during axonal transport. This might also involve HSC70, which is not restricted to neuronal cells. After synaptic vesicle fusion, the proposed axonal binding protein would be released from the inserted sequence allowing triskelia to polymerize and form coated vesicles for membrane retrieval. The paucity of LCB.2 and X16 immunoreactivity in white matter together with the intense neuropil and neuronal perikarya staining in the same reactions raises the possibility that LC_a and LC_b may be transported in association with other proteins or in a modified form such that antibody

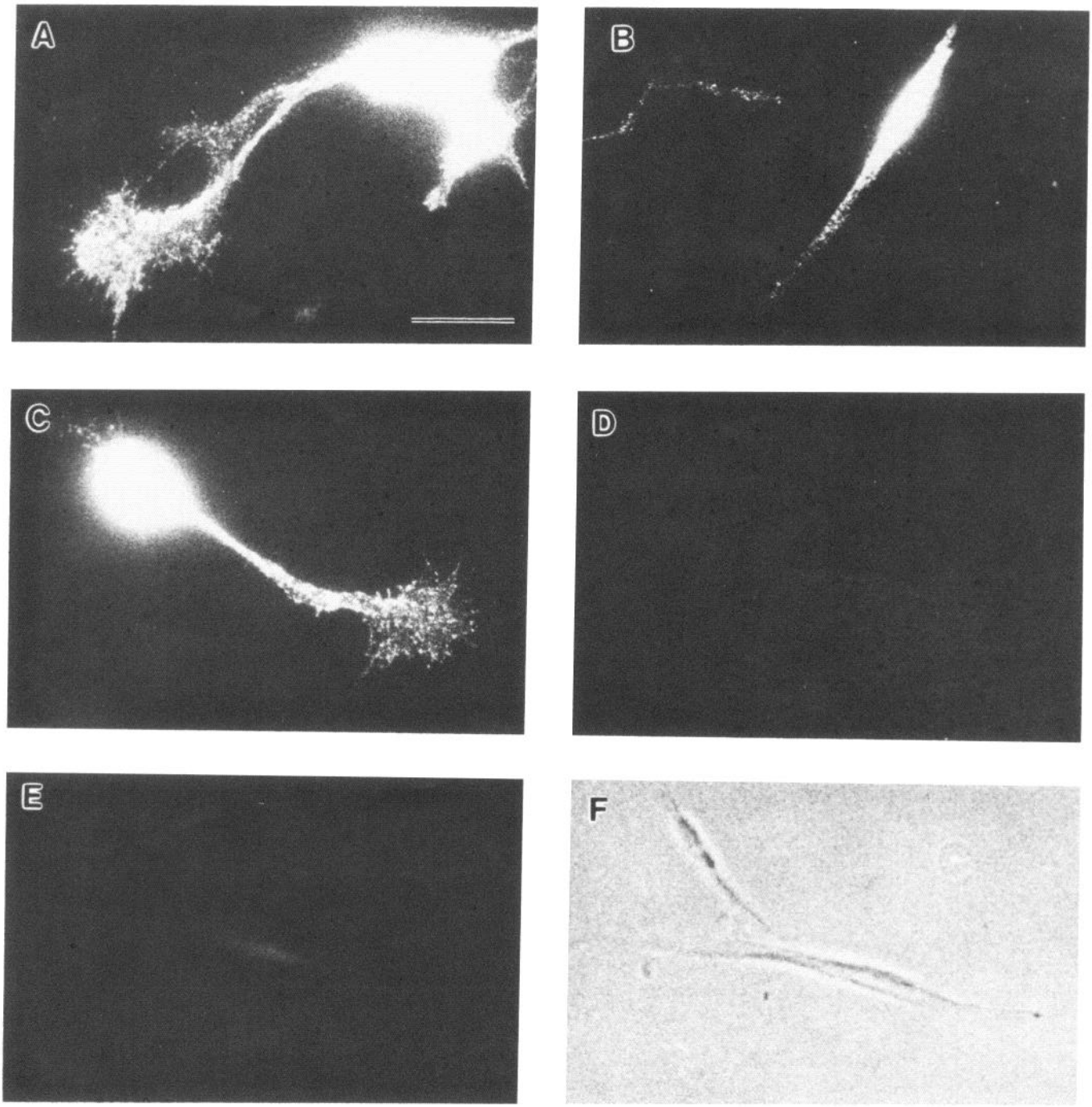

Figure 5. High-molecular-weight clathrin light chain LC_b is expressed in cultured chicken neurons and not in Schwann cells. Scale bar, 10 μm. (*A*) mAb X22 staining dorsal root ganglia (DRG), (*B*) mAb X22 staining Schwann cell, (*C*) mAb LCB.2 staining DRG, (*D*) mAb LCB.2 staining Schwann cell, (*E*) control mAb 29B5 staining Schwann cell, (*F*) phase-constant image of panel *D*. Note in panel *D* that the Schwann cell does not stain with LCB.2. mAb binding was detected with fluorescein- (*A*) or rhodamine-conjugated (*B–E*) anti-immunoglobulin.

binding is reduced. The 100 kDa components of clathrin-coated vesicles, known as adaptins (Glickman et al., 1989), also have "brain-specific" insertion sequences (Robinson, 1989; Ponnambalam et al., 1990), which might play a role in mediating cytoskeletal binding and/or preventing polymerization during axonal transport for these proteins as well. Investigating the specialized role of the clathrin light chain insertion sequences and those of other coated vesicle components in neuron-specific functions provides a focus for future studies.

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