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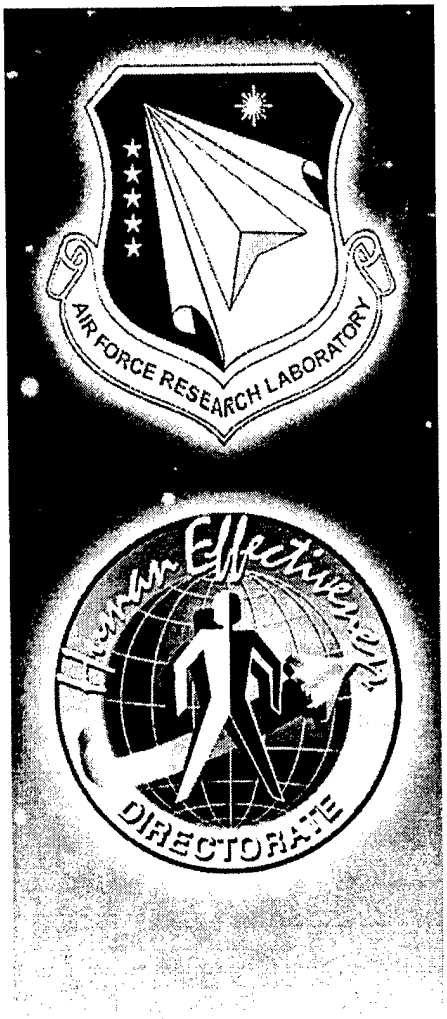

United States Air Force Research Laboratory

ELECTRON MICROSCOPY ANALYSIS OF ALBUMIN LEAKAGE ACROSS BLOOD-BRAIN BARRIER FOLLOWING MICROWAVE EXPOSURE

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14. ABSTRACT

This effort was to develop the histological techniques required to process brain tissue from microwave exposed rats to identify the brain regions affected. Immunocytochemistry was utilized to detect endogenous albumin that had passed through the blood-brain barrier into the brain tissue surrounding the affected vessels. The four key objectives were met: 1) Test for ultrastructural preservation with new perfusion apparatus and when using the specialized fixative; 2) Test that the specialized fixative would allow for immunocytochemical staining with recommended, commercially available antibody to rat albumin, and especially that the albumin staining was equivalent when a gold labeled antibody was or was not included in the traditional indirect immunocytochemical procedure; 3) Test if gold-labeled immunocytochemical staining was detectable in the electron microscopic samples; 4) Evaluate ultrastructure of brain blood vessels and blood brain barrier. The proposed novel histological approach to detect extravasated albumin in brain tissue at both the light and electron microscopic levels was successfully developed and implemented in positive control subjects.

15. SUBJECT TERMS
Albumin Blood Brain Barrier (BBB) Electron Microscopy Electromagnetic Fields Microwaves Rats

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Table of Contents

Figures

Table

Final Performance Report

Submitted by: Clyde F. Phelix

Project Title: Electron Microscopic Analysis of Albumin Leakage across the Blood Brain Barrier Following Microwave Exposure.

Number: F33615-01-1-6074

1.0 Scope, Outcomes, and Conclusions

1.1 The scope of this effort was to develop the histological techniques required to process brain tissue from microwave exposed rats to identify the brain regions affected. Immunocytochemistry was utilized to detect endogenous albumin that had passed through the blood-brain barrier into the brain tissue surrounding the affected vessels. These efforts were extended to electron microscopic analyses of the affected vessels to elucidate the mechanism(s) of action of the microwaves on altering the structure of the blood-brain barrier cellular components.

1.2 The four key objectives were met: 1) test for ultrastructural preservation with new perfusion apparatus in AFRL Brooks facility and when using the specialized fixative; 2) test that the specialized fixative would allow for immunocytochemical staining with recommended, commercially available antibody to rat albumin, and especially that the albumin staining was equivalent when a gold labeled antibody was or was not included in the traditional indirect immunocytochemical procedure; 3) test if gold-labeled immunocytochemical staining was detectable in the electron microscopic samples; 4) evaluate ultrastructure of brain blood vessels and blood brain barrier. The proposed novel histological approach to detect extravasated albumin in brain tissue at both the light and electron microscopic levels was successfully developed and implemented in positive control subjects.

1.3 The ultimate conclusions from the light and electron microscopic analyses of albumin leakage across the blood brain barrier are: 1) extravasated albumin is found within the interstitial space in the extracellular matrix and within vesicles of endothelial cells, astrocytes, and neurons; 2) when vessels are associated with albumin extravasation, two distinct changes are seen in the endothelial lining, i.e., appearance of intercellular clefts and increased occurrence of transcytotic vesicles (caveolae); 3) the ultrastructural data on gold-labeled albumin localization is most consistent with a transcytotic mechanism for albumin leakage across the blood brain barrier when extravasation occurs.

2.0 Requirements

2.1 The Offerer/Principal Investigator shall accomplish the following:

2.1.1 Design and implement the fixative and histological methods for detection of albumin in rat brain tissue slices concomitantly at light and electron microscopic levels.

Specialized Fixative Protocol: (6.5-11 Variable pH 4% Parformaldehyde-0.5% glutaraldehyde)

*Prepare fixative for 15 rats.

Note: For the low pH fixative (i.e., flush) solution prepare 200 mls per rat and for the high pH fixative solution prepare 500 mls per rat.

For 6.5: 200 X 15 = 3000 ml
For 11 : 500 X 15 = 7500 ml
TOTAL 10,500 ml

Then make 16% PFA (i.e. 4X conc. of final desired 4% ParaFormAldehyde)

Calculate: 10,500 / 4 = 2,625 ml of 16%PFA requires 420 grams of powdered PFA (Sigma Chemical Co. St. Louis, MO)

PREPARE 16% PFA: **(Within chemical fume hood with safety procedures)**

1. Heat 2,625 ml distilled water in a 4,000 ml beaker on a hot/stir plate (set heat at high)
2. Add the 420 grams PFA while stirring continuously
3. Stir and measure temperature until 56° C and then add 1 N sodium hydroxide until temperature drops about 3 or more degrees.
4. Stir with heat turned off until the solution clears (from white milky suspension to 'water' clear)
5. Filter with a general filter paper into a 4,000 ml Erlenmeyer flask
6. Leave in hood to cool and rinse the beaker with tap and distilled water for use in making low pH fixative solution

PREPARE LOW pH 6.5 FIXATIVE SOLUTION: **Within chemical fume hood with safety procedures.**

1. Place 750 mls of 16% PFA in 4000 ml beaker and qs (i.e., add quantity sufficient) to 3000 ml (i.e., add 2250 ml distilled water).
2. Place on stir plate and add 60 grams of sodium acetate (anhydrous, Sigma Chemical Co. St. Louis MO) while stirring continuously. This is a 2% sodium acetate buffer.
3. Adjust pH to 6.5 with glacial acetic acid (can use more dilute acetic acid if not experienced)
   *Careful because approaches 6.5 slowly and then can overshoot really easily
4. Transfer to flush container of perfusion apparatus and ready to use.
   *Can be stored at 4° C for 24 to 48 hours before use.

PREPARE HIGH pH 11.0 FIXATIVE SOLUTION: **Within chemical fume hood with safety procedures.**

1. Usually make this as needed for some number of rats FRESH before use.
   ***This example is for 8 rats so prepare a total of 8X500= 4,000 mls.**
2. Within a 4,000 ml Erlenmeyer flask place 1000 mls of 16% PFA and 3000 mls of distilled water.
3. Place on stir plate and stir continuously while adding: (both anhydrous, Sigma Chemical Co.)
   - **For every 500 mls of high pH fixative solution add:**
     Sodium Carbonate = 6.175 grams
     Sodium Bicarbonate = 0.3375 grams

- In this example 49.4 g Sodium Carbonate and 2.7 g Sodium Bicarbonate would be added to the 4,000 ml solution, i.e. 8 times the amount for 500 mls.

4. ADD GLUTARALDEHYDE IMMEDIATELY BEFORE USE!

- Use only EM Grade 50% glutaraldehyde from Sigma Chemical Co.
- For every two rats add one bottle of 10 mls of 50% glutaraldehyde, i.e., in this example for 8 rats we would add 4 bottles.
- So 20 ml of pure glutaraldehyde are added to 4000 ml solution giving a 0.5% concentration.

[This concentration of glutaraldehyde provides acceptable ultrastructural preservation of brain tissue and still permits immunocytochemical detection of many antigens, i.e., endogenous albumin in this case.]

HISTOLOGICAL METHOD FOR DETECTION OF ENDOGENOUS ALBUMIN:

To test if fixative would allow immunocytochemical detection of endogenous rat albumin in positive control animals, we used the Avidin Biotin Complex (ABC) method as provided by the AFRL Brooks team.

Reagent Sources:
Primary Antibody – Rabbit Anti-ratAlbumin, Code #55728, ICN Cappel, Costa Mesa, CA
ABC Kits, Rabbit -- Vector Laboratories, Burlingame CA
DAB -- Sigma Chemical Co, St. Louis MO

Dilution of Primary Antibody: According to ICN Cappel specification sheet for free floating sections the working dilution range is 1:50 to 1:200. The first staining series attempted, as reported February 12, 2002, demonstrated that a 1:50 working dilution worked best with the special fixative used in these studies.

PHELIX-LAB ABC-PEROXIDASE IMMUNOCYTHOCHEMISTRY PROCEDURE:

1. Wash sections (100 micrometer Vibratomed sections seemed to work best) in 0.1 M PBS for 45 minutes
2. Soak sections in 0.2 % Triton X-100 in 0.1M PBS solution for 20 minutes
3. Wash sections in 0.1 M PBS for 15 minutes
4. Incubate sections for 48 hours at 4°C in primary antibody (1:50 dilution in standard antibody diluent, which is a 0.1 M PBS, with 1.0% normal goat serum, and 0.1% sodium azide.)
5. Wash sections in 0.1 M PBS for 45 minutes
6. Incubate sections overnight at 4°C in biotinylated-goat antirabbit IgG solution (one drop in 10 mls 0.1 M PBS)
7. Wash sections in 0.1 M PBS for 45 minutes
8. Incubate sections overnight at 4°C in ABC-peroxidase solution (two drops of bottle A and two drops of bottle B into 10 mls 0.1 M PBS)

9. Wash sections for 45 minutes in 0.1 M PB (NOTE: no sodium chloride so as not to cause DAB precipitation)
10. Develop for 20 minutes in substrate chromogen solution (*preparation stated below)
11. Wash sections in 0.1 M PB for 30 minutes, inspect under dissection microscope for staining reaction.
12. Transfer sections to 1.0% sodium azide in 0.1 M PBS for storage at 4°C.

*SUBSTRATE CHROMOGEN SOLUTION: Prepare **immediately** before use.

1. 100 mls of 0.1 M PB (Note: no sodium chloride because it precipitates DAB)
2. 10 mg diaminobenzidine (one tablet from Sigma foil pack)
3. 20 µl of 35% hydrogen peroxide
4. Filter before use

2.1.2 Stain the sectioned brain tissue for the presence of albumin utilizing a combination gold and color chromogen reaction product that will allow visualization and localization of albumin at both the light and electron microscopic levels of anatomical structure.

NOVEL CONTRIBUTION BY OFFERER: (Credit given to my friend and colleague, Lothar Sievers unfortunately debilitated with schizophrenia.)

Reagent Sources:
Primary Antibody – Rabbit Anti-ratAlbumin, Code #55728, ICN Cappel, Costa Mesa, CA
Secondary Antibody - 4nm Colloidal Gold-Affinipure Goat Anti-Rabbit IgG (H+L) (LM Grade); Code # 111-185-144, Jackson Immunoresearch Laboratories Inc., West Grove, PA.
PAP – Rabbit Peroxidase Anti-peroxidase, Code # 323-005-024, Jackson Immunoresearch Lab...
DAB -- Sigma Chemical Co, St. Louis MO
PRE-EMBEDDING IMMUNOGOLD-PAP-DAB IMMUNOELECTRON MICROSCOPIC METHOD:

1. through 5. same as listed above with ABC METHOD.
6. Incubate sections overnight at 4°C in gold-secondary antibody at 1:40 dilution in standard antibody diluent **(must prepare fresh and can use only once, i.e., within 24 hrs)**
7. Wash sections for 45 minutes in 0.1 M PBS
8. Incubate sections overnight at 4°C in PAP at 1:600 dilution in special PAP diluent (i.e., 0.1 M PBS with 0.1% merthiolate as preservative)
9. Wash section for 45 minutes in 0.1 M PB (Note NO sodium chloride)
10. Develop in substrate chromogen solution as with ABC METHOD.
11. Wash sections in 0.1 M PB
12. Evaluate staining with dissecting microscope and use a razor blade to cut out selective regions of interest.
13. These small dissected samples are processed for electron microscopy in a routine manner. NOTE: A TEM Technician must be consulted for any special embedding procedure for orientation of tissue sample such that the immunocytochemical stain is not lost in the initial trimming of the plastic block face. Consult an expert.

2.1.2.1 Document through light microscopic, black and white photography the distribution of blood vessels in positive-stained animal brains and then in similar brain regions of negative-stained animal brains.

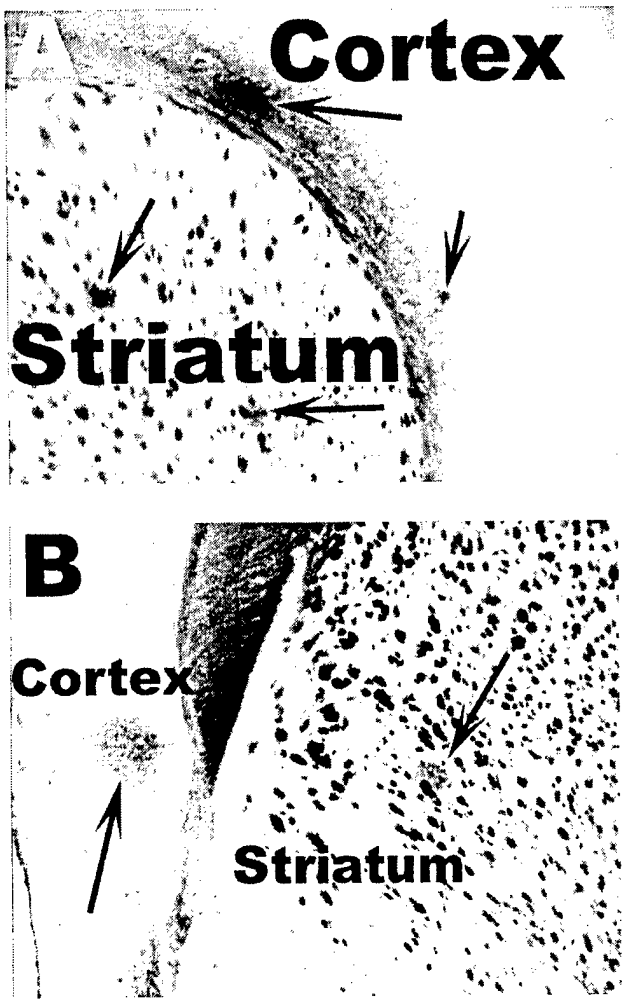
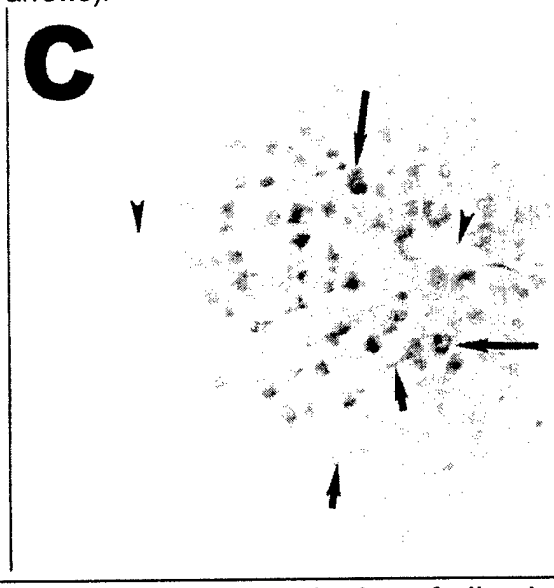


Figure 1: Wet mount digital image capture of striatum and cortex showing positive immunostaining for albumin in rat #186-05. A) At 4X original mag, both the dorsolateral neostriatum and somatosensory cortex show punctuate zones of albumin leakage around blood vessels (Arrows). B) At 4X original mag. Both the ventrolateral neostriatum and piriform cortex also reveal albumin leakage around vessels. (arrows) C) At higher magnification, 20X original, immunoreactivity for albumin is present diffusely within the neuropil around a blood vessel (arrowheads) and is seen in both neuronal soma (long arrows) and dendrites (short arrows).



2.1.2.2 Quantitate the frequency of positive stained vessels in the brains of all animals in the study groups.

TABLE 1: MORPHOMETRICS ON BRAIN VESSELS IN MICROWAVE EXPOSED RATS: LIGHT AND ELECTRON MIROSCOPY					
Sample No.	Vessel Diameter	cleft index	vesicle density	Avg. Spot No	Avg Spot Diam
188-10	3.27	0.773196	28.44	34.50	215.67
226-11	4.06	0.798387	29.33	48.25	239.07
280-01	4.48	0.744638	15.44	0.10	263.33
280-03	4.23	0.583333	23.55	3.00	106.20
280-04	5.26	0.680276	12.68	0.82	161.79
280-06	6.02	0.787879	19.74	0.11	54.67
280-07	5.21	0.547619	16.57	1.53	153.22
280-08	3.81		15.01	9.86	319.51
280-09	5.68	0.710066	27.02	17.32	198.74
280-12	5.09	0.666667	24.02	8.00	292.17
280-13	5.28	0.702128	14.88	5.64	158.34
280-27	5.66	0.437451	16.94	4.48	165.99
280-28	4.31		22.74	2.92	249.27
Vessel and Spot diameter in units of micrometers.					
Vesicle density in units of number per μm^2.					
Cleft index is proportion of interendothelial junction not occupied by tight junctions.					

2.1.3 Provide ultrastructural evidence for cellular mechanism(s) through which microwave exposure effects leakage of endogenous albumin across the blood-brain barrier in rat brain.
2.1.3.1 Procure services of TEM Facility Technician to process tissue samples from experimental animal brains that contain vessels of interest from 8 brain regions from each of the rats in the treatment groups. TEM Technician will generate ultrathin plastic sections of each sample as the final product.

With prior verbal approval of Technical POC, Patrick Mason, Ph.D., only the striatum was examined at the ultrastructural level.

TEM Technical services were procured through the UTHSCSA Department of Pathology.

2.1.3.2 Examine ultrathin plastic sections of each sample and make at least two photographs of each of at least three vessels in each of the 8 sample areas of each animal in the treatment groups.
2.1.3.4 Make qualitative descriptive analyses of electron micrographs.

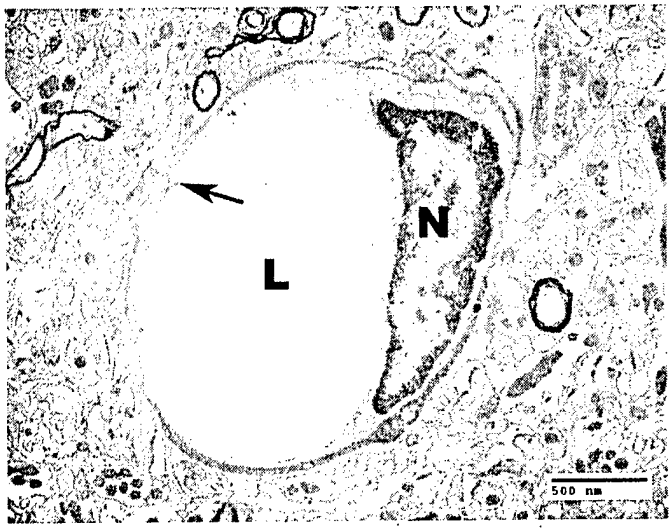


Figure 2: Transmission electron micrograph (digital image) taken at original mag. of 10,000 X. An immunonegative capillary is lined by a single endothelial cell surrounding the lumen (L). The nucleus (N) of the endothelial cell is located on one side of the capillary and the tight junction (arrow) is seen where its cytoplasmic extensions join on the opposite side. The cytoplasm of the endothelial cell has sparse vesicles and the capillary is surrounded by a pericyte (dark circular ring) and astrocyte foot processes. The blood brain barrier is intact in this sample, #188-14.

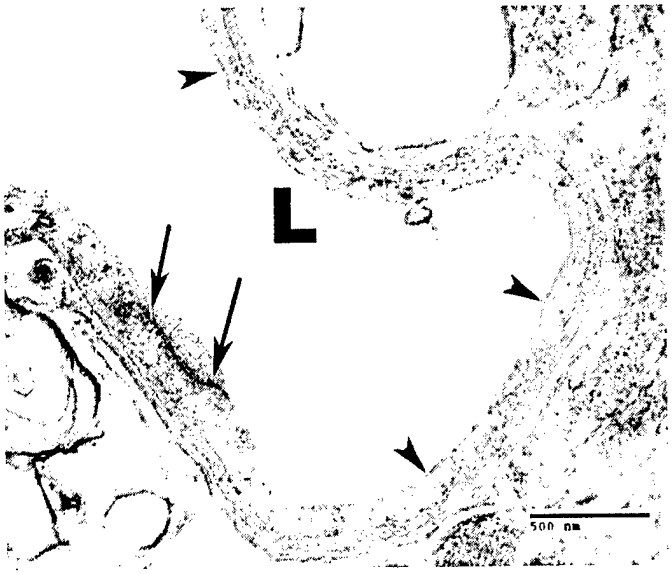


Figure 3. At higher magnification, 28,000 X original mag., another capillary from another immunonegative sample, #186-03, is seen. The cytoplasm of the endothelial cell (arrowheads) contains very few vesicles. The tight junction can be seen clearly as the dense bands (arrows) extending from the lumen (L) along toward the surrounding neuropil.

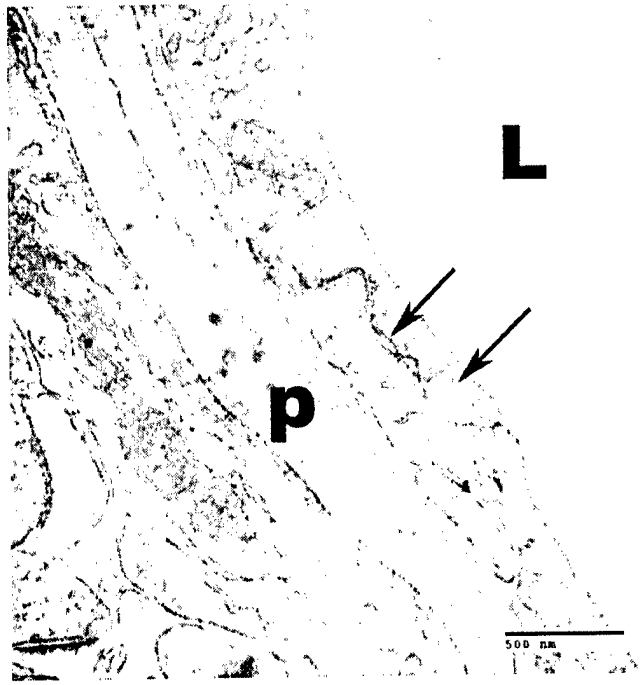


Figure 4. Electron digital micrograph taken at 28,000 X mag. An intercellular cleft (arrows) is evident extending from the lumen (L) of this capillary to the pericapillary space (p). The blood brain barrier in this sample, #186-05 has been disrupted.

This section was NOT processed for immunocytochemical detection of albumin.

The specialized fixative very nicely preserved ultrastructural integrity, even when the vasculature of the brain has been compromised.

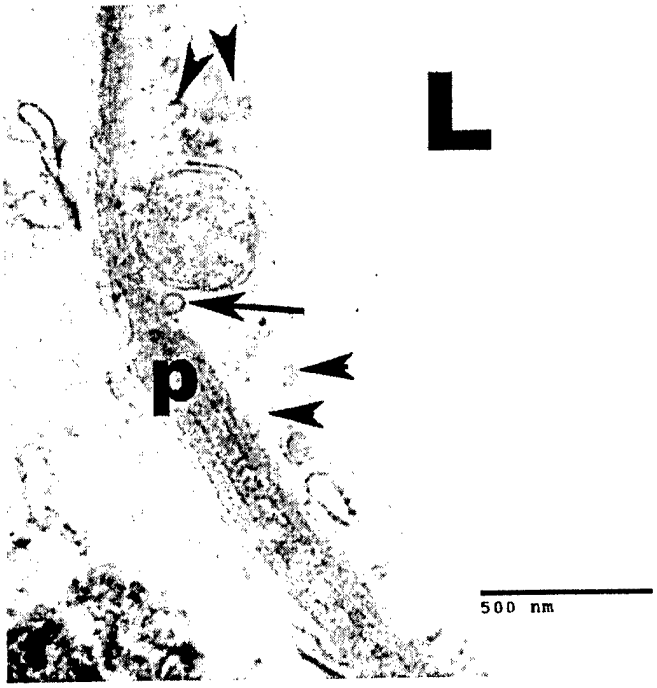


Figure 5. Also taken at an original magnification of 28,000X, this electron micrograph shows increased numbers of vesicles that are filled with the electron opaque material similar to that seen within the lumen of the capillary (L). The vesicles can be seen spanning from the endothelial cell membrane near the lumen, i.e., adlumenal (arrowheads) and even at the ablumenal membrane forming an omega figure (arrow) where the electron opaque material is continuous with the pericapillary space (p).

This image is taken from sample #188-10.

This section was NOT processed for immunocytochemical detection of albumin.

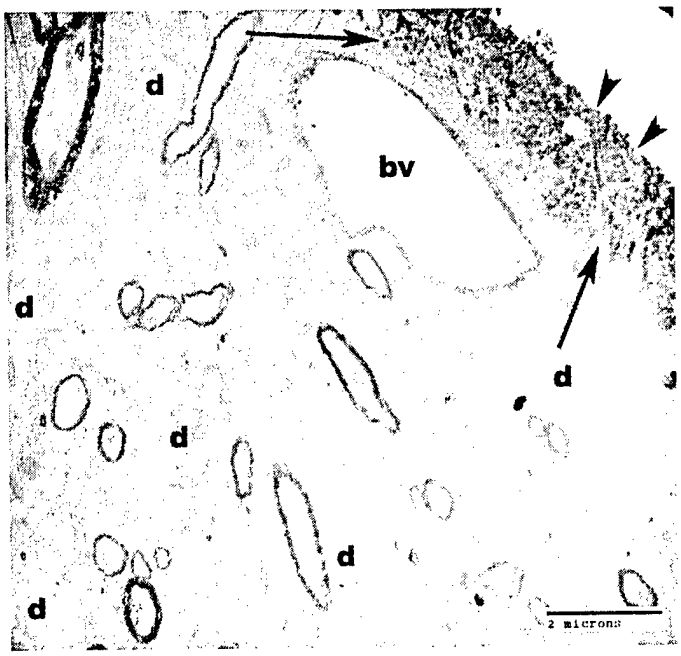


Figure 6. A low magnification electron micrograph taken at 5,600 X of an immunopositive stained section. The ultrathin plastic section was taken perpendicular to the Vibratomed edge of the fixed brain (arrowheads). The dark, electron dense material penetrates nearly 2 microns into the section (arrows) approaching a blood vessel (bv). The endothelial cell lining the bv is intensely immunopositive for albumin. Many unstained dendrites (d) are seen deeper within the section.

This section was stained before embedding with the gold labeled antibody method but only the DAB reaction product can be seen at this magnification.

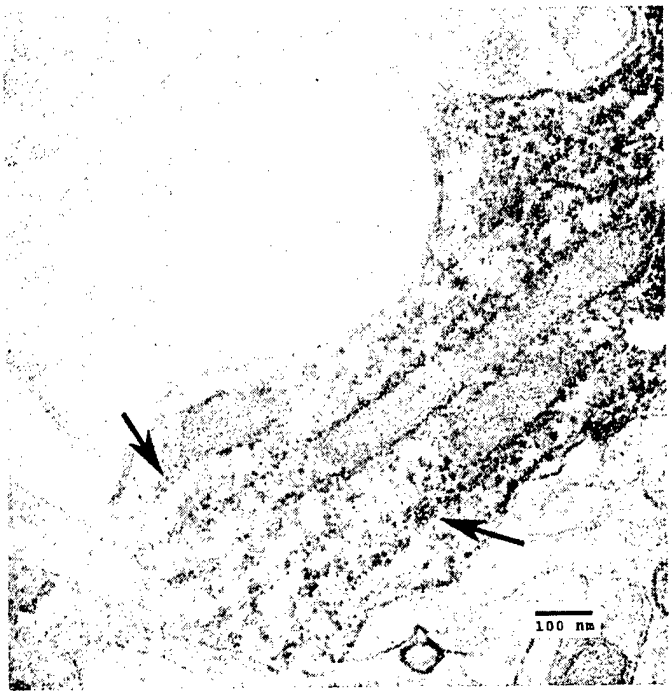


Figure 7. An immunopositive dendrite is obvious due to the electron dense DAB reaction product. A higher magnification of 28,000 X original allows the visualization of the 4 nm gold particles (arrows) dispersed throughout the DAB reaction product. All immunolabel is limited within the dendrite and the surrounding neuropil is immunonegative.

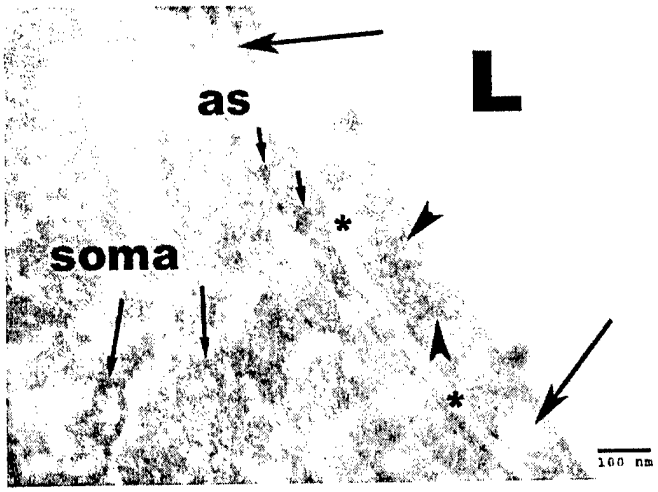


Figure 8. A strongly immunoreactive endothelial cell is seen at the lumen (L) of a capillary with an original magnification of 22,000 X. Ultrastructural clarity is compromised when the immunoreactivity is so strong. Intercellular clefts (large arrows) do not seem to contain any gold-DAB-labeled albumin. Whereas several transcytotic vesicles (arrowheads) are present within the endothelial cell cytoplasm and are immunopositive for albumin. The gold-DAB-labeled albumin is seen within an astrocyte (short small arrows) just beyond the pericapillary space (asterisks) and within a nearby neuronal soma (long small arrows).

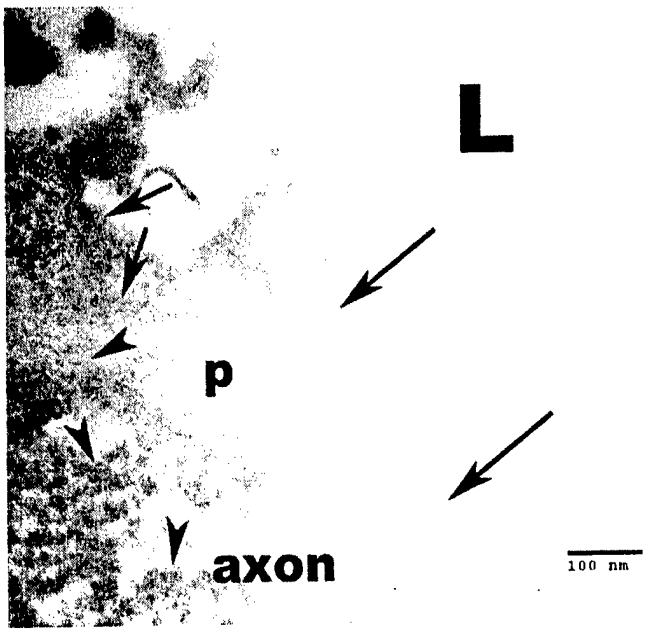


Figure 9. Another strongly immunoreactive endothelial cell shows decrement of ultrastructural integrity at 36,000 X original magnification. An intercellular cleft is present (long arrows) and seems not to contain gold-DAB-labeled albumin. Many gold particles are present throughout the cytoplasm of the endothelial cell (short arrows) extending from the lumen (L) through the pericapillary space (p) and into a nearby axon (arrowheads).

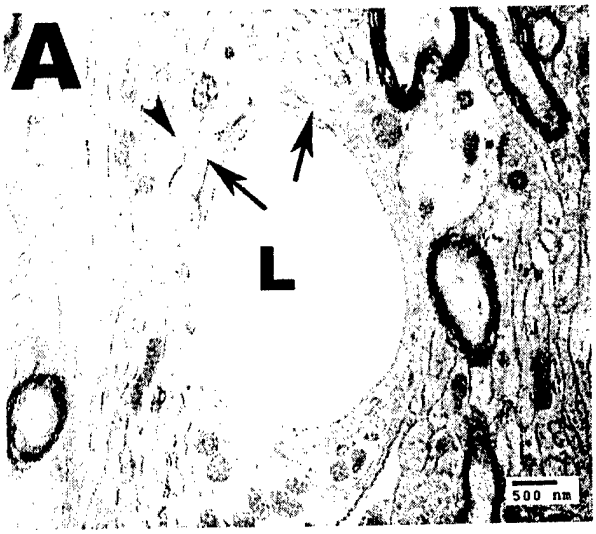
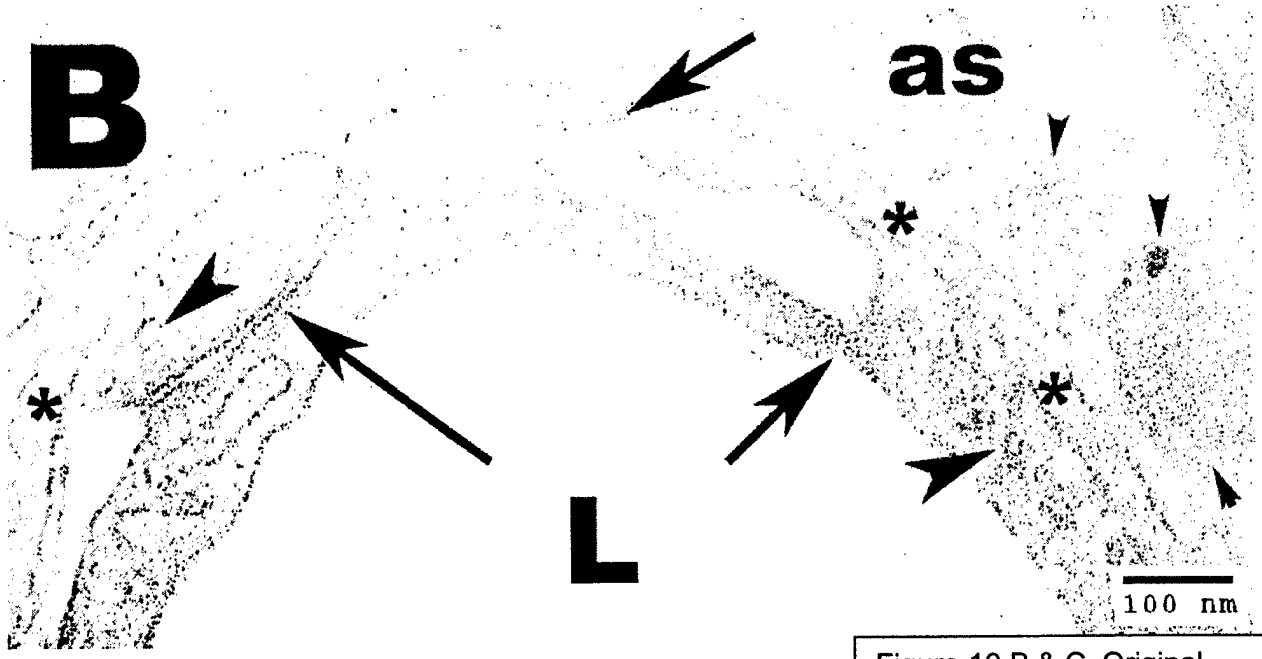


Figure 10 A). 8,900 original magnification. Slightly deeper into the section is a lightly immunolabeled capillary with a distinct lumen (L) and two large intercellular clefts (arrows) that are shown at higher magnification in panel B. The area where gold-DAB-labeled albumin can be followed between astrocyte foot processes into the surrounding neuropil (arrowhead) is shown at higher magnification in panel C.

Panels B & C are on the next page .



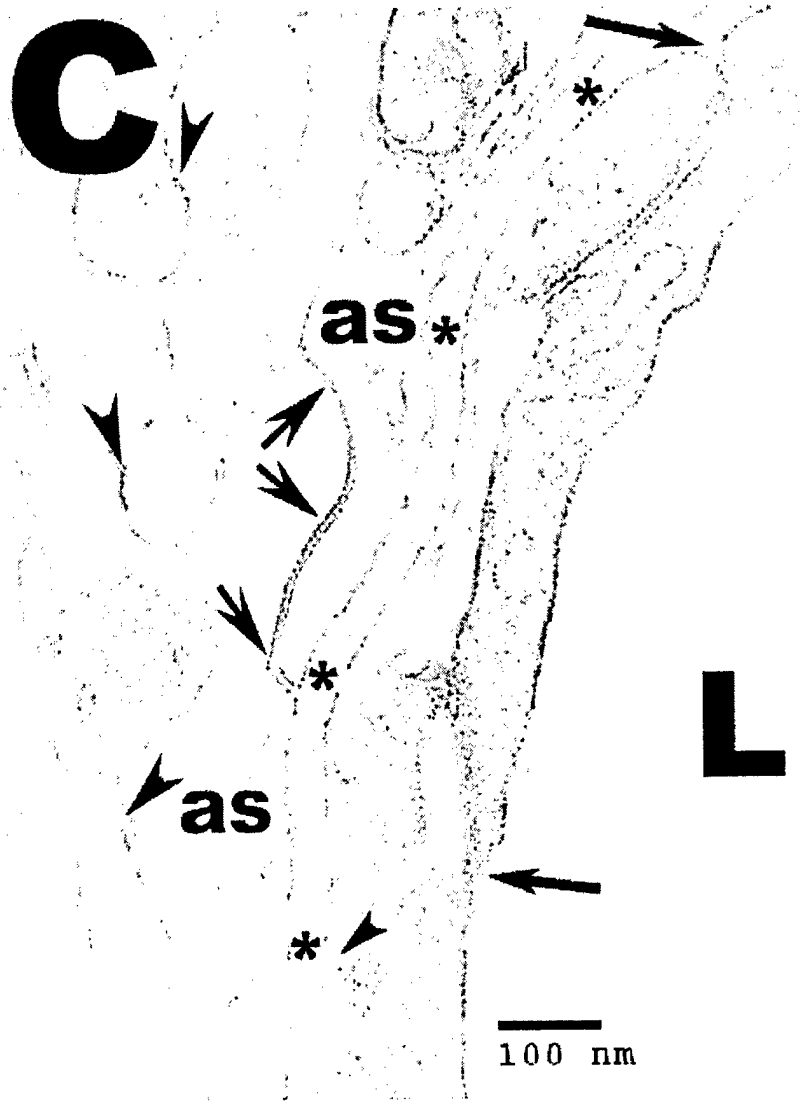


Figure 10 B & C. Original magnifications of 28,000 X. In B, numerous transcytotic vesicles are present within the cytoplasm of the endothelial cell and the 4 nm gold particles (large arrowheads) are evident. Apparently less gold-DAB labeled albumin is present within a dilated intercellular cleft (short arrows) that extends from the lumen (L) of the capillary to the pericapillary space (asterisks). Many gold particles are accumulated at the remnants of a tight junction (long large arrow) at the ablumenal end of another intercellular cleft. On the right there is evidence of albumin (small arrowheads) within an astrocyte (as).

In C, the full length of an intercellular cleft is seen (small long arrows). A gold-labeled transcytotic vesicle is seen fused to the ablumenal cytoplasmic membrane (small arrowhead) indicative of albumin release into the pericapillary space (asterisks). The gold particles can be followed through the interstitial space (short large arrows) between two astrocyte processes (as). Gold particles are also seen in the extracellular, interstitial, space (large arrowheads) within the general neuropil nearby.

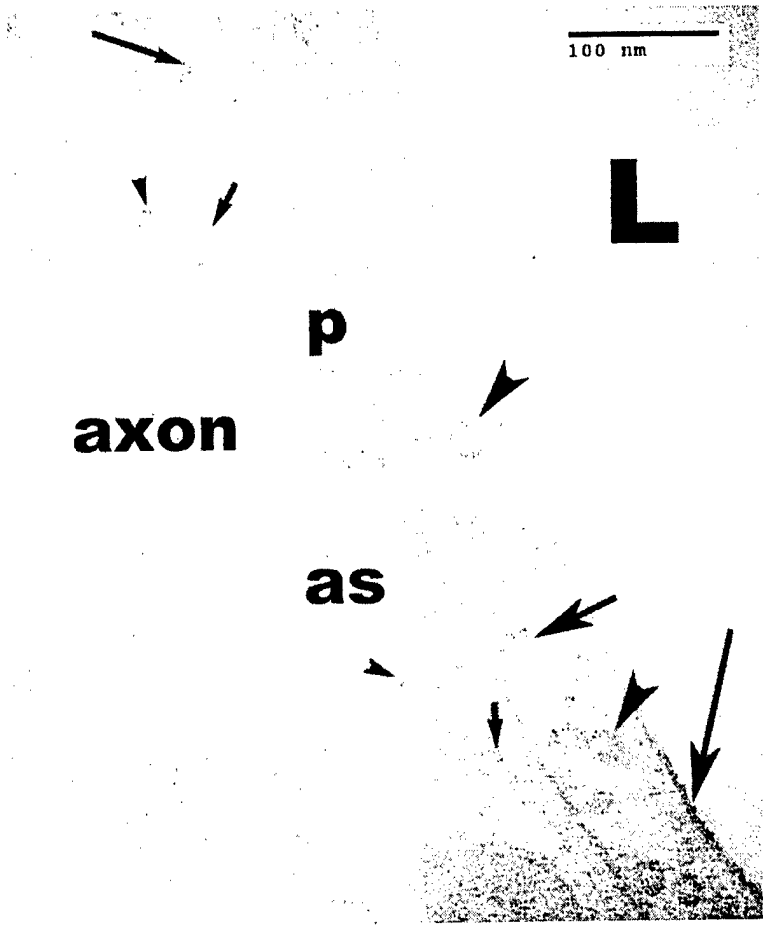


Figure 11. Additional evidence, at high magnification of 56,000 X, that albumin crosses the blood brain barrier via the transcytotic mechanism. Gold particles are seen on the adlumenal cytoplasmic membrane of the endothelial cell (long large arrow). Transcytotic vesicle immunopositive for albumin are seen near the lumen (L) (large arrowheads) and close to the pericapillary space (p) (short large arrow). Gold particles are seen at site of endocytotic uptake (short small arrows) at edges of an astrocyte (as) and axon, with extension into nearby vesicles (small arrowheads). Within the astrocyte the vesicles seem to reach the deeper cytoplasmic area (long small arrow) indicative of release into the nearby interstitial space of the neuropil.

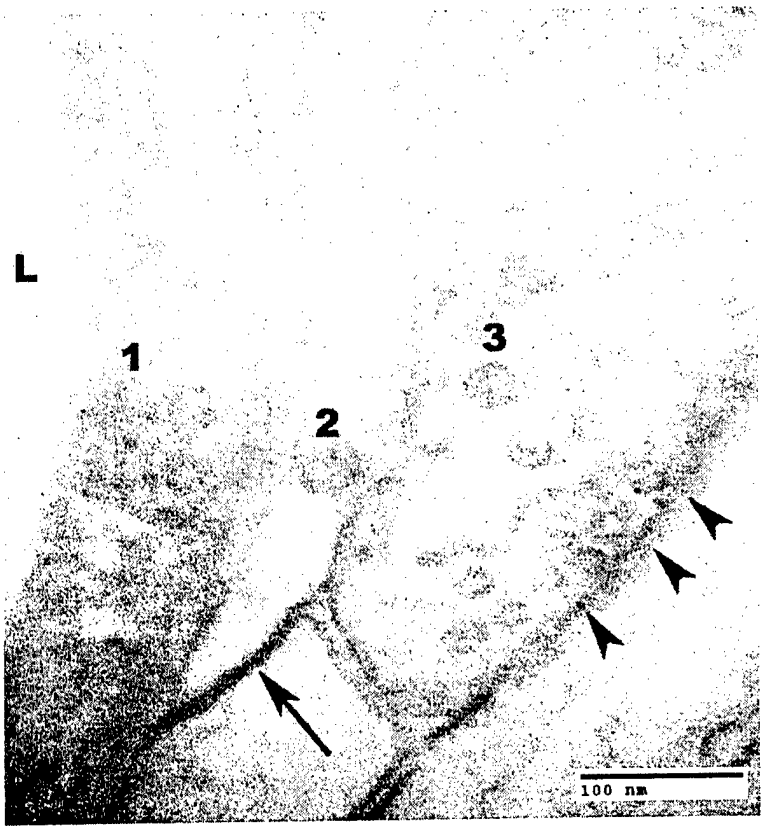
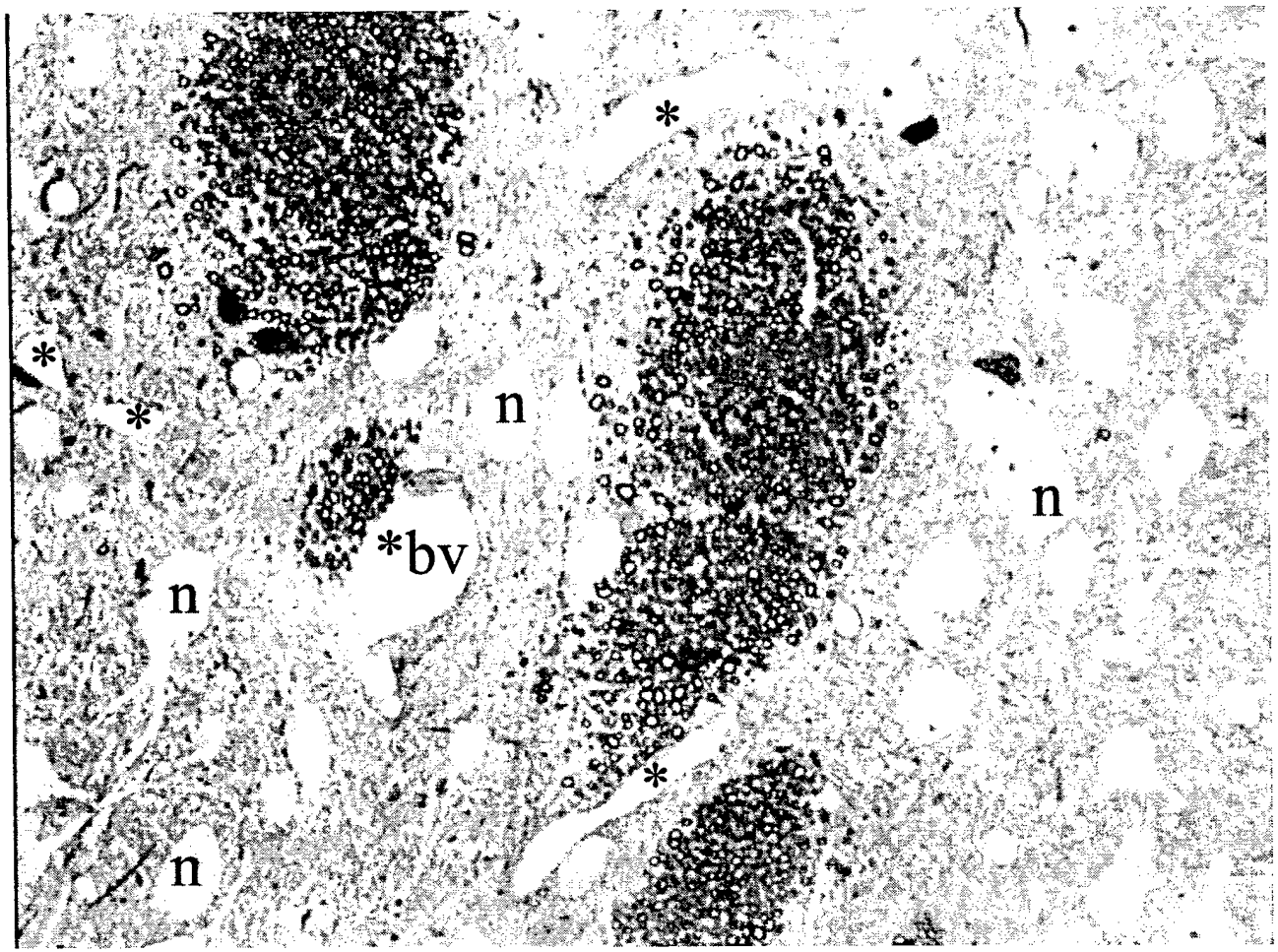


Figure 12. At even higher original magnification of 71,000 X, the vesicular transport of albumin is more evident. Numerous gold-labeled (i.e., albumin containing) vesicles are seen within the cytoplasm of the endothelial cell (1) and then of the astrocyte (2) and finally of an axon terminal (3) which is forming an axodendritic synapse (arrowheads). The general diffusion of albumin within the interstitial space, between astrocyte processes (arrow) is evident also.

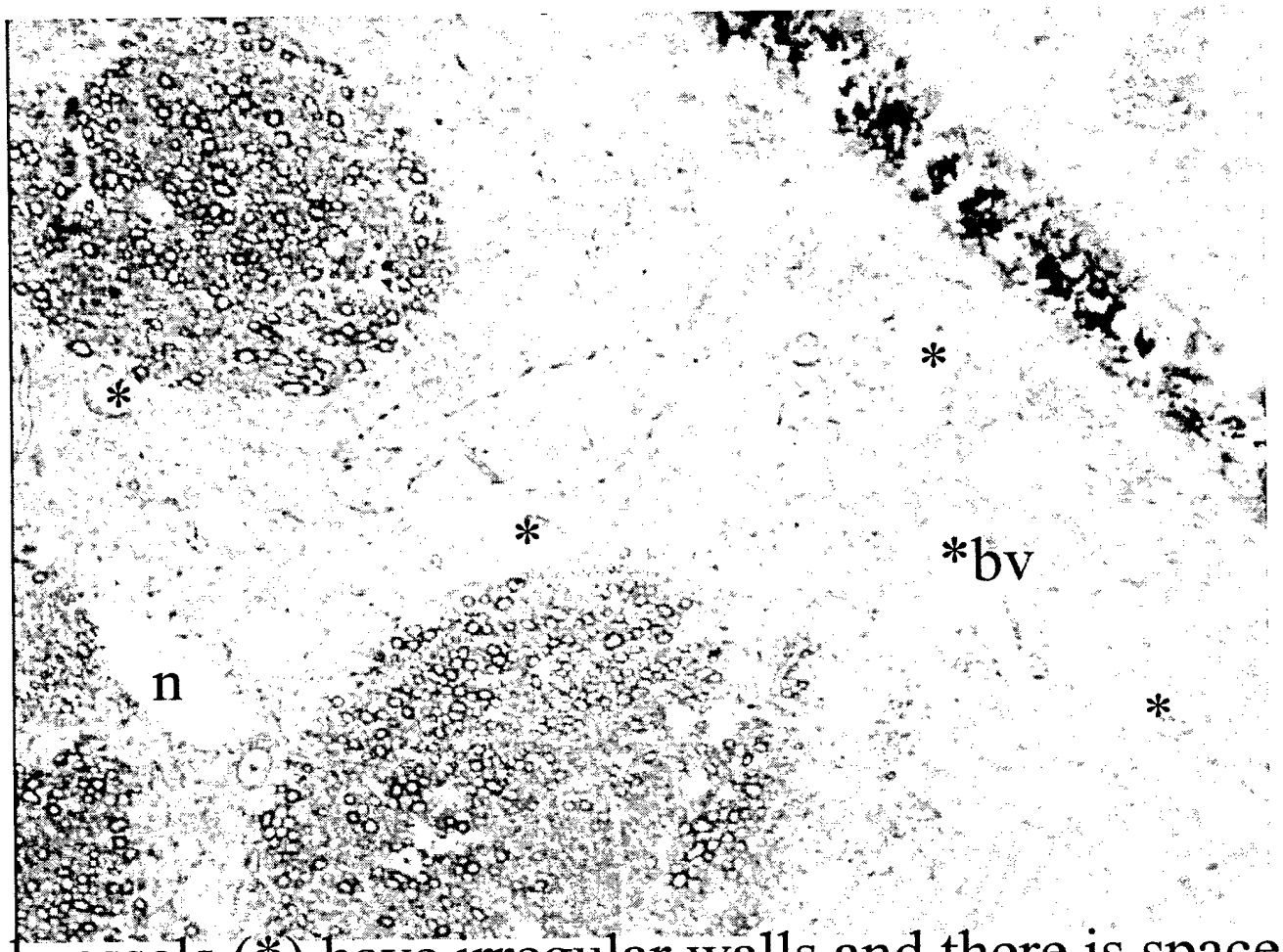
Appendix

186-01 1μm plastic section
Toluidine blue stain



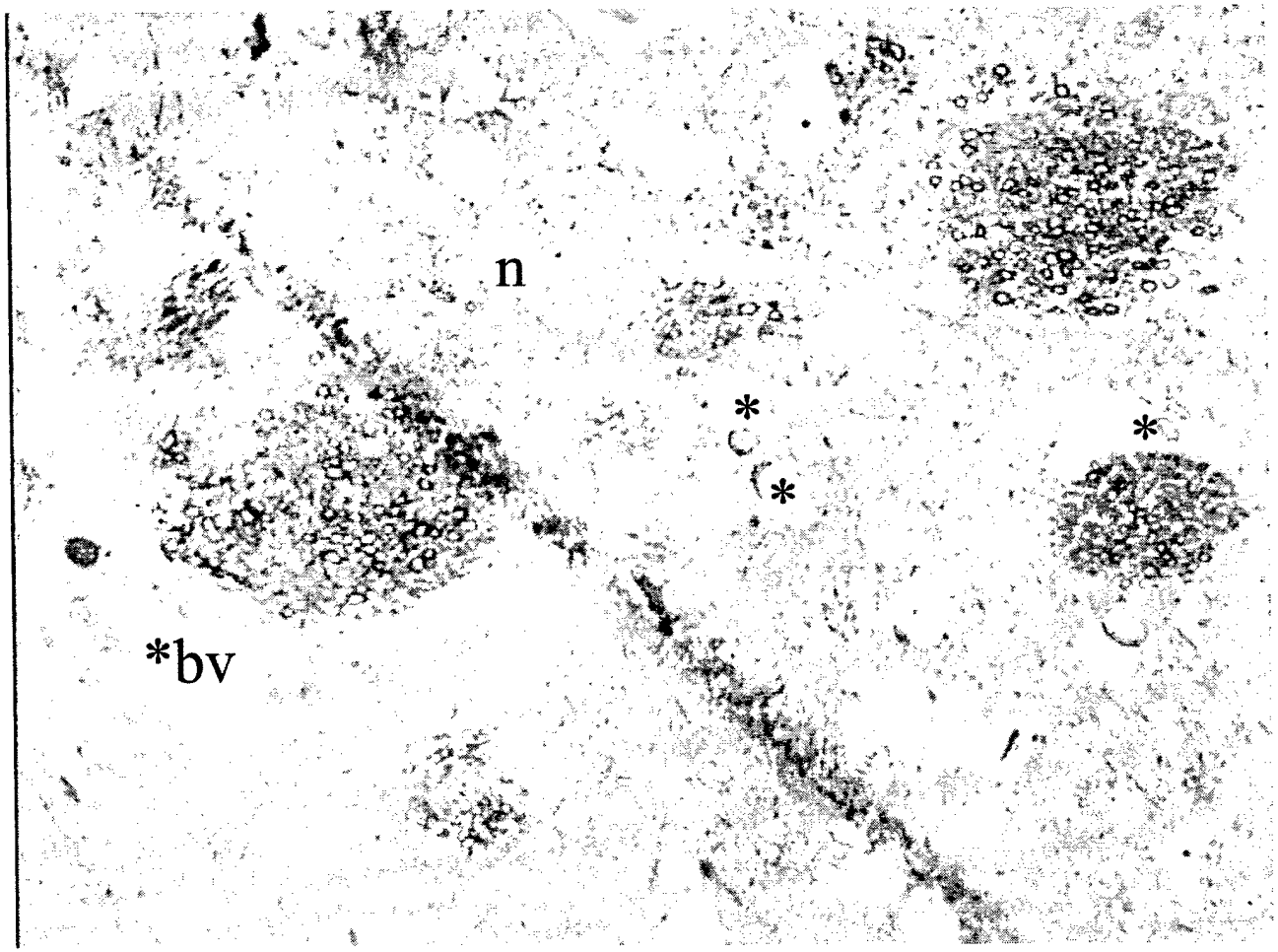
Note the margins of the blood vessels (bv and asterisks) are smooth and tight against the nearby nerve tissue. n - neurons

186-05 1 μm thick plastic section
Toluidine blue stain



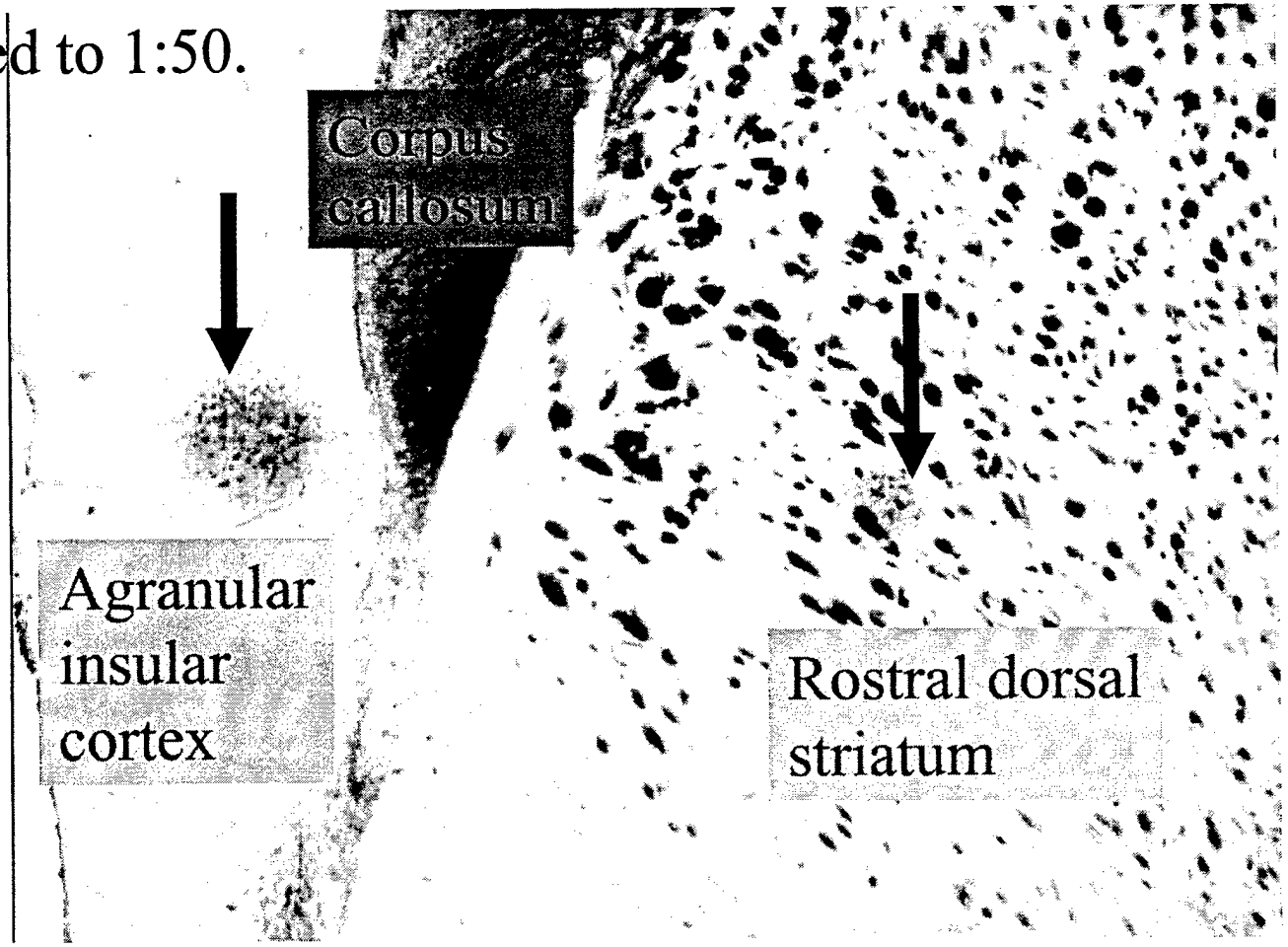
The blood vessels (*) have irregular walls and there is space at the edge of the nearby nerve tissue (neuropil). This neuron does NOT look healthy - vacuoles are obvious.

186-05 1 μm thick plastic section
Toluidine blue stain



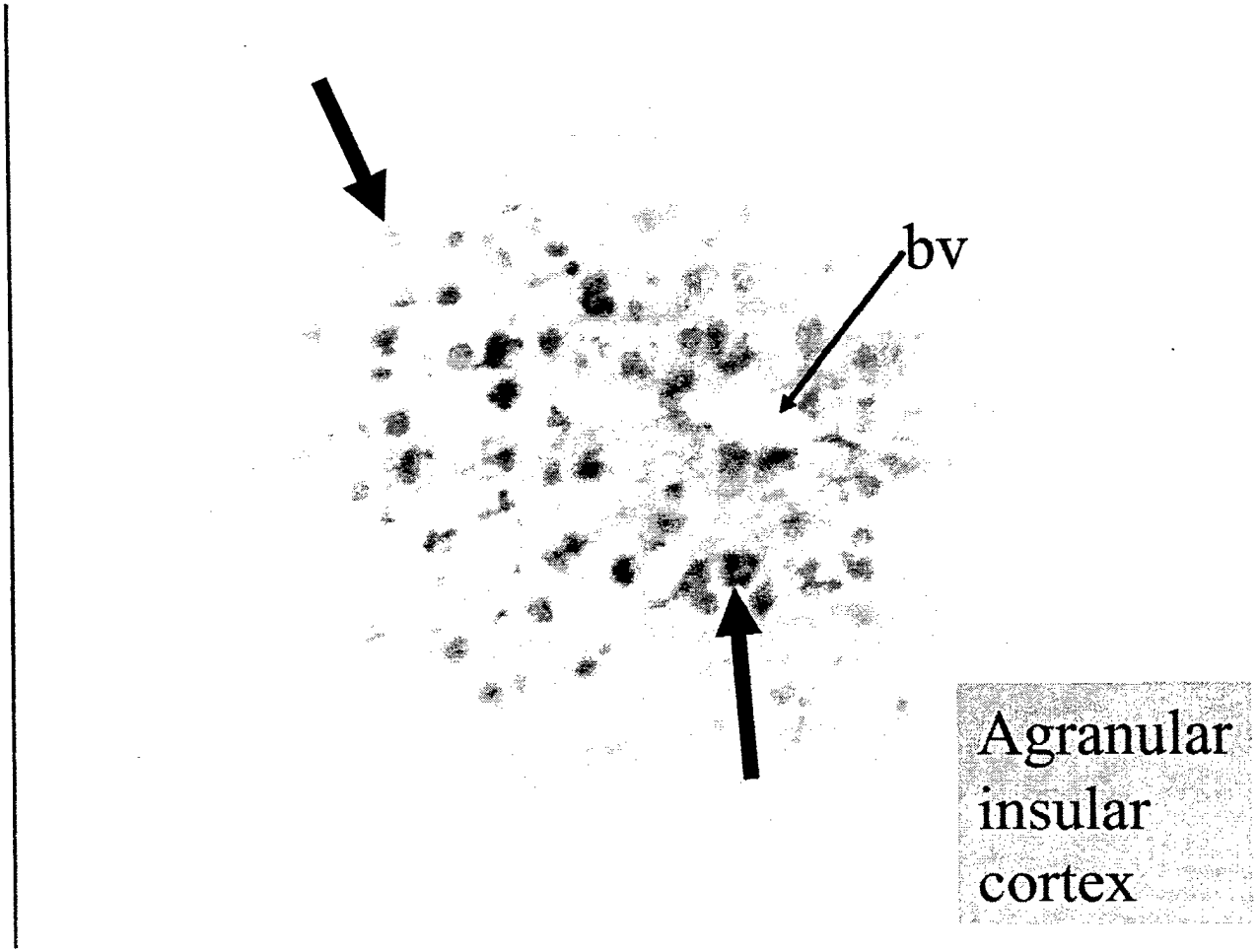
bv - a large blood vessel is 'in bad shape'. These neurons (n) show NO gross structural alterations.

186-05: Wet mount (so myelin is dark and neuropil light) of coronal sections, 50 μm. Stained with rabbit anti-albumin$_{rat}$ diluted to 1:50.



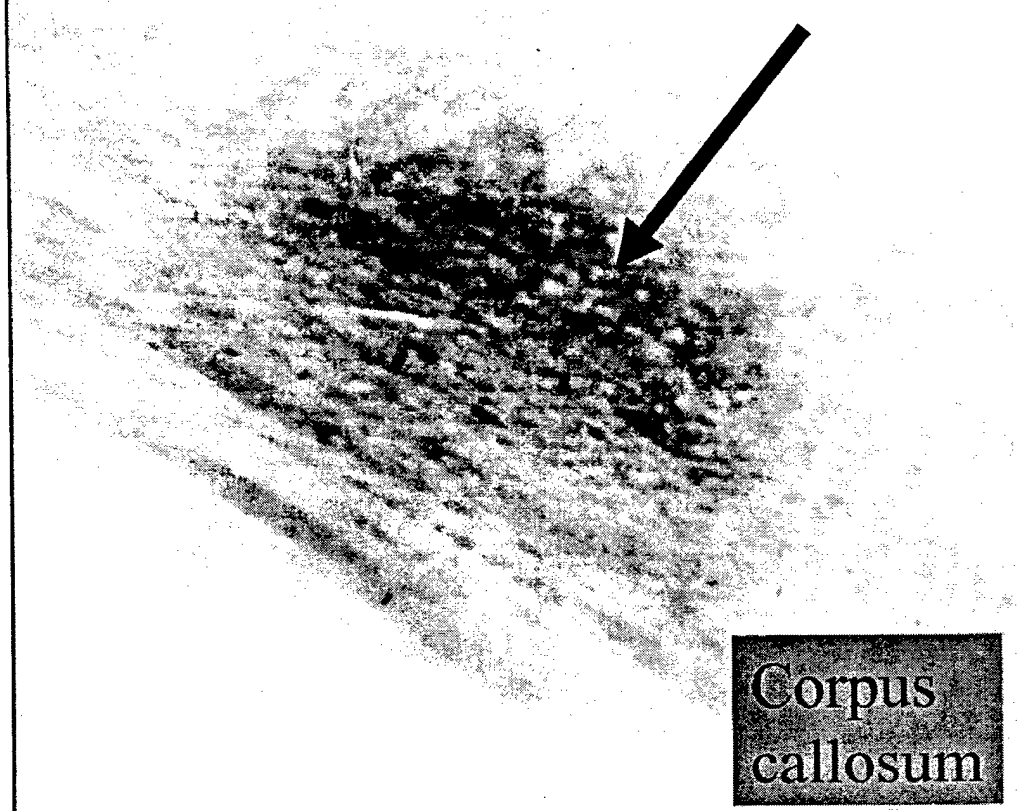
Arrows point to circular deposit of diaminobenzidine localizing albumin in perivascular 'halo'. 4X objective

186-05: Wet mount of coronal sections, 50 μm. Stained with rabbit anti-albumin$_{rat}$ diluted to 1:50.



Note perivascular 'halo' of albumin-immunoreactive (IR) material around the blood vessel (bv) at tip of arrow. Many neurons are also alb-IR.

186-05: Wet mount (so myelin is dark and neuropil light) of coronal sections, 50 μm. Stained with rabbit anti-albumin$_{rat}$ diluted to 1:200.



At 1:200 dilution no neurons stain for albumin. Interstitial 'halo' staining effect is obvious. Low level neuronal staining was observed in nearby cortex and striatum also.