

ABSTRACT

Paulette Hahn. **FUNCTIONAL MORPHOLOGY OF THE TUBULAR LATTICE IN THE CRAYFISH, PROCAMBARUS CLARKII, MEDIAL GIANT AXON**

(under the direction of Dr. Edward Lieberman, Dr. Gerhard Kalmus, and Dr. Jack Brinn), Departments of Physiology, Biology, and Anatomy, 1989).

The periaxonal space is the extracellular environment immediately adjacent to the axon. Ionic homeostasis, in general, and potassium homeostasis, in particular, of the extracellular microenvironment is important in neuronal function and synaptic integration. Potassium accumulation in the periaxonal space, which can occur during neural excitation, can adversely affect the resting membrane potential, action potential amplitude, action potential propagation along the nerve cord, synaptic transmission, and the excitation threshold.

This study of the medial giant axon of the crayfish was undertaken to investigate the functional morphology of structures that may be involved in potassium clearance from the periaxonal space, particularly, the role of the tubular lattice. As a first step, an electron microscopic study including a morphometric analysis of the neuronal structures was conducted to provide quantitative data about the structure.

From the morphometric data two structurally different-

glial layers were identified, the inner (adaxonal) and outer glial layers. The tubular lattice was found to be predominantly in the adaxonal glial layer. The average length of the mesaxonal intercellular pathways was calculated. It was found that the length of the intercellular pathways was reduced by 12% via tubular lattice connections. A structural model of the tubular lattice in the adaxonal glial layer was formed from morphometric data.

In order to relate the morphometric data to physiological events we measured electrophysiological parameters including action potential amplitude, action potential duration, and resting membrane potential in parallel with electron microscopic studies.

Lanthanum proved to be a good extracellular potassium ionic diffusion space marker. Examination of electron micrographs showed lanthanum at the axon surface and bound in the tubular lattice. Stimulation and current pulse experiments, except for an apparent osmotic effect, showed no conclusive change in lanthanum uptake into the tubular lattice. Drugs known to act on glia membrane electrophysiological properties, i.e., carbachol and forskolin, did not alter lanthanum distribution around or in the tubular lattice adjacent to the axon.

The result of these studies suggest that:

1. The tubular lattice is accessible to small ions and molecules from the extracellular space and,

2. The long tortuous intercellular diffusional length may be short circuited by the presence of the tubular lattice to account for a small change in the time constant of potassium clearance.

FUNCTIONAL MORPHOLOGY OF THE TUBULAR LATTICE
IN
THE CRAYFISH, PROCAMBARUS CLARKII, MEDIAL GIANT AXON

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Presented to
the Faculty of the Department of Biology
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In Partial Fulfillment
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Master of Science in Biology

by
Paulette C. Hahn
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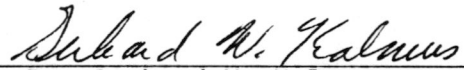
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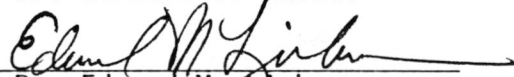
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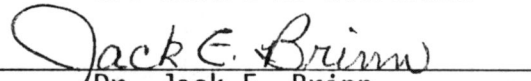
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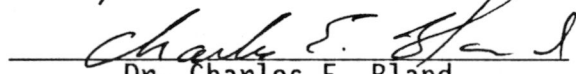
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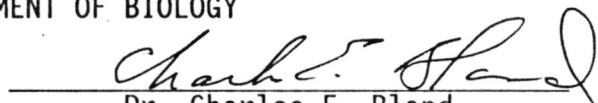
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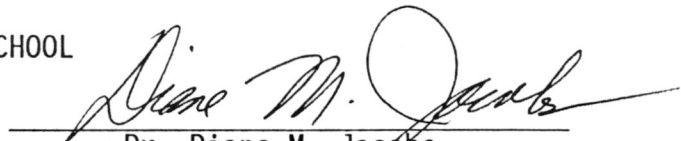
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TO GRAM, DAD, AND AUNT NAN

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ABBREVIATIONS

A	axoplasm
AD	adaxonal glia
cm	centimeter
CS	crayfish solution
C	collagen matrix
D	average diameter
GC	glia-collagen interface
GG	glia-glia interface
H	average thickness
HRP	horseradish peroxidase
Hz	Hertz
J_v	length density
KCl	potassium chloride
K^+	potassium ion
L	average length
$La_2(SO_4)_3$	lanthanum sulfate
La^{3+}	lanthanum ion
$LaCl_3$	lanthanum chloride
M	mitochondria (on figures)
M	molar (in text)
MA	mesaxonal opening
mm	millimeter
mM	millimolar
ms	millisecond
mV	millivolt

Na_2SO_4	sodium sulfate
Na^+	sodium ion
NCS	normal crayfish solution
nm	nanometer
N_v	numerical density
$^{\circ}\text{C}$	degree centigrade
ohm.cm^2	ohm centimeter squared
op	openings
OsO_4	osmium tetroxide
P	periaxonal space
PS	perineurial sheath
S	Schwann cell nuclei
S_v	surface density
T	average barrier thickness
TL	tubular lattice
T. L.	tubular lattice
V_v	volume density
$[\text{K}^+]_o$	potassium ion concentration outside
μl	microliter
μm	micrometer

REVIEW OF THE LITERATURE

Frankenhaeuser and Hodgkin (1956) first described potassium accumulation within the periaxonal space of the squid, Loligo, during a train of action potentials. The effect of increased external potassium concentration on the positive afterpotential was compared with 20-50 Hz repetitive stimulation. During stimulation the size of the undershoot could be related to extracellular potassium concentration, since there is a direct relationship between the amplitude of the undershoot and the K^+ equilibrium potential. It was thought that excess ions accumulate in an aqueous space between the glia and the axon, the periaxonal space. After repetitive stimulation, excess potassium is removed from the periaxonal space exponentially within a time constant of 30 - 100 ms, that is, 63% of the potassium is removed in 30 - 100 ms. To have this time constant, it was estimated that the periaxonal space must be approximately 30 nm wide and the electrical resistance across the glial layer would be approximately 1 ohm cm^2 .

In an attempt to better understand the physiology of potassium movement, it was important to relate the physiology to the structure of the squid. The ultrastructure of the squid giant axon has been described by Villegas and Villegas (1960; 1963; 1981; 1984) and recently by Zwahlen, et al. (1988). As in the crayfish, the Schwann cell investment is

formed by several Schwann cells layered around the axon. The thickness of the Schwann cell layer varies among squid species from 0.2 - 0.8 μm thick in Dorytheutis plei to 1.5 - 6.0 μm thick in Sepiotheuthis sepioidea and Dosidicus gigas (Villegas, 1981). The Schwann cell layer is crossed by long, tortuous intercellular clefts. These tortuous pathways found in the adaxonal glial layer are approximately 5 μm in length. The width of the Schwann cell clefts were in the range of 7.5 - 11.5 nm. The periaxonal space width ranges from 8 - 12 nm (Zwahlen et al., 1988).

The glial cytoplasm contains a three dimensional network of tubules known as the tubular lattice (Peracchia and Robertson, 1971; Peracchia and Dulhunty, 1976; Nordlander and Singer, 1972; Shivers and Brightman, 1976; Lieberman et al., 1981; Shrager et al., 1983; Cuadras and Marti-Subirana, 1987; Pichon et al., 1987; Zwahlen et al., 1988). It was previously thought that the tubular lattice did not exist in the squid, however, using glutaraldehyde fixation instead of osmium tetroxide, as in earlier work, the tubular lattice was identified in the squid (Zwahlen et al., 1988). It was found that portions of the tubular lattice open into the periaxonal, mesaxonal clefts, and the extracellular space. The relative tubular lattice opening area and mesaxonal area to the total periaxonal Schwann cell face is 0.42% and 0.15% respectively (Zwahlen et al., 1988). The mean diameter of the tubules is approximately 40 nm and the density of the

tubules is estimated to be 3.3 per μm^3 .

Among the invertebrates the crayfish also contains a large easily accessible axon which like the squid has made it a useful model for the study of both axon morphology and physiology. The gross morphological study of the crayfish nervous system made by Johnson (1924) laid the foundation for future structural and physiological studies on the crayfish medial and lateral giant axons. Robertson (1961) expanded the information on crayfish morphology with his investigation of the ultrastructure of excitable membranes, with an emphasis on the medial giant axon of the crayfish.

The ventral nerve cord of the crayfish consists of several paired ganglia including one supraesophageal or brain, one subesophageal, seven thoracic, and six abdominal (Appendix 1). These ganglia are joined by two longitudinal interganglionic connectives. Each connective contains a medial and lateral giant axon. The medial giant axon diameter ranges from 100 - 250 μm . The nerve cord is covered superficially by a sheath, the perineurium, that serves as a protective and structural blood - "brain" barrier.

The microstructure of the medial giant axon of the crayfish consists of 5 - 10 concentric glial layers alternating with layers of connective tissue that invest the giant axon (Hama, 1961; Nordlander and Singer, 1972; Lieberman et al., 1981). Nordlander and Singer (1972) reported the width of the glial layers to be in the range of 2 - 5 μm and

the adaxonal glial layer width 1 - 4 μm . Shrager et al. (1983) estimates the adaxonal glial layer width between 0.2 - 1.0 μm . The adaxonal glial layer of the crayfish has been estimated to contain between 15 - 20 interdigitated glial cells around the axon perimeter (Lieberman et al., 1981). The glial cells are separated from each other by tortuous intercellular clefts which communicate with the periaxonal space and connective tissue layer. The crayfish periaxonal space is estimated to be 10 - 30 nm wide (Shrager et al., 1983).

As previously noted for the ultrastructure of the squid glial cell, the crayfish glial cell also contains the tubular lattice system. Nordlander and Singer (1972) reported that the tubular lattice network was more abundant in the adaxonal glial layer of the crayfish compared to other glial regions and Peracchia and Robertson (1971) observed the lattice to open into the extracellular space. Freeze fracture preparations of the crayfish giant axons established that the lattice was made up of glial membrane invaginations (Shivers and Brightman, 1976). The diameter of the tubules was estimated to be approximately 40 nm. It was also noted that each lattice appeared separated from adjacent ones, however, it was not determined if there was any consistent spacing between the lattices.

The permeability properties of the glial layers and associated structures such as the tubular lattice, have been

studied ultrastructurally by electron opaque extracellular tracers including lanthanum (Abbott et al., 1977; Lane et al., 1977) and horseradish peroxidase (HRP) (Holtzman et al., 1970; Kristensson et al., 1972; Nordlander et al., 1975). Lanthanum penetration into the crayfish nervous system indicates that the perineurium is a major site of restriction for the entry of large ions and molecules. In crayfish nerve cord preparations with the perineurial sheath intact, lanthanum penetrated into the perineurium slowly and was not found in the glial tubular lattice. The desheathed preparation showed a rapid entry of lanthanum into the extracellular space and tubular lattice. The lanthanum deposits were found at the level of the axon surface.

Horseradish peroxidase, which also may be used as an extracellular marker, is an enzyme with a molecular weight of 40,000 whose presence is revealed by incubating it with peroxide and diaminobenzidine. The route that peroxidase follows may indicate the mechanisms by which proteins are transported from the sheath to the axon (Nordlander et al., 1975). Holtzman, et al. (1970) found that HRP penetrated the tubular lattice of the lobster walking limb axons suggesting that the tubular lattice is part of the extracellular pathway. Kristensson, et al. (1972) studied the fourth ganglion of the nerve cord of the crayfish, Astacus astacus using HRP. Although the nerve sheath was a barrier to the diffusion of the tracer, some HRP penetrated the peripheral nerve

sheath near the ganglion. HRP was then found in the extracellular spaces between glial membranes eventually reaching the axonal surface. Nordlander, et al. (1975) studied the claw nerves of the crayfish, Cambarus and Procambarus. In some nerves, HRP did not penetrate the nerve sheath. However, in those nerves in which HRP was able to cross the sheath, HRP was found between and within the glial layers. In the adaxonal glial layer, HRP was found in the tubular lattice.

At physiological conditions of 5.4 mM external potassium concentrations, the resting membrane potential in the crayfish giant axon is about -85 mV (Lieberman and Lane, 1976). During an action potential potassium moves out of the axon into the periaxonal space. Prolonged stimulation will significantly increase K_o^+ . Since a large potassium accumulation in the periaxonal space may alter the resting membrane potential and generation of an action potential, the system requires a mechanism to restore K^+ to normal levels (Peracchia and Robertson, 1971; Abbott and Pichon, 1976; Abbott et al., 1977; Abbott, 1979; Lieberman et al., 1981; Shrager et al., 1983; Orkand, 1980; Brunder and Lieberman, 1988).

Several hypotheses have been proposed to explain the removal of excess potassium from the periaxonal space. Orkand, et al. (1966) studied the role of glia in potassium clearance with work done on the leech and amphibian nervous

system. They proposed a mechanism of "spatial buffering" whereby potassium is removed from areas of high K^+ concentration via a voltage gradient generated by the depolarization of glial cells initiated by the excess K^+ . In order for spatial buffering to be an efficient mechanism, two characteristics must be present: first, the glial membrane potential must elicit a Nernstian response to potassium, that is, the membrane potential has a high sensitivity to small changes in $[K^+]_o$; and second, there must be gap junctions or electric coupling between glial cells (Abbott et al., 1977). It was noted that spatial buffering probably does not play a primary role in removal of excess potassium in the squid and crayfish since both have low glial membrane potentials and are not especially sensitive to K^+ .

The tubular lattice found in lobster and crayfish and most recently in squid has been suggested to provide an additional extracellular diffusion route for the clearance of potassium from the periaxonal space and may also serve as a pathway for movement of large molecules between the periaxonal space and the extracellular matrix. Shrager, et al. (1983) studied periaxonal potassium homeostasis in the medial giant axon of the crayfish, Procambarus clarkii using voltage clamp analysis. Potassium tail currents were measured after depolarizing the axon. It was found that the time constant was 2 ms for excess potassium to be removed from the crayfish periaxonal space. Shrager postulated that if

excess potassium left the periaxonal space by diffusion with a time constant of 2 ms, the length of diffusion should be no greater than 1.4 μm . Shrager also studied electron micrographs of the crayfish glial layers and observed openings of the tubular lattice to the periaxonal space and to the bulk extracellular fluid space. He proposed that the tubular lattice may provide a low resistance path for removal of excess potassium, although the series resistance in crayfish axons was estimated to be 6 ohm cm^2 which is higher than found in the squid. The series resistance has been largely attributed to the Schwann cell layer.

The function of the tubular lattice is yet to be confirmed. The purpose of this investigation is to study the tubular lattice system by morphological, electrophysiological, and pharmacological methods and thereby provide better understanding of the functional morphology related to the perineurial ionic homeostasis.

STATEMENT OF THE PROBLEM

The primary objective of this study was to establish a relationship between glial-neuronal structure and function as a basis for interpreting physiological interactions between axons and their associated glia.

Physiologically, the resting membrane potential is greatly influenced by the periaxonal potassium concentration. During an action potential, potassium moves down its electrochemical gradient from the axon into the extracellular space adjacent to the axon, the periaxonal space. Potassium that accumulates in the periaxonal space will depolarize the membrane and subsequently alter excitability properties of the axon.

There are four hypotheses for the path of potassium clearance:

1. Potassium moves into the axon from the periaxonal space by the action of the $\text{Na}^+\text{-K}^+$ pump.
2. Potassium clears via the mesaxonal intercellular pathways.
3. Potassium is taken up by the glia cell via various transport systems.
4. Potassium clearance through the glia is facilitated by the tubular lattice.

The purpose of this study was to investigate the structures that may be involved in potassium clearance, particu-

larly, the role of the tubular lattice. In order to accomplish this aim the study was divided into two areas of investigation:

1. Morphometric analysis of the neuronal structures to provide quantitative data about the structure.
2. Electrophysiological methods in order to relate the morphometric data to physiological events.

MATERIALS AND METHODS

Animals

The medial giant axon of the ventral nerve cord of the crayfish Procambarus clarkii was used in this study. Adult freshwater crayfish were obtained from Waubun Labs in Louisiana and maintained in a well-oxygenated aquarium at a water temperature of 12-15°C. The nerve cord was dissected and isolated using the method described by Wallin (1967). After isolation, the nerve cord was transferred to a plexi-glass chamber and secured by metal clamps at both the cranial and caudal ends. The nerve cord was continuously bathed by gravity flow with normal crayfish solution (NCS) (Van Harreveld, 1936) (Appendix 2). With the aid of a zoom binocular microscope, the perineurium or outer sheath surrounding the nerve cord was removed between the thoracic ganglionic segments 2 and 6 using fine forceps.

Preparation of tissue for electron microscopy

Initially a protocol using 1/4 strength Karnovsky fixative in phosphate buffer was used to fix the crayfish nerve cord, however, this fixative did not preserve the tubular lattice and the tissue showed evidence of osmotic shock. In order to determine the optimum fixation method for

the crayfish ventral nerve cord, various fixative solutions, buffers, and time of fixation, as shown in Appendix 5, were investigated.

It was found that the optimum fixative for the control preparation was 2% glutaraldehyde in crayfish solution (CS) containing veronal acetate. After each experimental procedure was completed, the nerve cord was immediately fixed for one half hour in the plexiglass chamber with 2% glutaraldehyde in CS containing veronal acetate buffer at pH 7.4, substituted for TRIS (Appendix 3). The plexiglass chamber was thoroughly rinsed with distilled water to remove any traces of glutaraldehyde after each experiment. The nerve cord was divided into interganglionic segments and fixed overnight at 4°C in 2% glutaraldehyde in veronal acetate buffered CS. Post fixation was achieved with 1% osmium tetroxide (OsO_4) in veronal acetate buffered CS for one hour. The details of the post fixation procedure are shown in Appendix 4.

The resin blocks, containing the ganglionic segment, were sectioned on a Reichart ultracut microtome using a Dupont Instruments diamond knife and mounted on 200 to 300 mesh copper grids. Sections were stained with lead citrate for 10 minutes at room temperature and examined with a Philips 201 transmission electron microscope. Electron micrographs were recorded on Kodak 4489 electron microscopy film.

Unprecipitated and precipitated lanthanum chloride (LaCl_3)

The trivalent cation, lanthanum, is unique in the ability to be located both physiologically and morphologically. When lanthanum is used without precipitation, lanthanum can be observed by electron microscopy at high affinity binding sites. Once lanthanum is precipitated, lanthanum can be found at low affinity binding sites as well, that is, globally in all lanthanum diffusion spaces (Doggenweiler and Frank, 1965; Bouldin and Krigman, 1975).

The desheathed nerve cord was perfused with 5 mM LaCl_3 diluted in Tris buffered NCS at varied time intervals: 5 minutes, 15 minutes, 30 minutes, and one hour. The duration of the action potential at 1/2 the action potential amplitude, and resting membrane potential were monitored during LaCl_3 perfusion. Two different methods were employed: unprecipitated LaCl_3 and precipitated La^{+3} to $\text{La}_2(\text{SO}_4)_3$. The protocol for the unprecipitated method included immediate fixation using the standard fixation protocol after LaCl_3 incubation. In order to prevent LaCl_3 washout when the fixative solution was applied, the precipitated method included a 15 minute precipitation step with simultaneous fixation in 2% glutaraldehyde and Na_2SO_4 solution (Appendix 6). After perfusion with the Na_2SO_4 solution, the nerve cord was further fixed using the standard fixation protocol. The

precipitation method was used for all experiments using LaCl_3 .

Attempts were made to determine if a redistribution of LaCl_3 occurred using biochemical and electrophysiological methods. The following methods were used, followed by transmission electron microscope examination of the tissue.

Pharmacological methods

Carbachol is an analog of acetylcholine which hyperpolarizes the membrane potential of the Schwann cells associated with the medial giant axon in crayfish. Forskolin has a similar effect by hyperpolarizing the membrane of the Schwann cell by selectively increasing the activity of the membrane bound adenylate cyclase (Evans *et al.*, 1984). Both carbachol and forskolin were separately used in conjunction with 5 mM LaCl_3 . The desheathed nerve cord was perfused with LaCl_3 in NCS for 30 minutes followed by a 5 minute perfusion of 5 mM LaCl_3 diluted in NCS with the addition of 10^{-6} M carbachol or 10^{-6} M forskolin followed by the standard precipitation and fixation protocol for LaCl_3 .

Electrophysiology

A) Electronics

The electronic apparatus used for membrane potential recording was previously described by Lieberman and Lane (1976). A glass electrode containing agar gel and filled with 2.5 M KCl was used as a reference electrode to monitor the external solution. The microelectrodes used to monitor the resting membrane potential and action potential propagation were made from rapid fill glass pipettes. The glass pipettes were pulled using a David Kopf Instruments vertical pipette puller model 700 C. The microelectrodes were filled with 2.5 M KCl and had a resistance between 10 and 30 megohms. The micropipette was placed on a micromanipulator and advanced at 30 degree angle to the axon until the electrode tip formed a dimple between the microelectrode and axon. Once the microelectrode was advanced through the axonal membrane, the resting membrane potential, after amplification by WPI 765 microprobe, could be monitored with a Keithley 130 digital multimeter and by a Gould digital storage oscilloscope model OS4020. Platinum external stimulating electrodes near the subesophageal ganglion were used to generate propagated action potentials. The stimulating pulse was initiated by a Grass S88 stimulator. Only those axons with a resting membrane potential of -80 to -90 mV and an action potential of 110 to 130 mV amplitude were used for

experiments.

B) High frequency stimulation

The desheathed nerve cord was perfused for 30 minutes with 5 mM LaCl_3 in NCS. The axon was checked for an adequate resting membrane potential and action potential after LaCl_3 incubation. A tightened ligature placed distal to the 4th ganglionic pair prevented the propagation of the action potential distal to the ligature. The segment distal to the ligature was used as a control after assuring that no action potential was generated in this segment. This was tested by impalement of the axon proximal and distal to the ligature. If high frequency stimulation generated an action potential proximal to the ligature, but only a stimulus artifact with no action potential distal to the ligature, the distal segment was used as a control. A train of stimuli, at 50 Hz for one minute was applied while the axon was exposed to 5 mM LaCl_3 . After stimulation, the tissue was fixed using the standard precipitation and fixation protocol for LaCl_3 .

C) Current pulse method

The following method was employed for both the hyperpolarizing and depolarizing current pulse experiments. The desheathed giant axon was impaled to confirm an adequate

resting membrane potential and action potential. Once a viable axon was confirmed, the impaling electrode was removed. A loose ligature was placed just proximal to the 4th ganglion pair. The cannulation electrode contained a 25 μ m tungsten wire threaded through the center of the electrode for current passing. The tungsten wire extended approximately 0.7 to 0.8 cm beyond the glass electrode tip. A Ag-AgCl plate electrode of 0.9 cm length was secured to the plexiglass chamber with wax and was grounded. The plate electrode allowed a parallel current pulse over most of the surface of the axon adjacent to the electrode. The axon was situated in the chamber so that the experimental segment between ganglionic pair 3 and 4 was parallel to the plate electrode. The cannulation electrode was mounted on a micromanipulator and connected to a second isolation unit through a 100,000 ohm resistor. The second isolation unit provided the ability to change the current pulse polarity to a hyperpolarizing or depolarizing current pulse. A small hole was cut in the axon with microscissors between the ganglionic pairs 5 and 6. The medial giant axon was carefully cannulated avoiding damage to the axon by contact of the cannulating electrode with the axon membrane. The tungsten axial wire was advanced between the ganglionic pair 4 and 5. The axonal membrane was then reimpaled between the ganglionic pair 3 and 4. The cannulating electrode and stimulating electrode were close or overlapping. It was important for the tips of the cannulating

and impaling electrodes to be near each other in order to insure the most accurate measurement of the effects of the current pulse amplitude on the axon membrane potential.

Once the medial giant axon was cannulated and the tungsten wire advanced, the nerve cord was perfused with LaCl_3 in NCS for one hour. The isolation unit was then set for either the hyperpolarizing or depolarizing direction. A -10 mV hyperpolarization or +10 mV depolarization test pulse was made followed by the respective experimental current pulse at a rate of two pulses per second, of 100 millisecond duration for two minutes. The axon was continuously superfused with LaCl_3 during the current pulse. After the completion of either the hyperpolarizing or depolarizing current pulse protocol, the tissue was fixed according to the standard protocol for LaCl_3 precipitation and fixation.

STEREOLOGY

Stereological methods were used to obtain quantitative information about the ultrastructure of the crayfish giant axon and its associated glia (Weibel, 1979). Data in the form of the number of intersections of a reference grid system with structures seen on electron micrographs were collected. Estimates were made of volume, surface, length, and relative number of organelles determined mathematically and statistically.

The crayfish nerve cord was cut into three interganglionic segments. Two grids of ultrathin cross sections were collected from each of the three interganglionic segments, for each animal, i.e., a total of six samples per animal. A field was randomly selected at low magnification (X1500) in order to avoid biasing the sample. The field selected for morphometric analysis was photographed at a primary magnification of X10,000 (final print magnification X25,000) showing the axon area from the axoplasm to the outer glial layers. This area was divided into three smaller sections so that the axon to the outer glial layers could be photographed at the magnification necessary for the morphometric design. The area was divided into inner (adaxonal), mid, and outer layers in reference to the axonal membrane. Micrographs were taken of these three areas at a primary magnification of X30,000 (final print magnification X45,000). There was some overlap between the inner and middle, and middle and outer photographs, but no overlap between the inner and outer.

The grid was carefully designed considering the magnification and size of the structures studied as well as the statistical requirements. The appropriate grid was determined to be a double square "lattice" which is a 10 cm X 10 cm square area divided into 10.05 mm X 10.05 mm squares ($d=10.05$ mm).

A point intersect was considered to be the intersection

of two grid lines which fell on the structure studied, such as, glial mitochondria. A line intersect was defined as the point where the structure, such as the axonal membrane, crossed a horizontal or vertical grid line. A line transection is the number of membrane segments in one square. The diameter of an opening, such as the diameter of the tubule opening of the tubular lattice, was measured using a micrometer eyepiece. The diameter of a cross-sectional structure, such as the diameter of a tubular lattice patch, was measured using a millimeter ruler to determine the long and short axis. The diameter was then calculated using the long and short axis dimensions.

Volume density (V_V)

The volume density is the fractional representation of two volumes, one containing the other, such as, the total mitochondrial volume related to the glial cell volume.

Equation 1.
$$V_V = P_a/P_t$$

in our example P_a and P_t are the number of point intersects on glial mitochondria and cell cytoplasm, respectively. The volume density of mitochondria, and the volume density of the tubular lattice per glial cell volume, were calculated.

Surface density (S_v)

The surface density is the fractional representation of surface to volume, for example, the glial cell membrane surface in relation to the glial cell volume.

$$\begin{aligned}\text{Equation 2.} \quad S_v &= (2 I_a) / (k_1 d P_t) \\ &= (2 I_a) / (2 d P_t) \\ &= I_a / (d P_t)\end{aligned}$$

and I_a = line intersects of membranes, P_t = Point intersects of volume; d = length of the grid block/magnification (0.23 microns); and, k_1 for double square lattice = 2.

The glial plasma membranes were divided into two categories: glial membrane opposed to collagen (glia-collagen interface) and glial membrane opposed to other glia membrane (glia-glia interface). The surface density of both the glia-collagen and glia-glia interface was calculated.

Average barrier thickness (T)

A barrier represents a mass of tissue separating two spaces. The arithmetic mean barrier thickness estimates the tissue mass forming the barrier.

$$\begin{aligned}
 \text{Equation 3.} \quad T &= (L_t P_b) / (2 I_b) \\
 &= (2 d P_b) / (2 I_b) \\
 &= (d P_b) / I_b
 \end{aligned}$$

and L_t = test line length associated with each test point of the grid lattice which is $2d$ for a square lattice; P_b = points of the grid on the glial or connective tissue volume, or both; I_b = line intersect of the glia-collagen interface forming the respective boundary. The arithmetic mean barrier thickness (T) of the total glial layers, adaxonal glial layer, outer glial layer, and glia-glia interface was calculated.

Length density (J_v)

The length density expresses the length of a curve in a three-dimensional space with respect to the volume the curve is contained within. The length density of the total glia-glia interface and total glia-collagen interface including the membrane facing the periaxonal space was calculated where:

$$\begin{aligned}
 \text{Equation 4.} \quad J_v &= (2 Q) / A_T \\
 &= (2 Q) / (P_T k_2 d^2)
 \end{aligned}$$

and A_T = test area (μm^2); Q = line transection; $k_2 = 1$; P_T =

test point number; and $d^2 = (\text{length of the grid block/magnification})^2$ where d^2 for low magnification prints = $0.176 \mu\text{m}^2$ and for high magnification = $0.053 \mu\text{m}^2$. The length densities (J_V) of the adaxonal and outer glial layer glia-glia interface both with and without tubular lattice connections were calculated on high and low magnification prints.

Average length (L) and Average thickness (H)

From the mean thickness of the outer layer the average length of the outer glia-glia interface with and without tubular lattice and average thickness were calculated.

$$\text{Equation 5.} \quad L = J_V T$$

$$\text{Equation 6.} \quad H = V_V T$$

and V_V is the volume density of glial cytoplasm.

Average diameter of an irregular structure

To determine the average diameter of the tubular lattice, both the short axis (X) and the long axis (Y) of the structure were measured using high magnification prints (final print magnification X45,000). The average diameter (D) was calculated, where:

Equation 7.
$$D = (X \cdot Y)^{1/2}$$

and X = mean value of short axis, and Y = mean value of long axis.

Numerical density (N_V)

The numerical density of the tubular lattice (N_V) represents the number of tubular lattice groups per glial cell volume.

Equation 8.
$$N_V = V_V / (k_2 d^2 D)$$

and $k_2 = 1$ for a simple double square lattice; d^2 = grid block size at $X1$ magnification (μm^2); D = average size of tubular lattice patch; and, V_V = average volume density of tubular lattice related to glial cell volume. The tubular lattice numerical density was calculated for the total and then subdivided into the numerical density in the inner adaxonal layer and outer layer using the respective average size of the tubular lattice patch.

An estimate of the total mean number of glial layers and glial cells was calculated from data obtained from low magnification prints. The glial cells were defined by following the path of the glia-glia interface and identifying this interface as the separation between glial cells.

In order to further explore the structure and relationship of the tubular lattice to diffusional pathways the following parameters were calculated using high magnification prints:

1. The mean number of openings to extracellular pathways per tubular lattice patch.
2. The number of tubular lattice openings per patch to glia-collagen interface in the adaxonal inner and outer glial layer.
3. The number of tubular lattice openings to the inner aspect and outer aspect of the glia-collagen interface per patch.
4. The mean total of tubular lattice openings to the glia-glia interface in the inner and outer glial layers.
5. A comparison of the number of tubular lattice openings in the adaxonal glial layer to the periaxonal space and glia-glia interface.
6. The number of tubular lattice openings to the periaxonal space compared to the number of mesaxonal openings to the periaxonal space.
7. The mean diameter of the tubular lattice cross-sectional openings.

The mean of the parameters were calculated from at least 32 micrographs. Significant difference between the adaxonal and outer glial layers was determined using a paired (two-tail) t-test as $p < 0.05$.

RESULTS

An initial goal of this study was to determine the optimal fixation method for the crayfish nervous system. The following criteria were used to identify a well preserved specimen: intact mitochondria, well defined membranes, intact Schwann cell nuclei, preservation of the tubular lattice, and minimal tissue shrinkage.

Karnovsky fixative, containing paraformaldehyde and glutaraldehyde (Karnovsky, 1965) has a higher osmolality than physiologic for mammalian and crayfish tissue. Despite the high osmolality, Karnovsky fixative usually minimizes tissue shrinkage and stabilizes tissue structure when used for mammalian preparation. However, when the medial giant axon of the crayfish, Procambarus clarkii, was exposed to Karnovsky fixative at varied concentrations including full strength, 1/2 strength, and 1/4 strength paraformaldehyde-glutaraldehyde at pH 7.4, the tissue showed evidence of osmotic shock. Following several attempts at using a variation of Karnovsky fixative, the optimal fixation method was found to be a combination of an initial fixation with 2% glutaraldehyde in veronal acetate buffered NCS overnight, followed by the postfixation protocol detailed in the Materials and Method section.

General Morphology

All criteria for a well preserved specimen were met as seen in Fig. 1. The axon is surrounded by concentric layers of glia with the greatest concentration of glia noted in the adaxonal layer. It appeared that there is a greater concentration of glial structures such as mitochondria and tubular lattice in the adaxonal layer. The tubular lattice in the adaxonal layer opens into the periaxonal space, glia-glia interface, and glia-collagen interface. However, in the outer layer it appears that the tubular lattice spans the glial cell opening to both glia-collagen interfaces. Concerning the glial mitochondria, it was noted that there was a greater concentration in the adaxonal glial layer, decreasing progressively through the remaining glial layers toward the outer neural lamella. At the outer neural lamella, close to the perineurium, there were often seen large patches of concentrated mitochondria (Fig. 2). It may be suggested that these highly concentrated regions of mitochondria in the outermost portion of the glial layers are involved in the active maintenance of the perineurium.

It was found that morphologically the tissue was well preserved, however it was important to ascertain whether veronal acetate elicited any physiologic effect on the resting membrane potential or action potential of the medial giant axon. If veronal acetate produced a change in the

membrane potential or action potential generation it would be eliminated as a suitable buffer in experimental solutions. The desheathed nerve cord, immersed in NCS, was checked for a viable resting membrane potential and action potential. After establishing the viability of the axon, the tissue was superfused with CS containing veronal acetate buffer, at pH 7.4. The axon was monitored at 10 minute intervals. After one hour superfusion of the axon-Schwann cell preparation with veronal acetate buffered CS there was no change from the baseline resting membrane potential or action potential.

Unprecipitated and precipitated lanthanum chloride

The lanthanum ionic diffusion space was determined by lanthanum superfusion of the axon-glial preparation followed by fixation and electron microscopic observation. The nerve cord was superfused with 5 mM LaCl_3 in NCS for 15 min, 30 min, and one hour. There was no evidence of the appearance of lanthanum along the glial intercellular space or in the tubular lattice for all unprecipitated preparations (Fig. 3). When LaCl_3 was precipitated, the 15 min (Fig. 4), half hour (Fig. 5), and one hour (Fig. 6) preparations showed evidence of lanthanum precipitation along the inner glial layer intercellular membranes as well as staining along the tubular lattice interior membrane and precipitation along

the periaxonal membrane. However, qualitatively the half hour and one hour preparations showed heavier precipitation formation along the periaxonal membrane, than shorter incubation periods.

During 5 mM lanthanum incubation the action potential duration and resting membrane potential were monitored. The initial action potential duration was 0.5 to 0.8 ms. The action potential duration increased within 5 min from 1.0 to 2.4 ms and after sufficient loading of the axon-glial preparation with LaCl_3 , the duration reached 2.0 to 5.0 ms. This increase in action potential duration was accompanied by no change in the membrane potential, and was indicative that La^{3+} diffused to the axolemma rapidly and was freely diffusible through the glial intercellular and periaxonal space.

Pharmacological and Electrophysiological Experiments

We attempted to determine if physiological, pharmacological or electrophysiological manipulations influenced the lanthanum distribution.

A) Carbachol and forskolin

When the nerve cord was incubated with lanthanum for one half hour followed by 10^{-6} M carbachol or 10^{-6} M forsko-

lin treatment, there was no evidence of lanthanum redistribution. Lanthanum precipitates found in the tubular lattice did not differ from the control preparation.

B) High frequency stimulation

The preliminary results of one half hour LaCl_3 incubation followed by a train of stimuli at 50 Hz for 2 min (Fig. 7) showed evidence of tissue disruption in the adaxonal glial layer, areas of disruption of the axon membrane, and increased vacuole formation throughout the tissue. These structural changes were observed in sections of the dorsal aspect of the axon, where only glia and perineurium were associated with the axon. Along the ventral aspect where smaller axons were adjacent to the giant axon disruption of the tissue was not observed.

C) Axial wire current pulse

The preliminary results of one half hour LaCl_3 incubation of one axon preparation followed by +10 mV depolarization of 100 ms duration, current pulse for 30 sec showed damage and a dramatic redistribution of lanthanum precipitate (Fig. 8 and 9). In one preparation, the adaxonal glial tubular lattice patches were completely filled with lanthanum precipitate. The outer glial layer tubular lattice did

not show tubular lattice darkening. This result occurred only in the glial layers in closest association to the perineurium, where supposedly the current density was highest. The area of the axon insulated by the smaller axons did not show this effect.

Stereology

Figure 10 is a cross section of the medial giant axon of the crayfish showing the glial layers from the axon to the outer neural lamella. The ultrastructural elements subject to morphometric studies are labeled. The glial layers were characterized morphometrically and may be described as consisting of three major components: the collagen matrix, glial cells, and the intercellular space (Fig. 11A). The collagen matrix makes up $56\% \pm 2\%$ of the total volume. The glial cells form $40\% \pm 2\%$ and the intercellular space comprised $4\% \pm 0.3\%$ of the total volume. An estimate of the number of glial layers and glial cells in the total glia volume were calculated. The mean number of glial layers was eleven with a range between 6 and 16. The mean number of total glial cells was 19 with a range between 12 and 28. These values correlate with previous estimates found in the literature (Lieberman et al., 1981).

Preliminary observations of the control axon-glial preparation suggested two structurally different glia based

on differences in osmium staining, distribution of glia and glial cellular components including the glial mitochondria and tubular lattice. The glial layers were divided into two distinct types of glia: the inner (adaxonal) and outer layers. This observation was supported by morphometric data. The barrier thickness was calculated for the total thickness of the glial layers and collagen matrix and then subdivided into the adaxonal and outer glial layer (Fig. 12). The total mean barrier thickness is $3.18 \pm 0.28 \mu\text{m}$. The mean barrier thickness of the adaxonal glia layer is $0.29 \pm 0.02 \mu\text{m}$. The mean barrier thickness of the outer layer is $2.44 \pm 0.21 \mu\text{m}$. The thickness of the space between glial cells or the inter-cellular space was calculated as $0.029 \pm 0.002 \mu\text{m}$. Interestingly, when the volume of the adaxonal and outer glial regions were compared, it was found that the adaxonal glia comprised 25% of the total glial cell volume (Fig. 11B). Also, only 2% of the total collagen matrix exists in the adaxonal layer.

The adaxonal glia appeared more tightly interdigitated compared to the outer glia. This observation was supported morphometrically by calculating the surface density of the glia-collagen and glia-glia interfaces. The horizontal and vertical grid line intersects of the glia-collagen and glia-glia interfaces were counted. The value obtained for the glia-collagen and glia-glia interfaces was related to the glial cell volume only. The surface density of the glia-glia

interface was found to be approximately 2 X greater in the adaxonal layer (6.47 ± 0.58) compared to the outer layer (3.77 ± 0.33) (Fig. 13). Furthermore, the adaxonal glia-collagen interface was subdivided into the membrane facing the axon surface and that facing the bulk collagen matrix. It was found that the surface density of the membrane facing the collagen matrix (6.68 ± 0.66) was approximately 5 X greater than that facing the axonal surface (1.24 ± 0.08) (Fig. 13).

At the glial cellular level, it was suspected that there may also be a difference in the intracellular organelles between the adaxonal and outer glial layers. This morphometric study concentrated on quantifying two structures, the glial mitochondria and the tubular lattice system. Particular emphasis was placed on the study of the tubular lattice.

Since it has been proposed that the adaxonal glia participate in transport mechanisms (Abbott and Pichon, 1976; Abbott et al., 1977; Abbott et al., 1988; Brunder and Lieberman, 1988; Gardner-Medwin, 1981; Hassan and Lieberman, 1988; Lieberman and Hassan, 1988) it was thought that there would be a greater volume of glial mitochondria in the adaxonal region compared to the outer region. The glial mitochondria volume was related to the glial cell cytoplasm volume. It was found that for the total glial volume, glial mitochondria comprise $3.8\% \pm 0.5\%$ of the volume. There was

approximately 2 X more glial mitochondrial volume in the adaxonal (0.036 ± 0.007) compared to the outer (0.018 ± 0.005) glial layer (Fig. 11B).

Qualitative examination of micrographs of the axon-glial preparation revealed that the tubular lattice appeared in all the glial layers (Fig. 14). The total average tubular lattice volume density was calculated using data from low magnification prints (final print magnification X25,000). The inner and outer average tubular lattice volume density was calculated using the high magnification prints (final print magnification X45,000). The systematic examination of the tubular lattice distribution showed approximately 5 X greater volume in the adaxonal layer (6.7 ± 0.2) compared to the outer layers (1.2 ± 0.5) (Fig. 11B). The tubular lattice was found to open into the extracellular space in the inner and outer glia regions. The number of tubular lattice openings per unit area of membranes, such as, openings facing toward the axon surface, between adjacent glia, and openings toward the collagen matrix were determined (Table 1). It was found that in the adaxonal layer for every 1 opening toward the axon, there were 4 openings between adjacent glia, and 3 openings toward the collagen matrix. The same parameters for the outer glial region showed 5 openings toward the axon, 1 opening between adjacent glia, and 6 openings toward the outer collagen matrix. Note that in the outer region the tubular lattice tends to open to the glia-collagen interface

rather than to the glia-glia interfaces between cells. When the relative number of tubular lattice openings directly to the periaxonal space (0.12) was compared to the number of mesaxonal openings per tubular lattice (0.32) (Fig. 15 & 16), there was no significant difference between the tubular lattice and mesaxonal openings.

In order to calculate the numerical density of the tubular lattice per glial cell volume, the size of the tubular lattice patch had to be determined. The mean cross-sectional diameter of the tubular lattice was $0.022\ \mu\text{m}$ as shown on a frequency bar graph (Fig. 17). The average size of the tubular lattice patch was $0.356\ \mu\text{m}$. There was a difference found in the calculated size of the tubular lattice patch, depending on their location in inner and outer glial regions. The average diameter of the tubular lattice patch in the inner region was $0.378\ \mu\text{m}$. The average size of the tubular lattice patch in the outer region was $0.305\ \mu\text{m}$ (Table 2). The calculated value of d^2 was $0.1764\ \mu\text{m}^2$ for the low magnification prints (X25,000) and $0.0529\ \mu\text{m}^2$ for the high magnification prints (X45,000). The value of (D) or the average size of the tubular lattice patch is an overestimate. Therefore, the numerical density calculation is an underestimate since (D) is in the denominator of the numerical density equation (see Equation 8 in the Methods). The numerical density of tubular lattice per μm^3 of glial cell volume for all data was 1.21 per μm^3 of glial cell volume.

The numerical density of tubular lattice in the inner region was 3.75 per μm^3 of glial cell volume. The numerical density of tubular lattice in the outer region was 1.25 per μm^3 of glial cell volume.

In the adaxonal layer, one tubular lattice patch has an average of 8 total openings per tubular lattice patch to any surface. The numerical density shows that there are 3.75 tubular lattice per μm^3 adaxonal glia, hence, there are 30 openings per unit volume to any surface in the adaxonal layer. The outer layer has a numerical density of 1.25 tubular lattice per μm^3 glia and thus has 15 openings per unit volume to any surface.

An estimate of the average three-dimensional pathway from one side of the barrier to the other per unit volume may be obtained by calculating the length density. The length density for the adaxonal and outer glia-glia interfaces was calculated from high magnification prints. The length density of the adaxonal glia-glia interface without tubular lattice was $14.9 \pm 2.5 \mu\text{m}^{-2}$ and with tubular lattice $13.1 \pm 2.2 \mu\text{m}^{-2}$ (Fig. 18). The length density of the outer glia-glia interface with and without tubular lattice connections was calculated. The length density of the glia-glia interface without tubular lattice was $5.9 \pm 0.4 \mu\text{m}^{-2}$ and with tubular lattice was $5.5 \pm 0.3 \mu\text{m}^{-2}$ (Fig. 18).

The thickness of the glial layers and the relationship of the thickness to the average length of glia-glia inter-

face further helps to quantitatively define the structure of the glial layers. The average length of the adaxonal and outer glia-glia interface with and without tubular lattice connections was calculated by incorporating both the barrier thickness and the length density of the glia-glia interfaces. The average length of the adaxonal glia-glia interface with tubular lattice was $3.79\ \mu\text{m}$ and without tubular lattice was $4.32\ \mu\text{m}$. The tubular lattice connection decreases the glia-glia interface length by 12%. Since the calculated volume density of the outer glia was 0.398, the thickness of the outer glia was 41% of the thickness of the calculated $2.4\ \mu\text{m}$, or $1.0\ \mu\text{m}$. This value can be related to the length density by calculating the average length of the glia-glia interface. The average length of the outer glia-glia interface with the tubular lattice was $5.5\ \mu\text{m}$ and without tubular lattice was $5.9\ \mu\text{m}$. The tubular lattice connecting to the outer glia-glia interface decreases the membrane length by 5%.

Figure 1. Untreated crayfish Procambarus clarkii medial giant axon (sheath intact) fixed overnight in 2% glutaraldehyde in veronal acetate buffered normal crayfish solution. The axoplasm, through the glial layers, to the outer sheath is represented in this electron micrograph collage: Schwann cell nuclei (S); tubular lattice (TL); mitochondria (M); periaxonal space (P); collagen matrix (C); perineurial sheath (PS); axoplasm (A). The marker equals 0.5 micrometers.

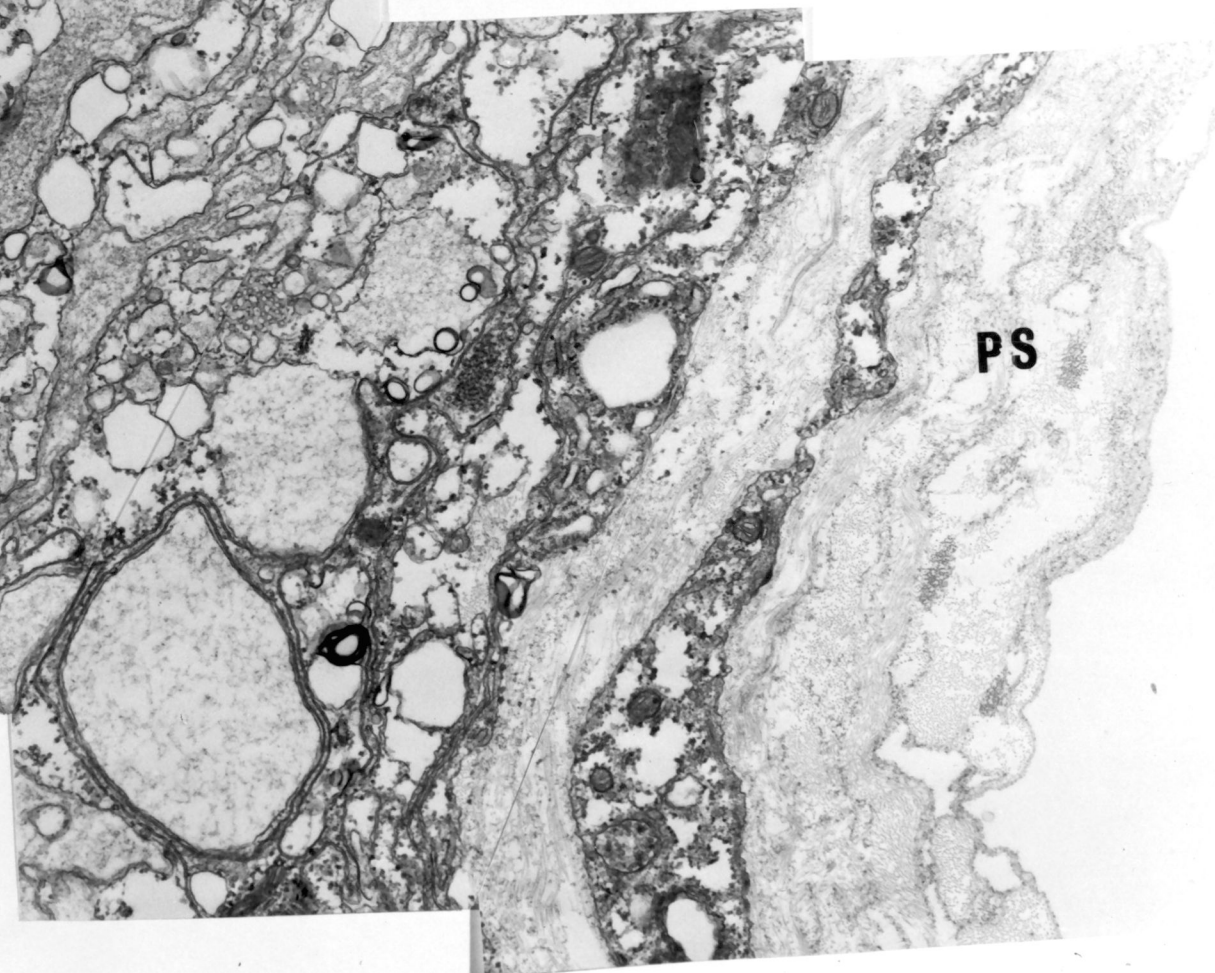
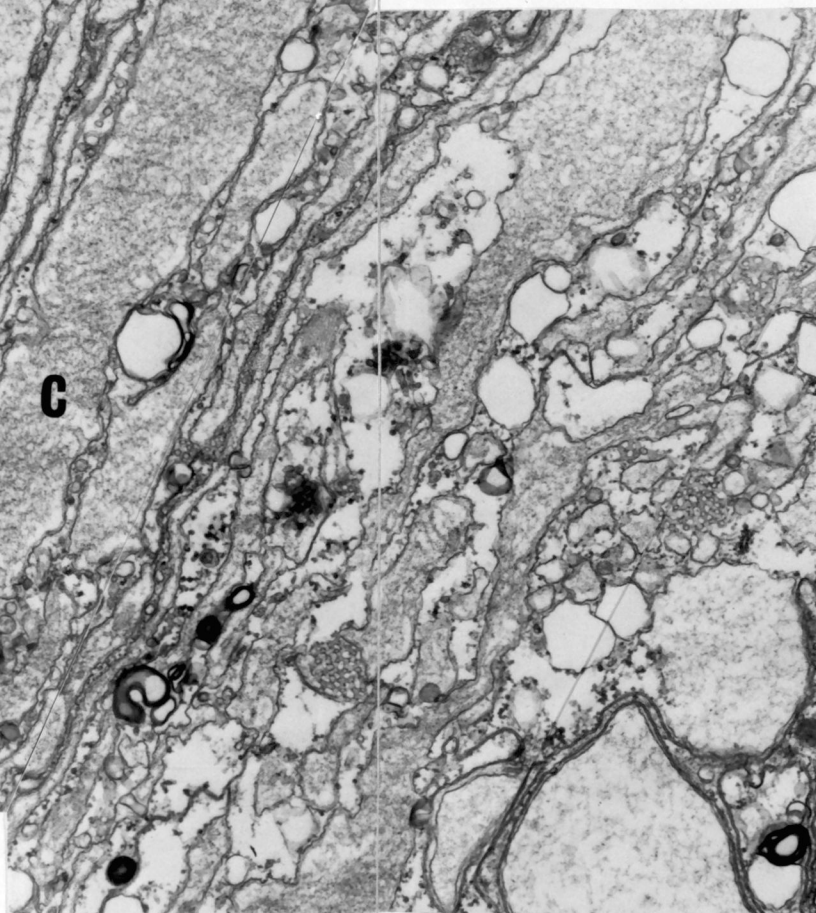
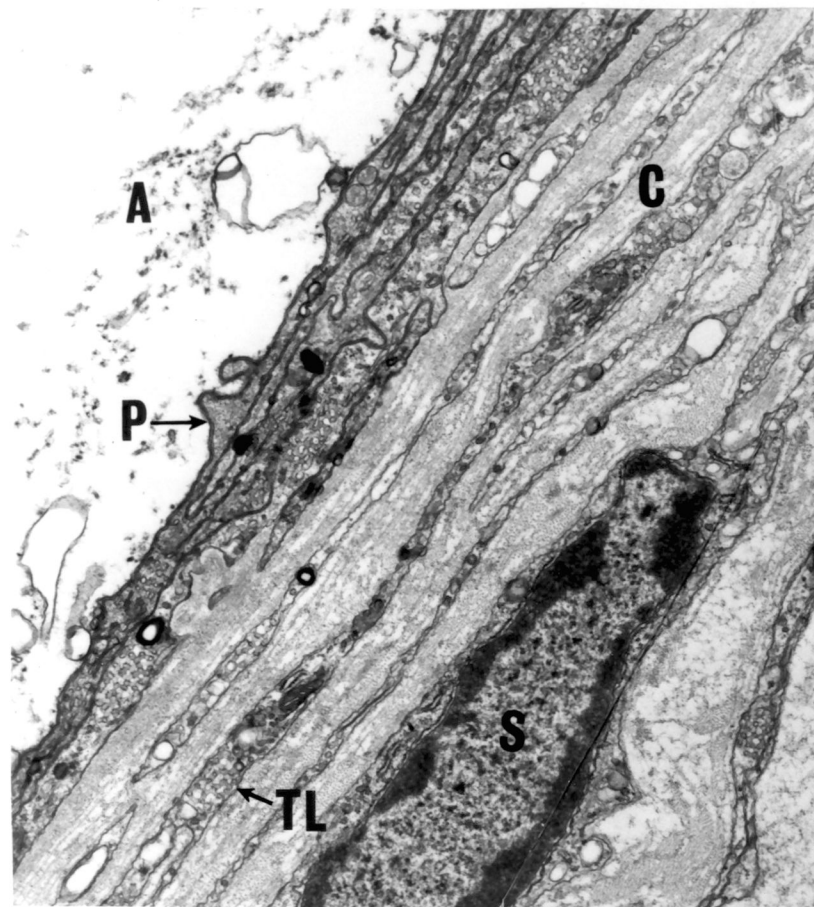


Figure 2. Electron micrograph of the perineurium showing an area of mitochondrial accumulation in the outermost glial layer: axoplasm (A); mitochondria (M). The marker equals 1.0 micrometer.



Figure 3. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for 1/2 hour. A precipitation step was not included: axoplasm (A); periaxonal space (P); tubular lattice (TL); collagen matrix (C). The marker equals 1.0 micrometer.

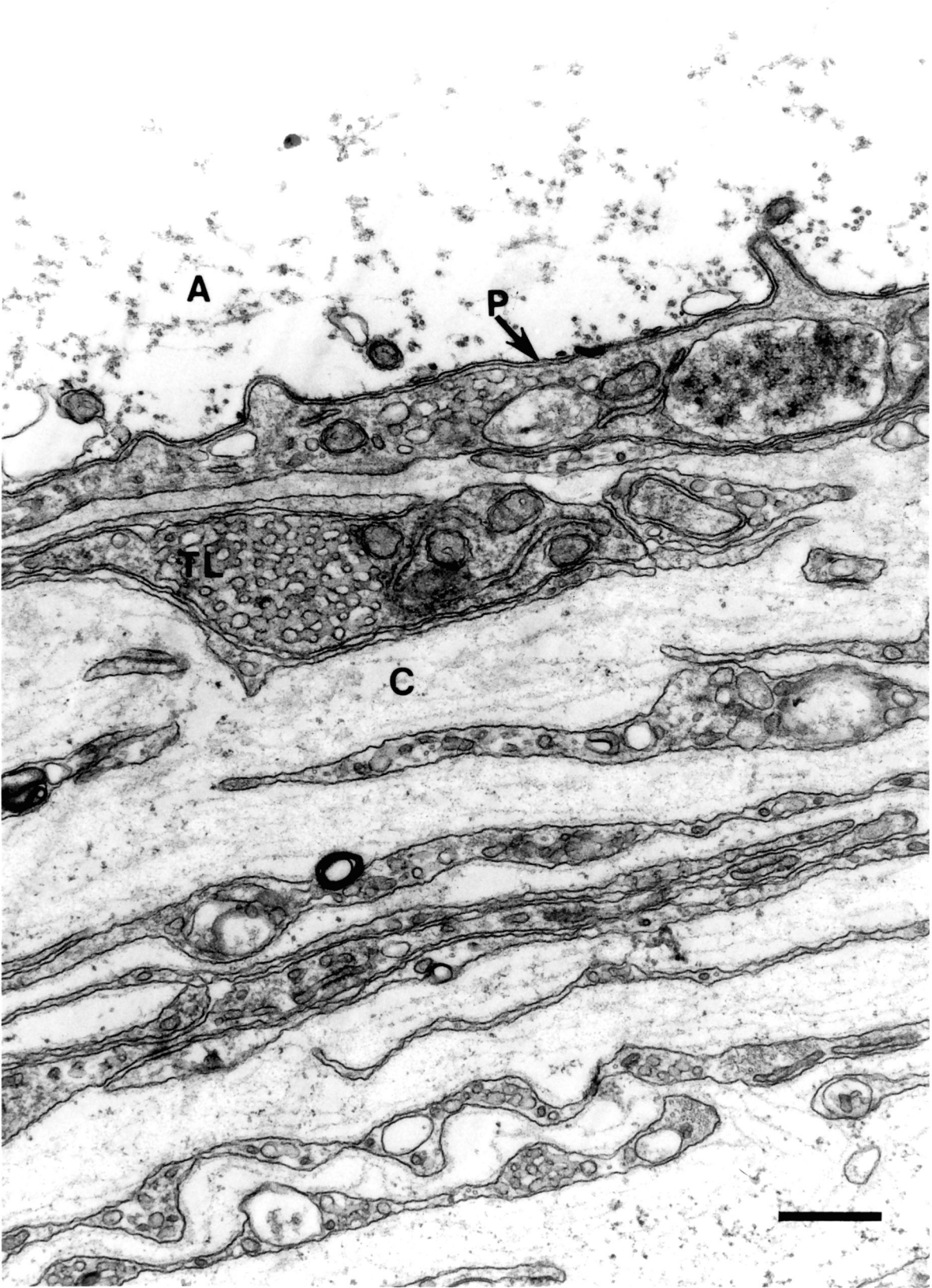


Figure 4. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for 15 minutes. A precipitation step was included and lanthanum precipitation was observed along the periaxonal and inner glial membranes, and within the tubular lattice: axoplasm (A); periaxonal space (P); tubular lattice (TL). The marker equals 1.0 micrometer.

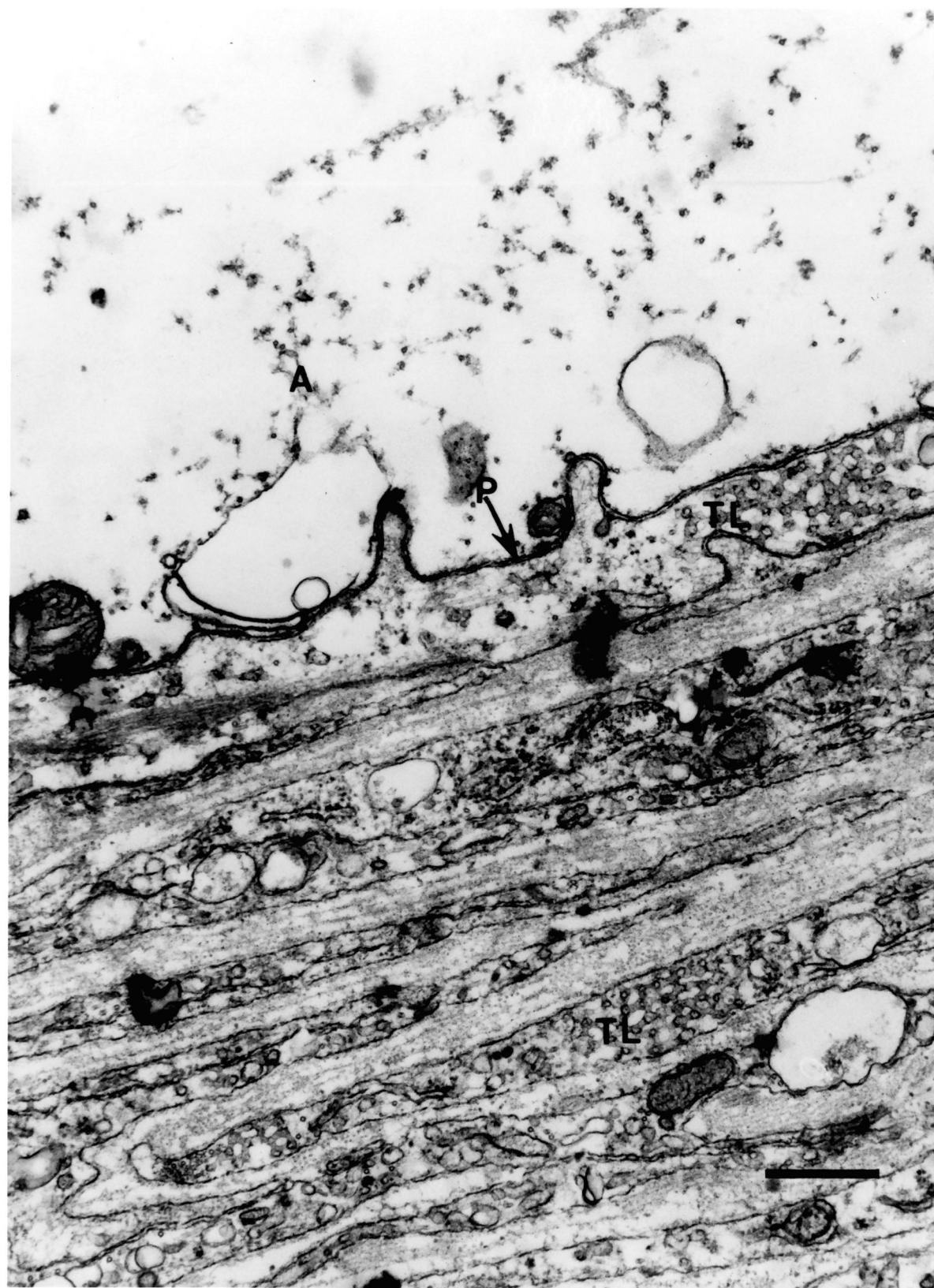


Figure 5. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for 1/2 hour. A precipitation step was included and lanthanum precipitation was observed along the periaxonal and inner glial membranes, and within the tubular lattice. Heavier precipitation was observed along the periaxonal membrane than found in the 15 minute incubation: axoplasm (A); periaxonal space (P); tubular lattice (TL). The marker equals 1.0 micrometer.

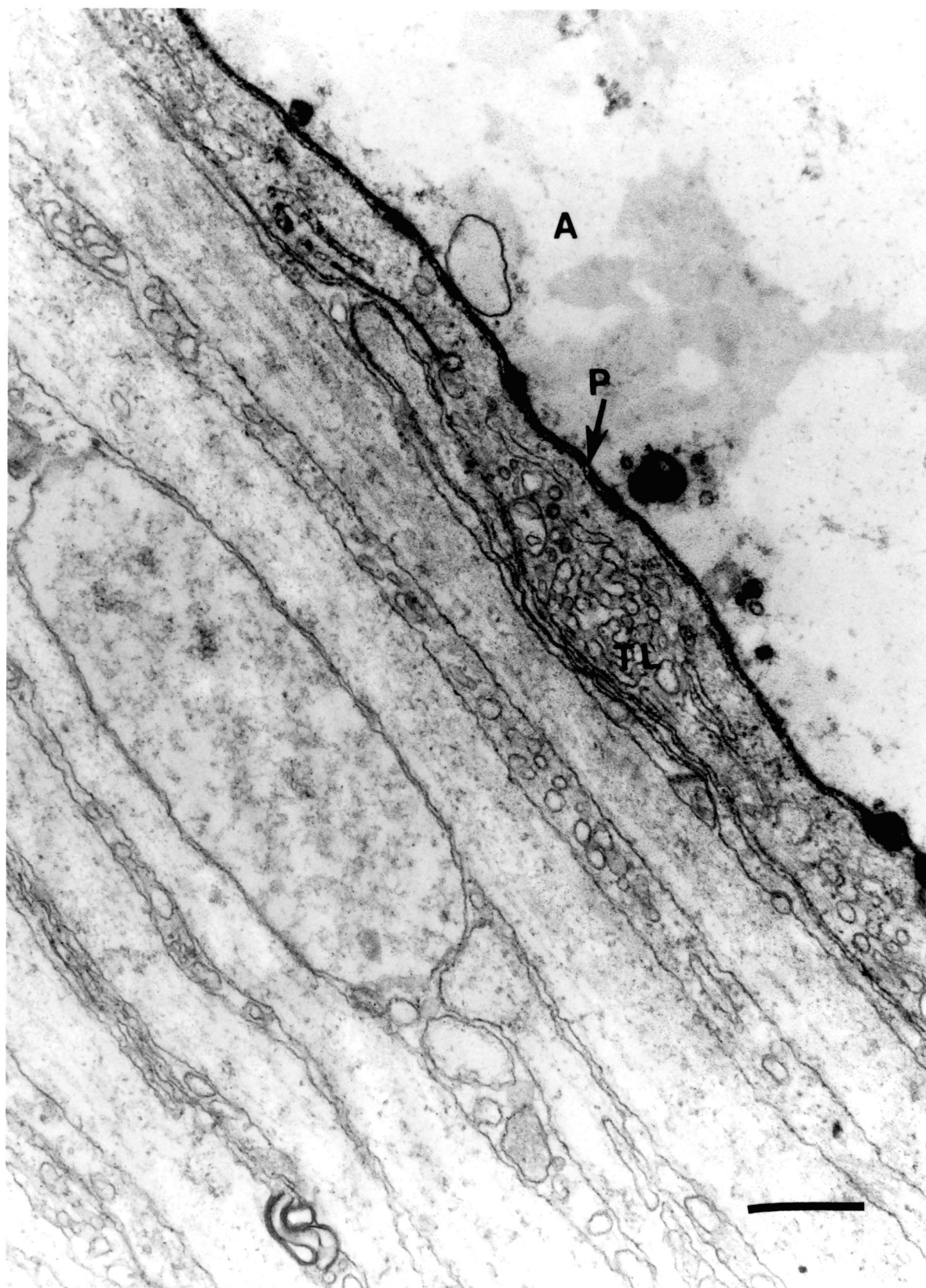


Figure 6. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for one hour. A precipitation step was included and lanthanum precipitation was observed along the periaxonal and inner glial membranes, and within the tubular lattice. Heavier precipitation was observed along the periaxonal membrane than found in shorter incubation periods: axoplasm (A); periaxonal space (P); glia-glia interface (GG). The marker equals 1.0 micrometer.

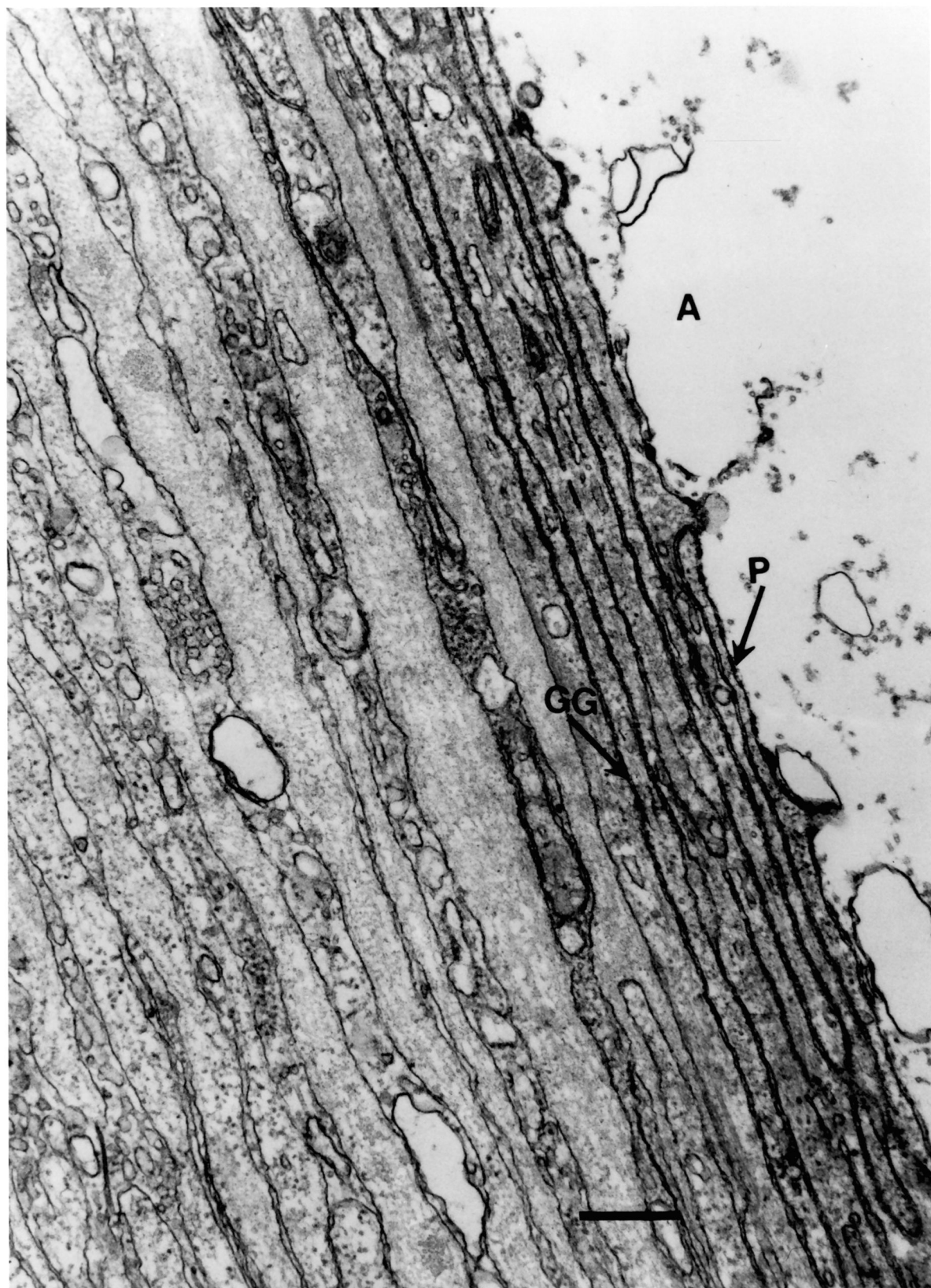


Figure 7. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for 1/2 hour followed by a train of stimuli at 50 Hz for 2 min and lanthanum precipitation. There was disruption of the periaxonal membrane, and increased vacuole formation throughout the tissue: axoplasm (A); adaxonal glial layer (AD); periaxonal space (P); tubular lattice (TL). The marker equals 0.5 micrometer.

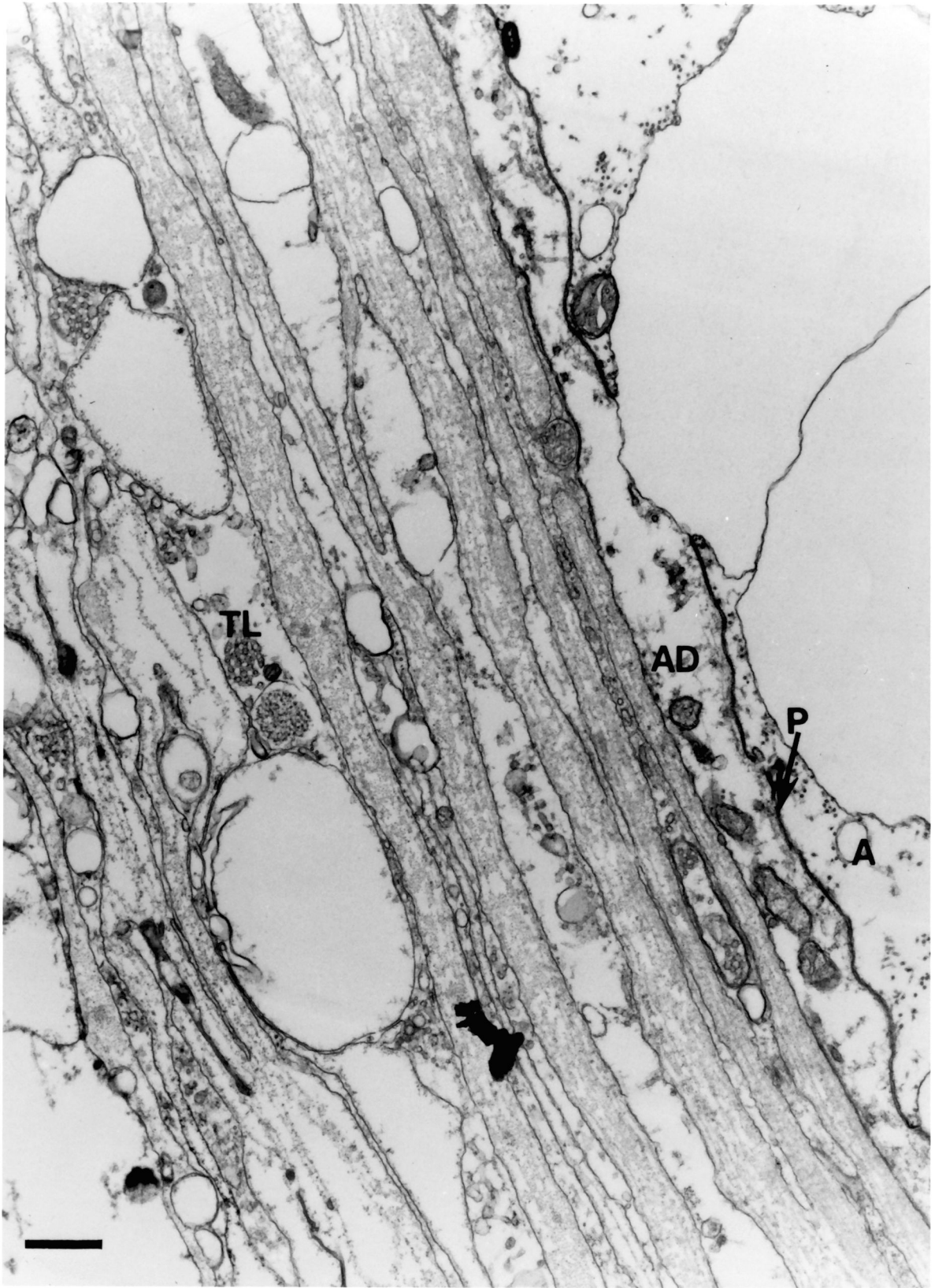


Figure 8. Distribution and binding of La^{3+} in the adaxonal and intermediate layers of glia. The tissue was incubated with LaCl_3 (5 mM) for 1/2 hour followed by +10 mV 100 millisecond depolarization current pulse for 30 seconds and lanthanum precipitation. There was damage to the periaxonal membrane and the adaxonal tubular lattice was completely filled with lanthanum precipitate: axoplasm (A); periaxonal space (P); adaxonal glia (AD); tubular lattice (TL). The marker equals 0.5 micrometer.

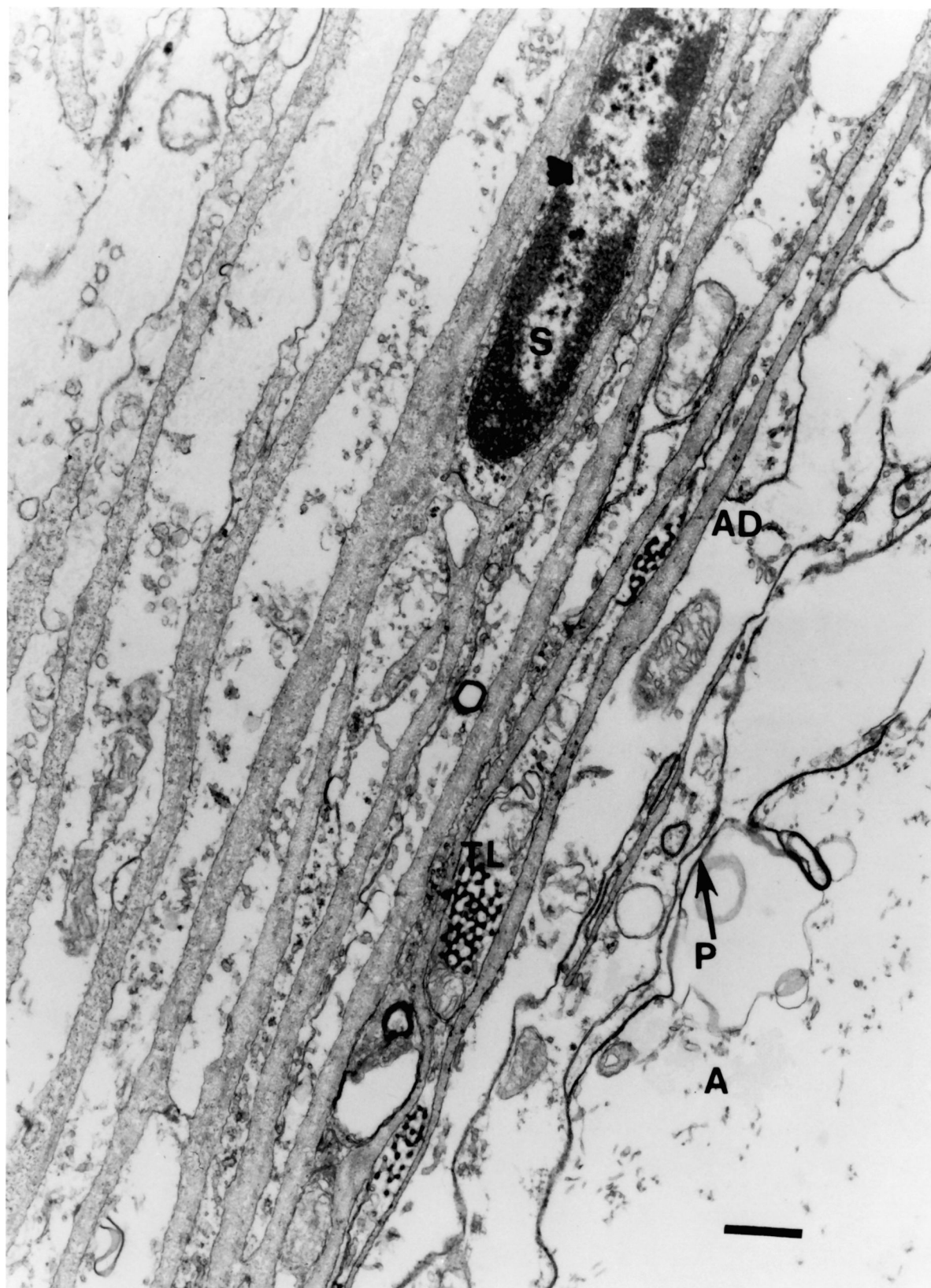


Figure 9. A high magnification electron micrograph of Figure 8 showing the disruption of the periaxonal membrane and tubular lattice filled with lanthanum precipitate: axoplasm (A); periaxonal space (P); adaxonal glia (AD); collagen matrix (C); mitochondria (M); tubular lattice (TL). The marker equals 0.25 micrometer.

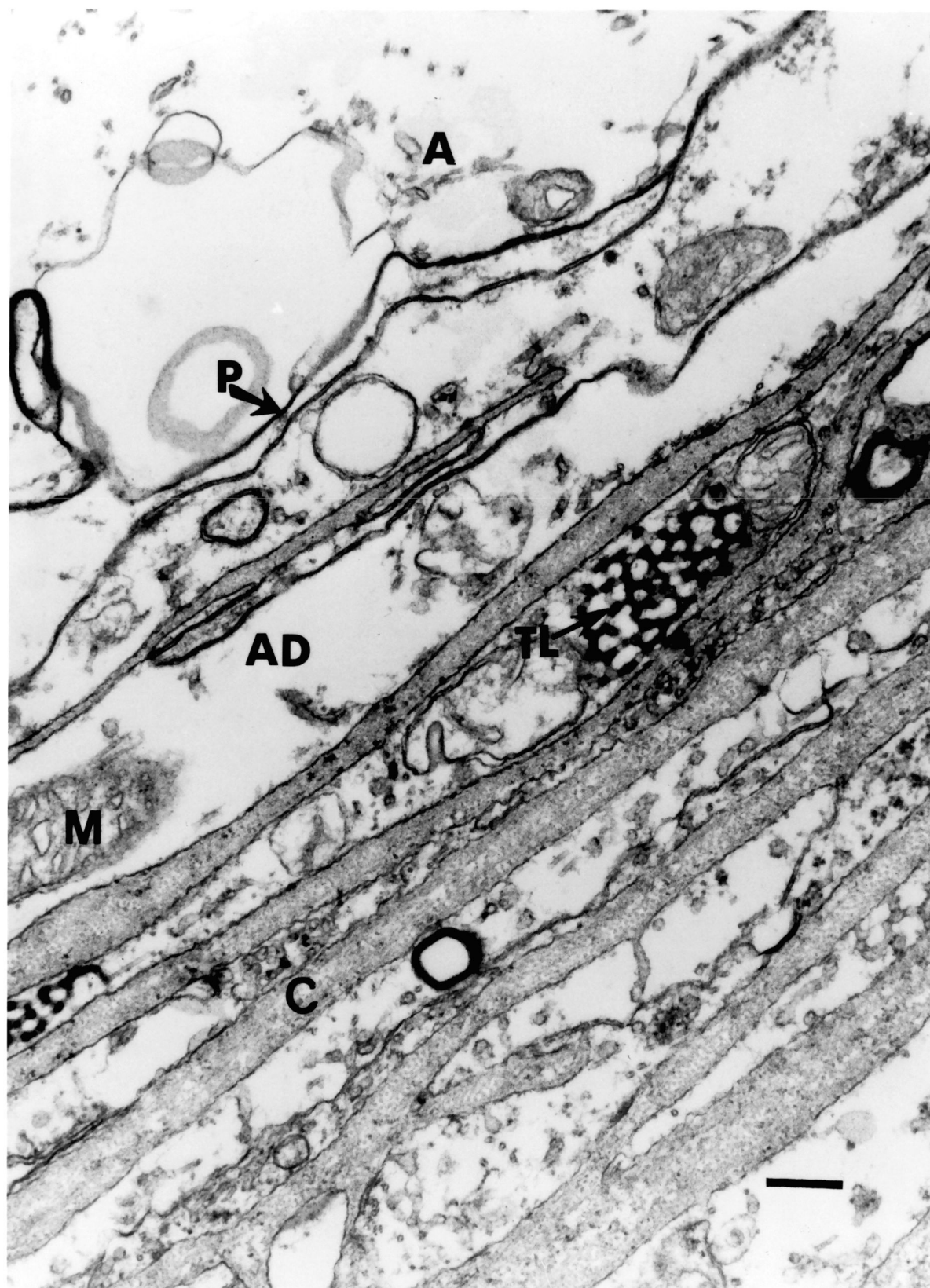


Figure 10. Electron micrograph cross section of the medial giant axon of the crayfish. The parameters measured in this morphometric study are labeled: axoplasm (A); collagen matrix (C); tubular lattice (TL); mitochondria (M); glia-collagen interface (GG); and glia-glia interface (GC). The marker equals 0.5 micrometer.

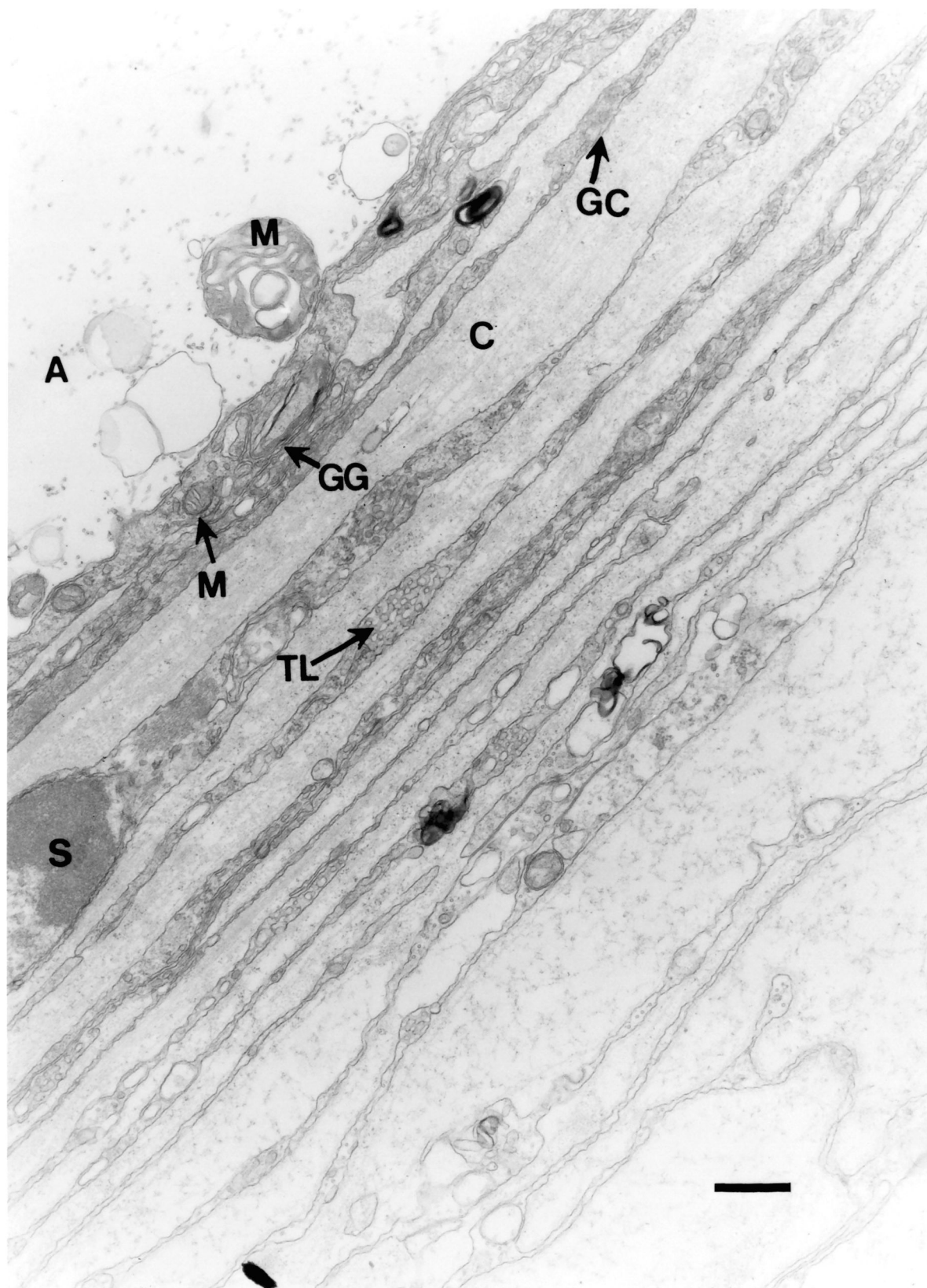


Figure 11 (A). The relationship between the total volume density of the collagen matrix, glia, and intercellular space (n = 34).

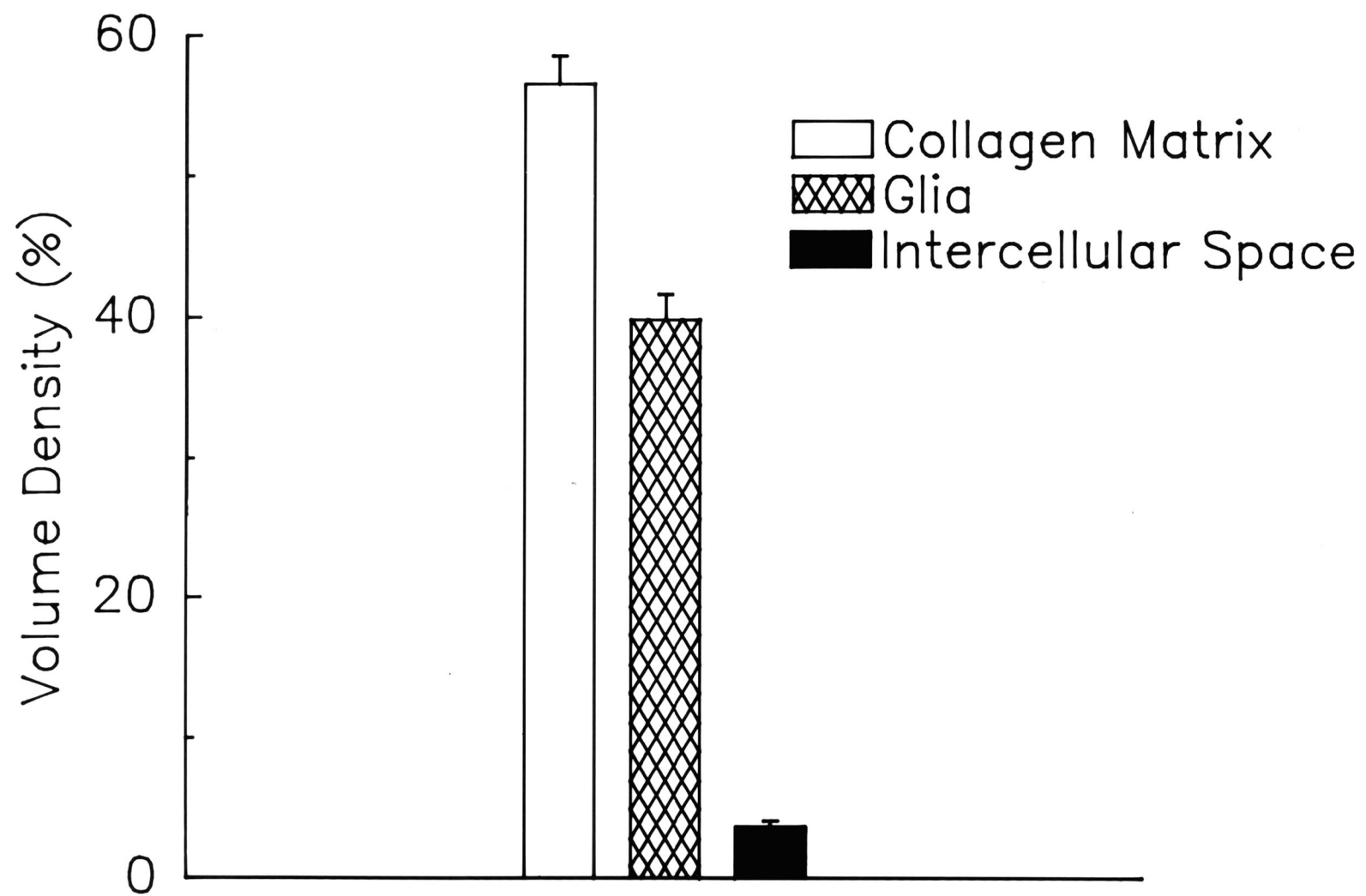


Figure 11 (B). The relationship between the total, adaxonal, and outer glia regions for the volume density of glia, mitochondria, and tubular lattice ($n = 34$).

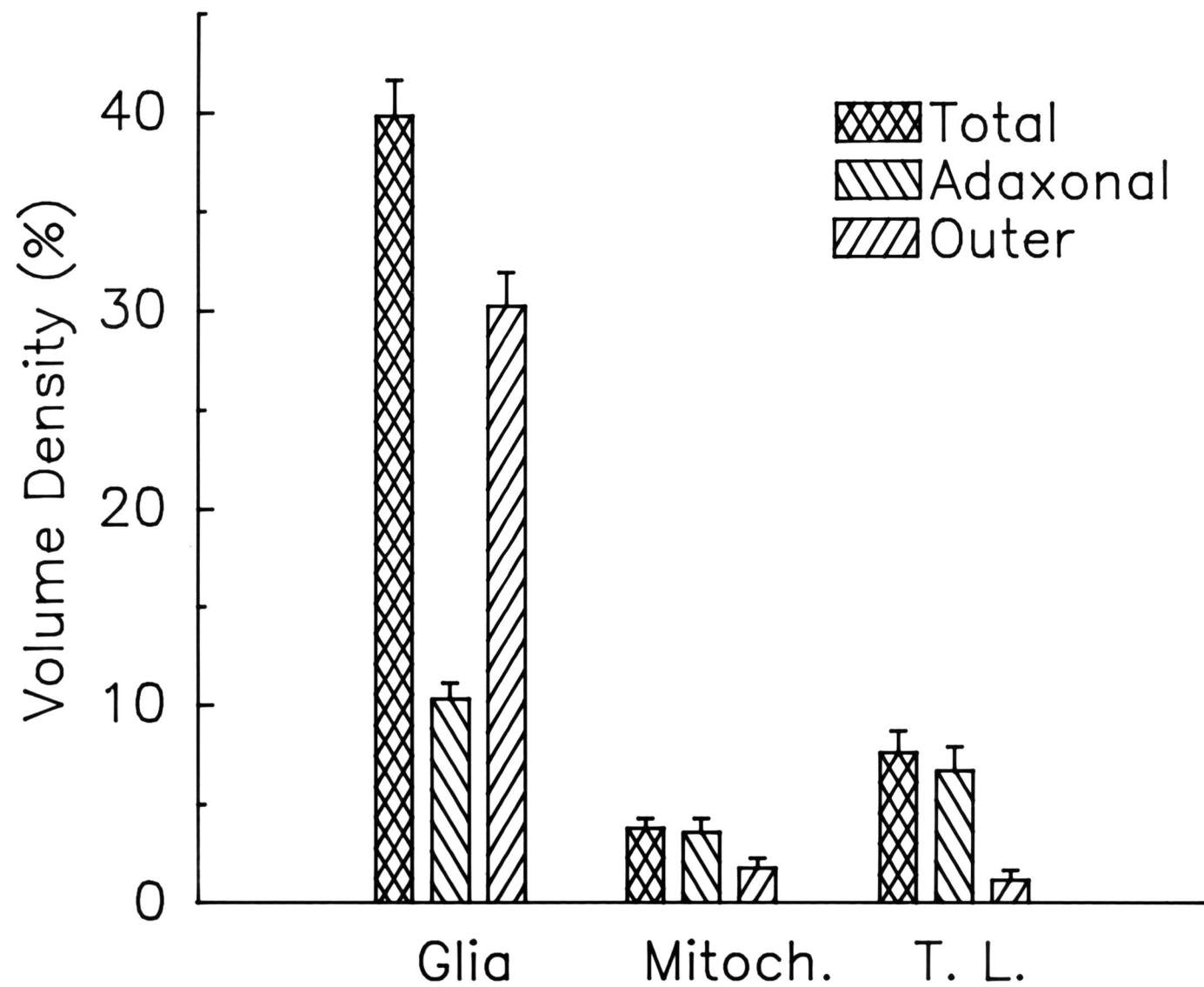


Figure 12. The relationship between the total, adaxonal, outer, and intercellular space mean barrier thickness (n = 34).

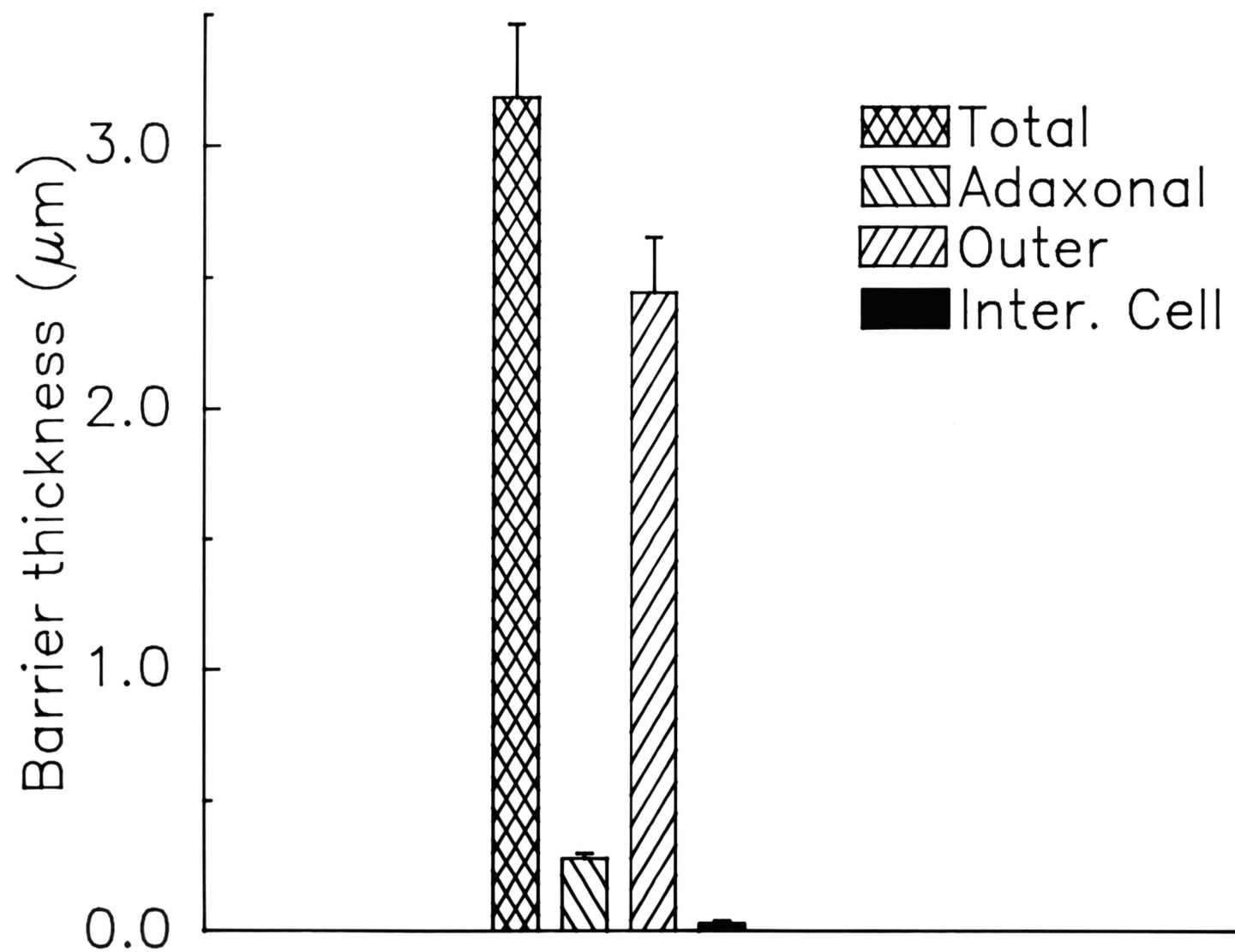


Figure 13. The surface density of glia-glia interface subdivided into the adaxonal and outer layers; and, adaxonal glia membrane facing the axon and facing the collagen matrix.

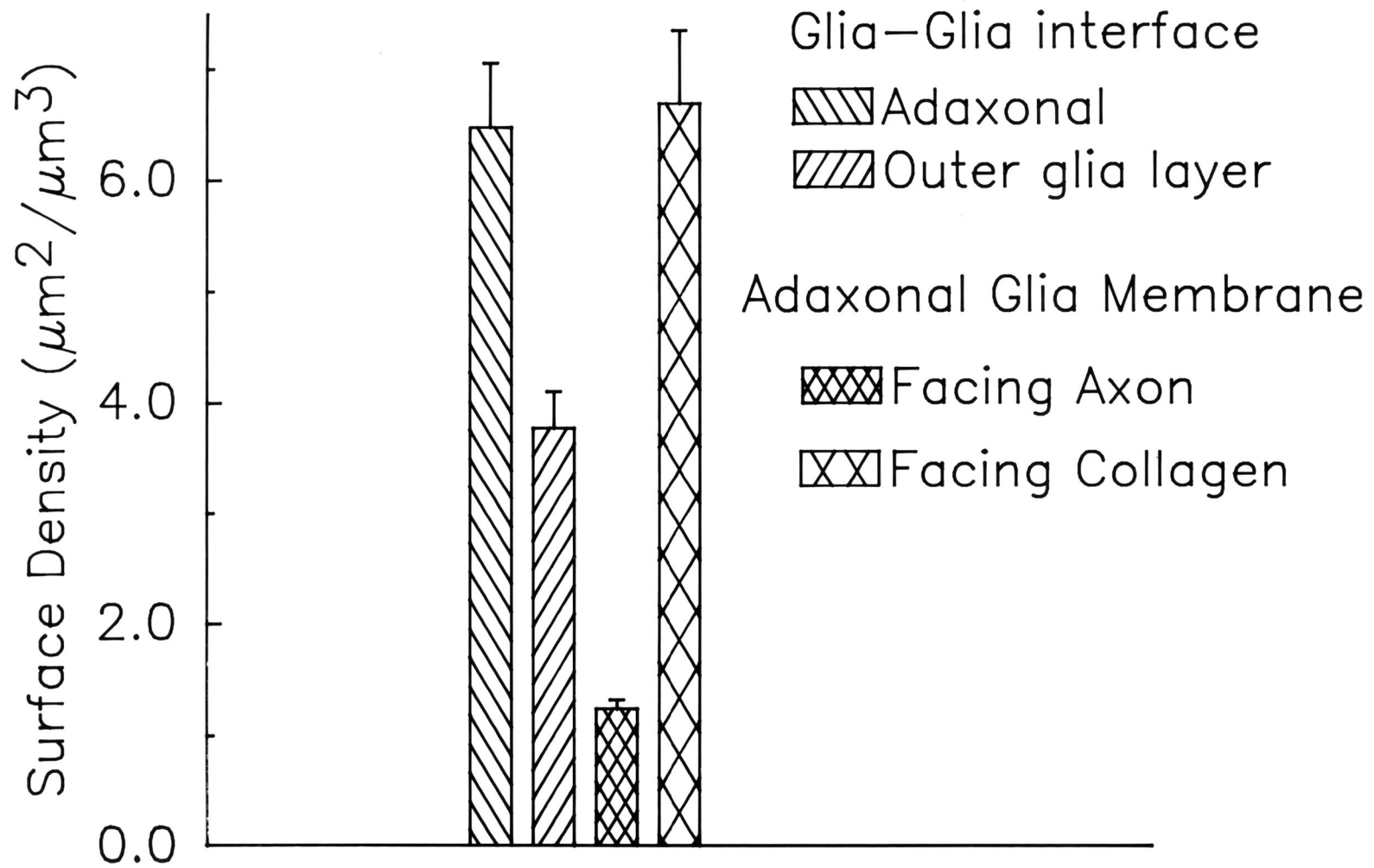


Figure 14. Electron micrograph of tubular lattice patch located in the outer glial layers: tubular lattice (TL); glia-glia interface (GG); glia-collagen interface (GC). The marker equals 0.25 micrometer.

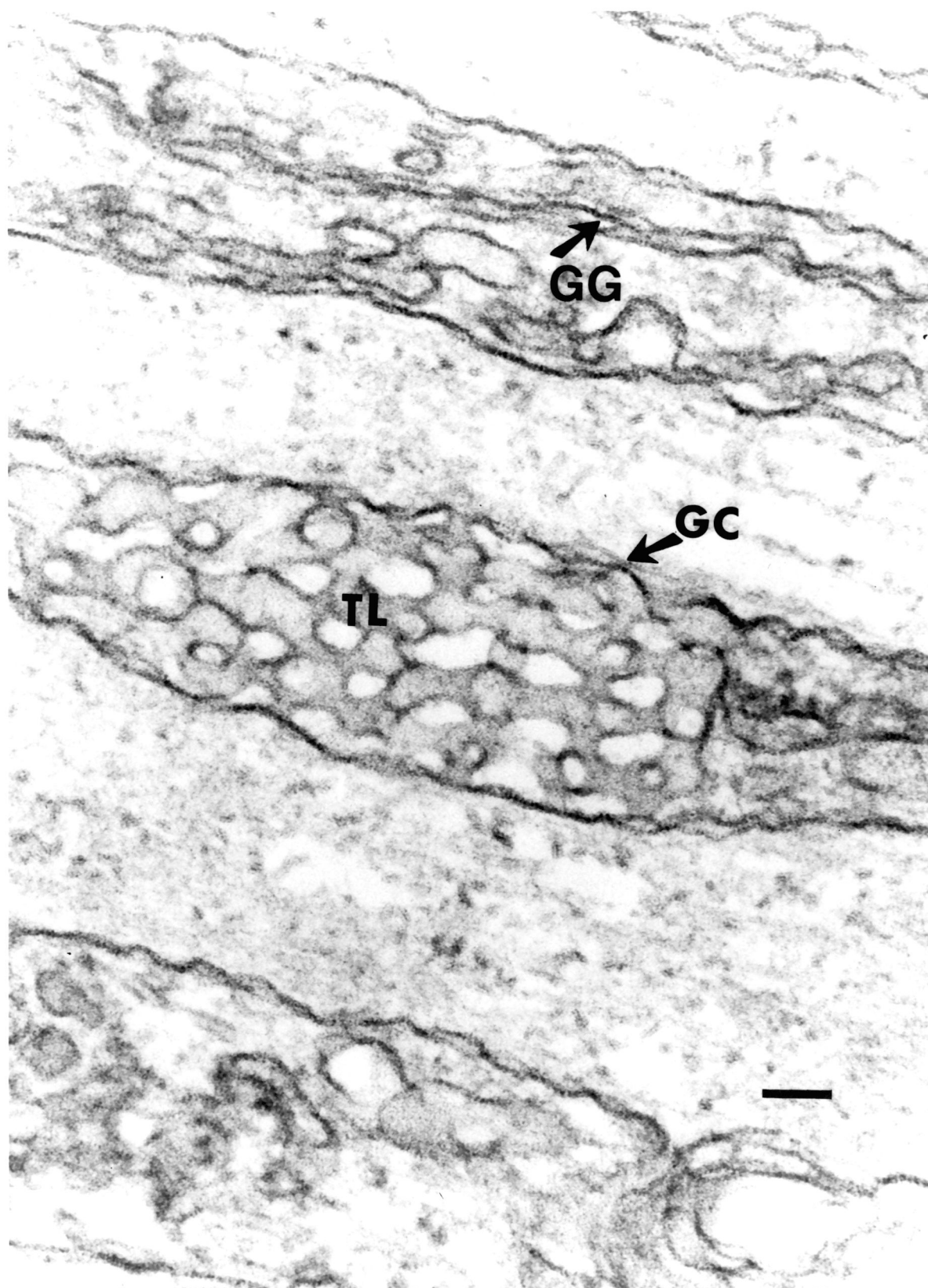


Figure 15. Electron micrograph of the adaxonal glial layer showing the long tortuous glia-glia interface with connections to the tubular lattice patch shown at the bold arrow: axoplasm (A); periaxonal space (AD); tubular lattice (TL); glia-glia interface (GG); mesaxonal opening to the periaxonal space (MA). The marker equals 0.5 micrometer.

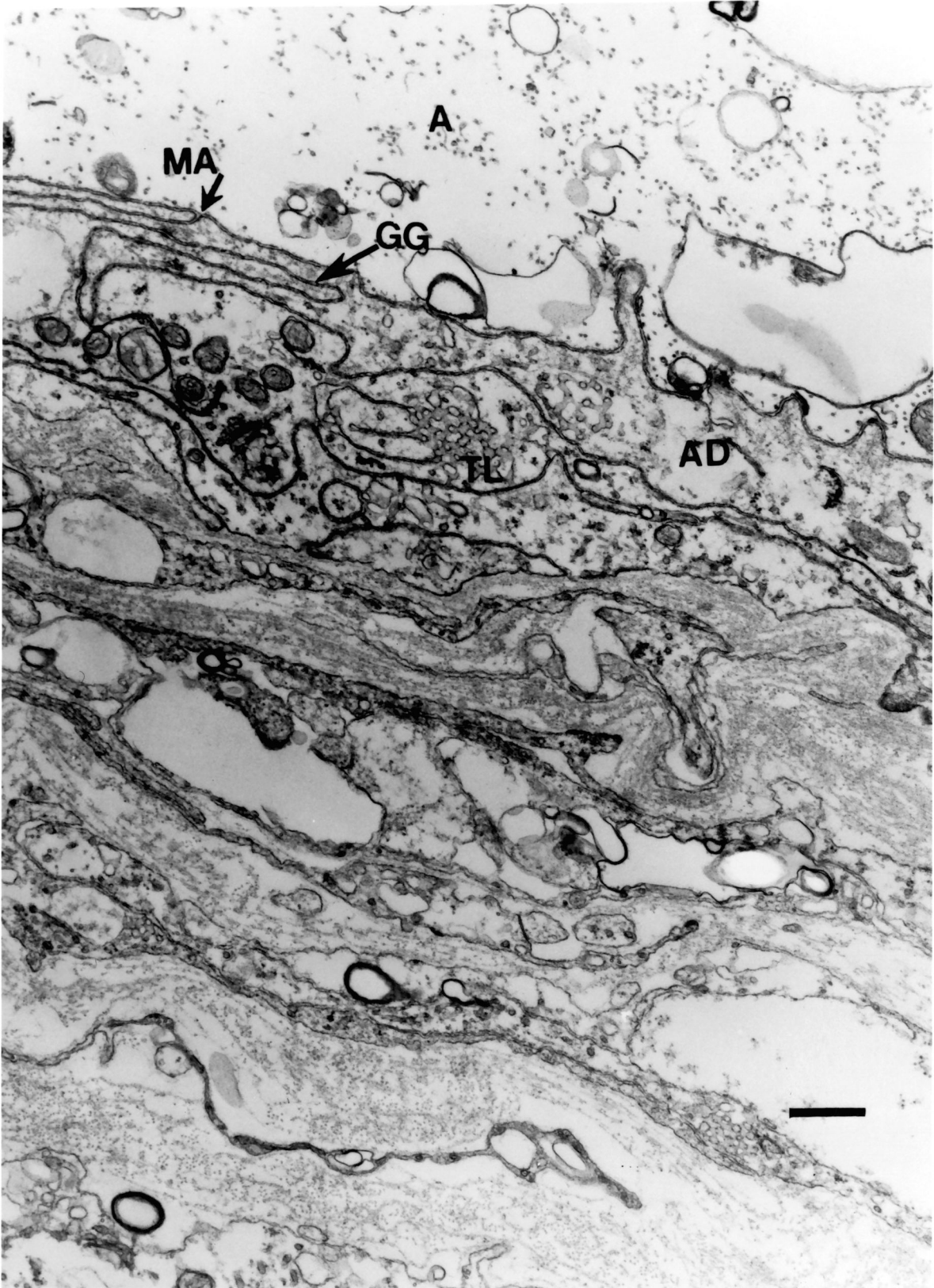


Figure 16. A high magnification electron micrograph of Figure 15 showing long projections from the tubular lattice patch. The opening of the tubular lattice to the glia-glia interface is shown at the bold arrow: tubular lattice (TL); glia-glia interface (GG). The marker equals 0.1 micrometer.

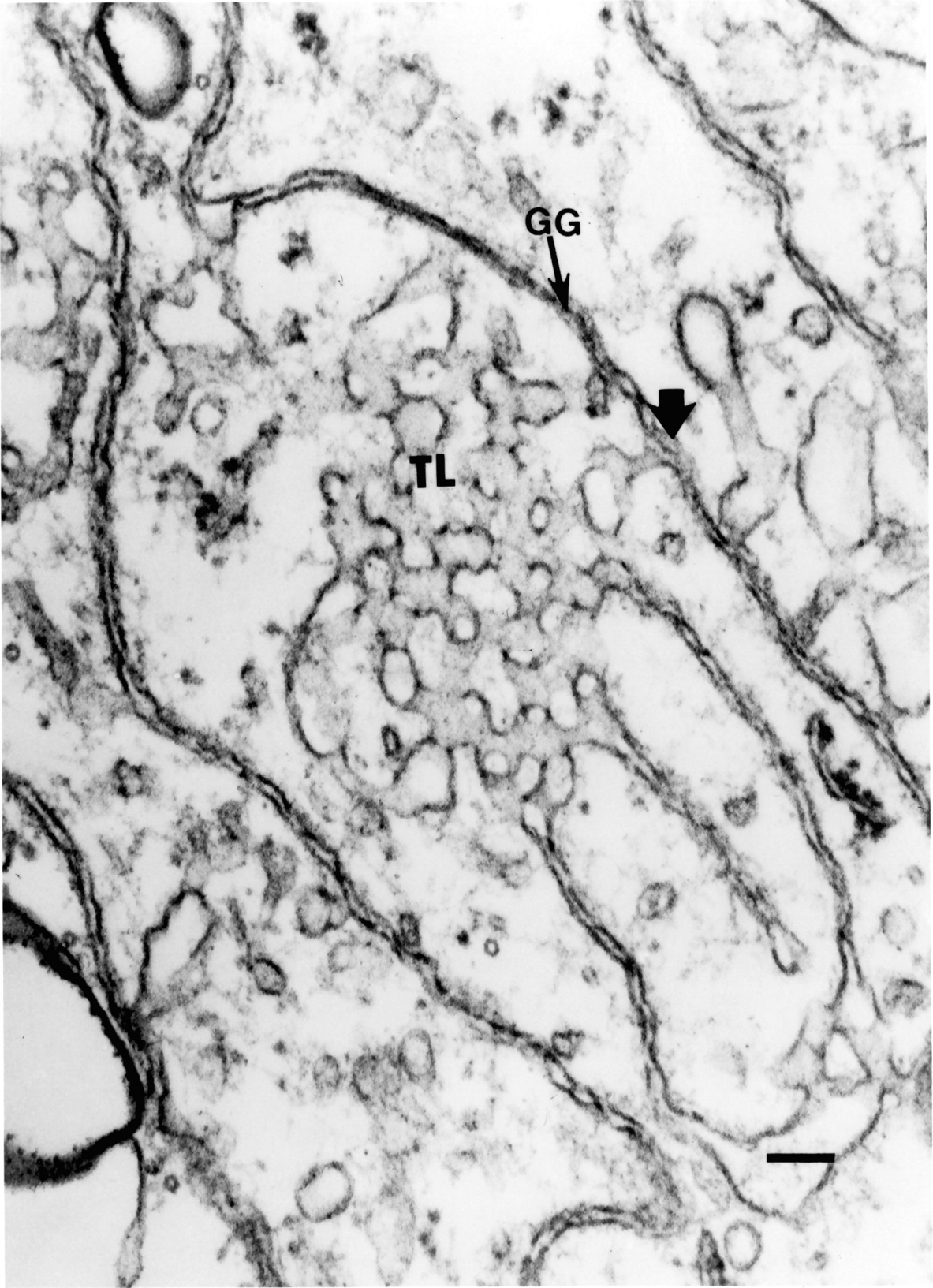


Figure 17. Frequency bar graph of the cross-sectional diameter of the tubular lattice. Data collected from 34 high magnification prints (X45,000).

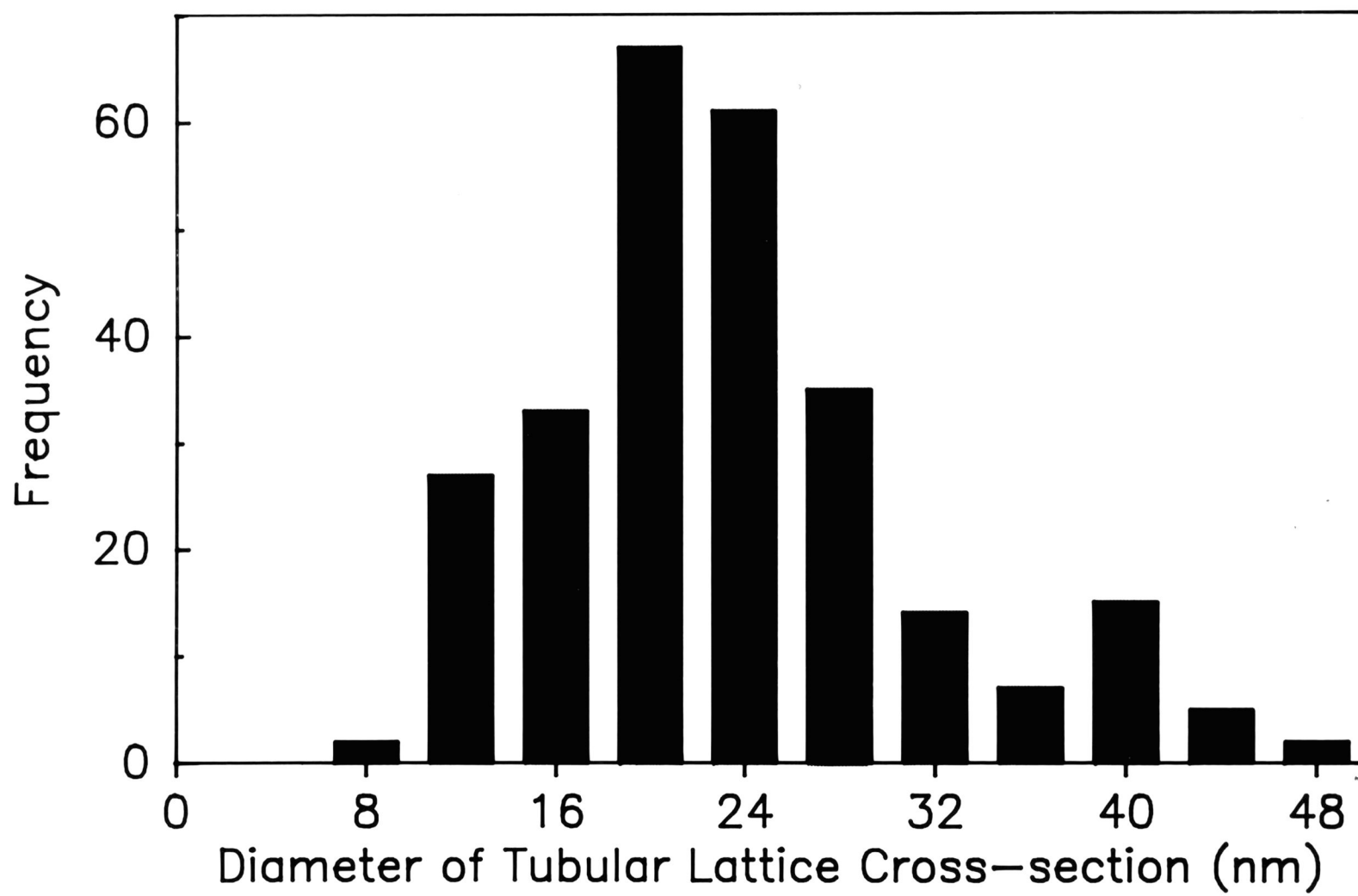


Figure 18. The relationship between the length density of glia-glia interface with and without tubular lattice connections for both the adaxonal and outer glial regions ($n = 34$).

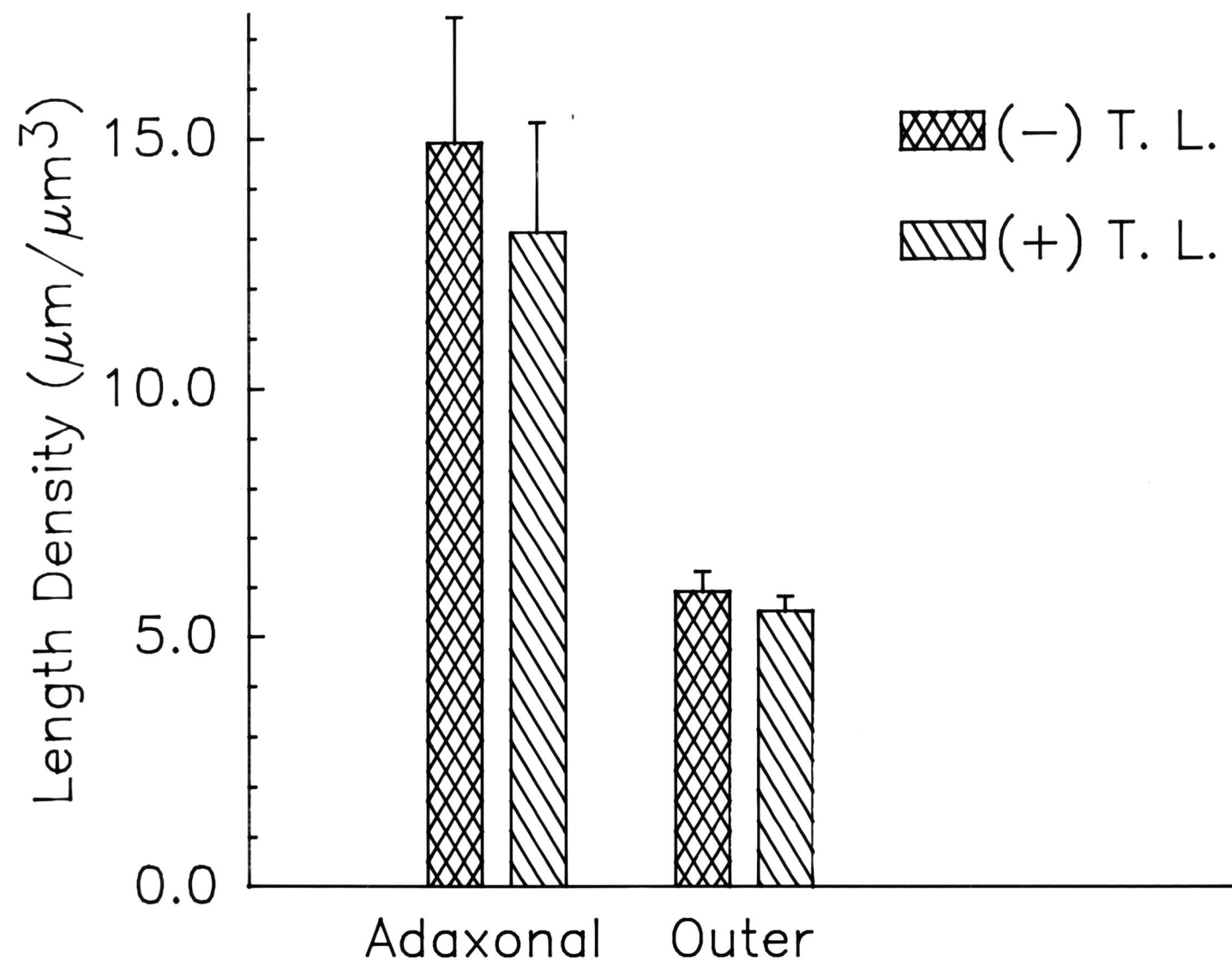


Figure 19. Electron micrograph of adaxonal glial layer. At the arrow is a tubular lattice projection connecting two tubular lattice patch groups. There are also connections of the glia-glia interface with the tubular lattice: glia-glia interface (GG); tubular lattice (TL); Schwann cell nucleus (S); mitochondria (M); periaxonal space (P); axoplasm (A). The marker equals 0.5 micrometer.

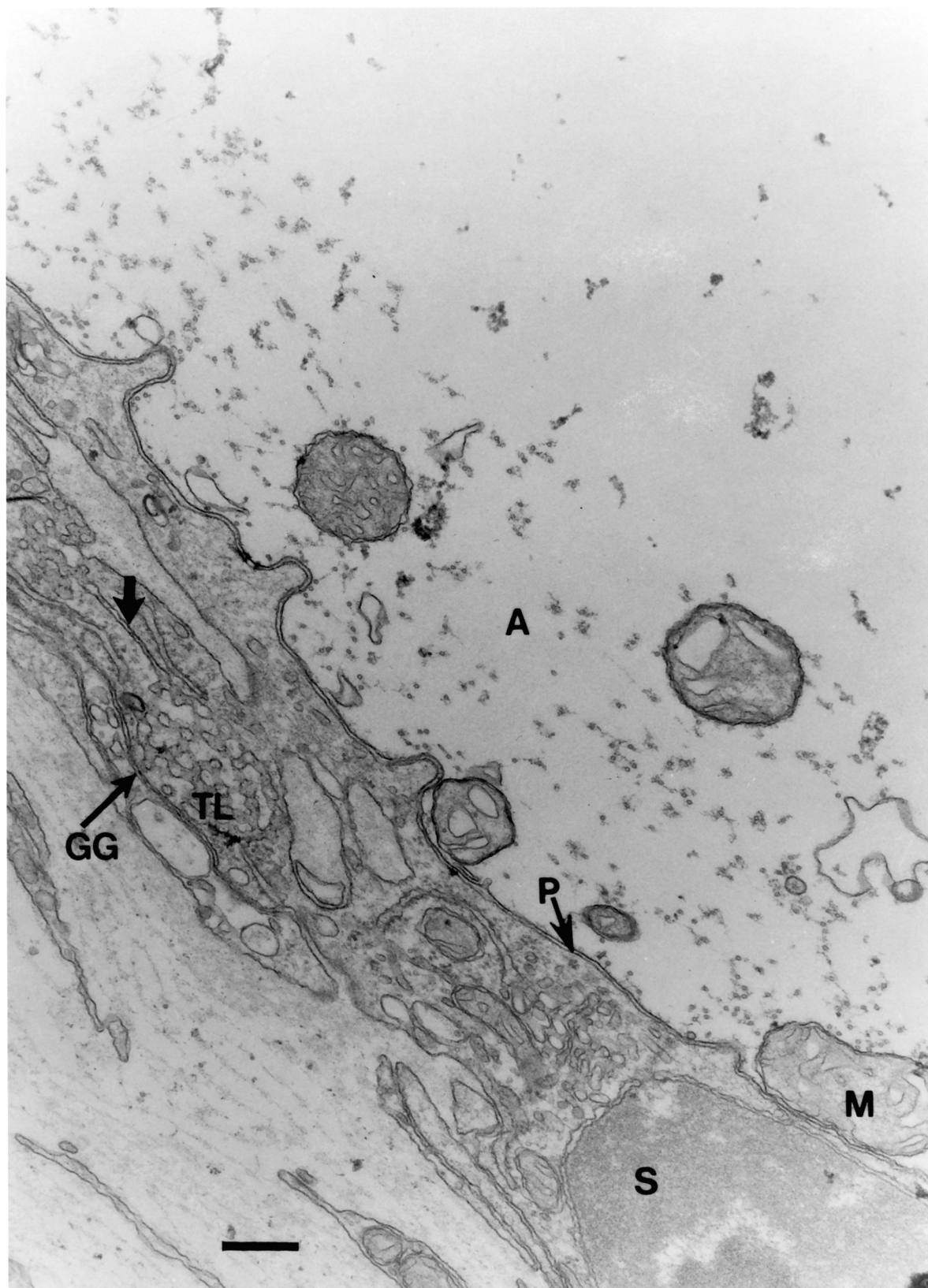


Table 1. Membrane surface density (S_v) related to number of openings (# op) per tubular lattice patch (T.L.)

ADAXONAL GLIA LAYER				
	S_v	# op/ T.L.	# op/ <u>surface</u> <u>area</u>	
Glia membrane facing axon	1.24	0.20	.16	(1)
Glia-glia interface	6.47	4.20	.65	(4)
Glia collagen interface	6.68	3.60	.54	(3)
OUTER GLIA LAYERS				
Glia membrane facing axon	4.90	3.97	.81	(5)
Glia-glia interface	3.80	0.80	.23	(1)
Glia collagen interface	4.90	4.77	.97	(6)

Table 2. Diameter* of tubular lattice patch

	TOTAL (μm)	INNER (μm)	OUTER (μm)
(X) SHORT AXIS	0.23 ± 0.01	0.24 ± 0.02	0.19 ± 0.02
(Y) LONG AXIS	0.56 ± 0.02	0.60 ± 0.04	0.50 ± 0.06
DIAMETER	0.36 ± 0.02	0.38 ± 0.03	0.31 ± 0.04

* diameter calculated: $(X Y)^{1/2}$

DISCUSSION

The purpose of this study was to investigate the functional morphology of the medial giant axon of the crayfish and its associated glia layers. The primary emphasis was the acquisition of morphometric data of structures that may be important in the ionic homeostasis of the crayfish giant axon. In order to maintain normal nerve function it is necessary to clear excess potassium from the periaxonal space that tends to accumulate during nerve excitation. Four major possible mechanisms exist for the clearance of potassium:

1. Potassium moves back into the axon from the periaxonal space by the action of the sodium-potassium pump which is thought to be a slow process.
2. Potassium diffuses through the mesaxonal intercellular pathways to the intermediate glial extracellular space.
3. Potassium clears through the adaxonal glial cell cytoplasm by spatial buffering to the intermediate glial extracellular space.
4. Finally, potassium clearance through the glial tubular lattice to the intermediate glial extracellular space.

Frankenhaeuser and Hodgkin, in 1956, first described the properties of accumulation of potassium within the periaxonal space of the squid during periods of action potential generation. They found that the build-up of potas-

sium in the periaxonal space is reduced within about 50 ms following cessation of excitation. Orkand, in 1966, studied the role of central glia of the leech and amphibia in potassium clearance. They proposed a mechanism given the name "spatial buffering" whereby potassium is removed from areas of its high concentration to areas of low concentration driven by a voltage gradient across the glial layer caused by the potassium gradient. Although the actual mechanism of potassium clearance is unknown, it has been speculated by Shrager, et al. (1983) that the tubular lattice may provide a pathway which allows rapid diffusion of excess ions from the axon surface. Shrager, et al. (1983) found that potassium clearance from the crayfish periaxonal space occurred with a time constant of 2 ms. Pichon, et al. (1988) have shown there is no difference between the squid and crayfish time constant which differs from the previous data by Frankenhaeuser and Hodgkin (1956). Although it has been suggested that the tubular lattice and glia may be involved in potassium homeostasis, there have been no physiological experiments designed to study the role of the tubular lattice in neuron-glial interactions in crayfish.

Determining the optimal fixation and postfixation method for the crayfish nerve cord proved to be a challenge since both structure and function had to be preserved until the last measurement of the experiment. Overnight fixation in 2% glutaraldehyde in CS buffered with veronal acetate at

pH 7.4, proved to be optimal for preservation of the tissue structure. Veronal acetate or sodium barbital-acetate buffer, at pH 7.4, has been reported to have no effect on the fixation properties of glutaraldehyde since the imido groups of veronal acetate do not react with the aldehyde groups of glutaraldehyde (Sabatini and DiPollo, 1968). In addition veronal acetate had no effect on the action potential or resting membrane potential of the crayfish axon. In the postfixation process, care was taken to maintain a physiologic osmolality and pH of solutions, including the graded dehydration of the tissue with ethanol. The only deviation from the physiologic pH was during en bloc staining with uranyl acetate in maleic acid at pH 5.2 and the pre- and post- rinses with maleic acid, at pH 5.2.

Once the nerve cord of the crayfish was fixed, the tissue was cut into thin sections and examined by electron microscopy. The electron micrographs of the control preparation were found to be comparable in quality to those already found in the literature (Lieberman et al., 1981; Shrager et al., 1983).

Preliminary observations of the untreated axon glial preparation suggested the presence of two structurally different glia around the giant axon. There were differences in osmium staining distribution of glia and glial cellular components including the glial mitochondria and tubular lattice, between the inner (adaxonal) and outer glia. The

glial layers in general are composed of three major components: collagen matrix, glial cells, and the intercellular space. The glial cells form 40% of the total volume surrounding the axon, excluding the perineurium. Since the thickness of the glial investment is approximately $3\text{ }\mu\text{m}$ with eleven glial layers and 20 glial cells the volume of one glial cell may be calculated. Using a 1 cm^2 reference for the axon surface the total volume is $3 \times 10^{-4}\text{ cm}^3$, the volume of total glia is $.40 \times 0.3\text{ }\mu\text{l}$ or $0.12\text{ }\mu\text{l}$. There are approximately 20 glial cells, hence, one glial cell is approximately $0.006\text{ }\mu\text{l}$ volume.

The thickness of the adaxonal glial layer was calculated as $0.29\text{ }\mu\text{m}$ which agrees with the estimate reported by Shrager, et al. (1983) of $0.2 - 1.0\text{ }\mu\text{m}$. The adaxonal layer generally is comprised of several glial cells tightly interdigitated (Fig. 19) as opposed to the outer layer which is usually only one glia cell thick. There is only a small amount of collagen matrix in the adaxonal layer further promoting the close contact between adjacent glia cells. The distance between adjacent glia cells (intercellular space) was calculated for the total tissue and not subdivided into adaxonal and outer regions. However, by observation the thickness appeared uniform throughout the tissue and was calculated as $0.029\text{ }\mu\text{m}$. To further support the observation that the adaxonal glial cells are tightly aligned, the surface density of glia membrane facing each other was

greater in the adaxonal layer.

The surface density of the glia-glia interface provides information about the potential cell to cell interaction between glial cells. The intercellular space may be one of several alternative pathways for potassium efflux from the periaxonal space. The surface density of adaxonal glia membranes forming the intercellular space was twice that found in the outer layer. Since these membranes may participate in interglial communication, it was expected that the adaxonal glial layer would have a greater proportion of glia surface facing each other than facing the extracellular matrix.

The ratio of the surface density in the adaxonal and outer layer for the glia-glia interface and the same ratio for the length density of this pathway were the same within the standard error of the mean, giving a value of approximately two. Since the surface and length densities are related, this gives the data an added measure of validity.

When the adaxonal glia membrane facing the axon and facing the collagen matrix was compared it was interesting to find that with only a $0.6\text{ }\mu\text{m}$ change in the axon-glial diameter there was a 5 X greater increase in the surface density of these membranes. From the increase of diameter one would expect only a 1.003 time change in surface density. The increase in surface density probably occurs because of infolding of the membrane facing the collagen matrix.

However, the adaxonal glia membrane facing the collagen matrix has approximately the same surface density compared to any of the outer glia-collagen interface. If the assumption is made that the channel and pump density of the membranes at the glia-collagen interface are the same for the adaxonal membrane facing the axon, it may be suggested that the net flux of potassium is increased with the surface. This structural observation along with tubular lattice openings to the boundary surface supports the idea that periaxonal potassium clearance is facilitated to the bulk collagen matrix.

At the cellular level, the glia in the adaxonal and outer layer were also found to differ. Two structures were studied: the glial mitochondria and the tubular lattice. It was found that there was a greater volume density of glial mitochondria in the adaxonal glial layer. The greater volume of mitochondria may signal more energy utilization, supporting ion transport mechanisms across the axonal membrane. If the adaxonal glia are actively involved in ionic homeostasis, glia may require more energy to maintain the ionic milieu surrounding the axon and glia.

It was also found that there is a greater volume density and numerical density of tubular lattice in the adaxonal layer, suggesting that this region might be involved in the maintenance of ionic homeostasis (Shrager et al., 1983). The tightly arranged glial cells found in the adaxonal layer and

the abundance of tubular lattice may provide a transcellular pathway for the rapid removal of excess potassium from the periaxonal space through or into the glial cells.

A model was constructed by relating the surface density to the number of tubular lattice openings to glia membrane facing toward the axon, the glia membrane facing toward the collagen matrix, and the glia-glia interface. It was found that for every one tubular lattice opening facing the axon, there were four openings facing toward adjacent cells and three facing the collagen matrix in the adaxonal layer. There was no difference in the number of tubular lattice openings to the periaxonal space compared to the number of mesaxonal openings to the periaxonal space. It appears that there is equal access to the tubular lattice and mesaxonal paths from the periaxonal space, but the structure of these openings are different. The tubular lattice is a network of tubules, most likely cylindrical in structure, whereas, the mesaxonal opening represents a cleft between two glial cells. In the adaxonal layer the increased number of tubular lattice openings to the intercellular space and collagen matrix may suggest a structural element facilitating removal of excess potassium from the periaxonal space to adjacent glia and to the collagen matrix in a "diverging" path, or analogous to an "inverted funnel". Removal of potassium from the periaxonal space may take several routes, i.e., potassium leaving via the mesaxonal path may enter the adaxonal

glia, enter the tubular lattice openings, or continue to the bulk collagen matrix. Potassium directly entering the tubular lattice at the periaxonal space may move into the corresponding glia cytoplasm, out of the tubular lattice into adjacent glia via the intercellular space or into the bulk collagen matrix.

The volume density of the tubular lattice in the adaxonal and outer glial layers was compared. The adaxonal glia contain approximately 6 X more volume of tubular lattice than found in the outer glia. In the outer layers the structure differs in that the tubular lattice tends to open to the glia-collagen interface toward the collagen matrix rather than toward adjacent glia cells. This would suggest that potassium clearance in the outer layers may be via the collagen matrix rather than into adjacent glia. In the outer layer, the tubular lattice openings to the glia-collagen interface are approximately equal whether facing toward the axon or toward the outer collagen matrix. There no longer exists the same structural elements for a diverging path of potassium clearance as seen in the adaxonal layer.

Shrager, et al. (1983) suggested that the time constant was 2 ms for excess potassium removal from the crayfish periaxonal space. Although it was previously reported that the squid time constant was 50 ms (Frankenhaeuser and Hodgkin, 1956), recently Pichon, et al. (1988) found the time constant for the squid to be 10 ms, only 5 times slower than

the time constant found in crayfish. However, Zwahlen, et al. (1988) noted that in squid, as the mesaxonal path opens to the periaxonal space a punctate density or tight junction exists which may account for the increase time constant in squid compared to the crayfish. A survey of the mesaxonal openings to the periaxonal space in this study of the crayfish showed no tight junctions. However, it must be noted that micrograph magnification may be a limitation.

Lanthanum chloride distribution, separately and with both pharmacologic and electrophysiological methods, was studied. Lanthanum is an electron dense ionic diffusion space marker and is unique in that it can be detected by electrophysiological means as well as by electron microscopy. The trivalent cation, lanthanum, has been found to stain intercellular membranes by binding to anionic sites on the membrane and has the ability to displace monovalent and divalent cations such as sodium, potassium, and calcium from bound sites due to its trivalent nature. Lanthanum is a potassium channel blocker which prevents inward potassium steady state rectification evidenced by a 2.5 fold increase in the action potential duration. Lanthanum was not found when 5 mM LaCl_3 was superfused without subsequent precipitation. The physiologic effects of La^{3+} could be seen on the action potential duration suggesting that La^{3+} was present at the axon membrane but following fixation and electron microscopic examination there was no evident lanthanum

staining. This may have been due to removal of lanthanum from low affinity binding sites when the tissue was superfused with glutaraldehyde. Once glutaraldehyde was superfused, lanthanum may have remained bound to high affinity binding sites, however, if the high affinity binding sites were few it would have been difficult to visualize by electron microscopy. La^{3+} precipitation overcame this problem.

Interesting results were obtained when high frequency stimulation and axial wire current pulse with LaCl_3 incubation were studied. Stimulation of the axon will increase the amount of potassium in the periaxonal space, therefore, structures involved in potassium homeostasis and clearance might be expected to show enhanced lanthanum content after stimulation.

When a 2 minute 50 Hz stimulation pulse was applied following LaCl_3 incubation, the electron microscope examination of the tissue revealed, what appeared to be osmotic shock, particularly in the adaxonal glial layer. There was also evidence of increased vacuolization and axonal membrane disruption. Two explanations for the occurrence of osmotic distortion following high frequency stimulation are possible: first, the tissue was damaged by the applied stimulation current; or second, an osmotic effect secondary to stimulation exists (Hassan and Lieberman, 1988). It is unlikely that high frequency stimulation destroys the tissue because previous studies have shown that the axon can be

stimulated with 7 min of 50 Hz stimulation pulse at much higher current density with no apparent effect on its ability to recover full electrophysiological viability. This alternating stimulation and recovery can be repeated for several hours and still obtain a good action potential. Therefore, it is more likely that an osmotic effect occurs secondary to stimulation. The glia may take up such high concentrations of potassium that the fixative solution optimal for resting conditions of the nerve cord is no longer osmotically suitable for the stimulated condition. The tendency toward osmotic disruption occurred only on the dorsal side of the axon may be explained by a decrease rate of diffusion and permeability of the test and fixative solutions to the axon level on the ventral side due to the diffusion restriction provided by the smaller surrounding axons. The +10 mV depolarization current pulse also caused a similar osmotic disruption of the axonal tissue, however, in addition to the disruption there was a complete filling of the tubular lattice with lanthanum precipitate. The presence of lanthanum in the tubular lattice may represent an increase in the diffusion of small ions as calcium, sodium, and potassium into the tubular lattice during an outward depolarizing current pulse. Prior to further experimentation, the fixation method to minimize osmotic damage during electrical stimulation needs to be resolved. Only then can a reliable interpretation of stimulation and current pulse

experimental data be made.

The mechanism for removal of potassium from the periaxonal space ideally should be energetically efficient, rapid, and structurally feasible. Although there may be some participation of ATP dependent transport processes, this mechanism may be minimal since first, an active transport process is not energetically economical, and second, active processes are considered slow. The mechanism of spatial buffering requires the presence of gap junctions as a structural component of cell to cell communication. It is unlikely that spatial buffering is the only mechanism for potassium homeostasis since there is minimal documented presence of gap junctions found between glial cells in the crayfish. The long, tortuous intercellular pathways or glia-glia interfaces as defined in this study, open directly to the periaxonal space in the adaxonal layer via the mesaxonal pathway suggests this as a possible path for removal of potassium from the periaxonal space. The average length calculated in this study of the glia-glia interface from the periaxonal space to the outer adaxonal glia-collagen interface is 4.32 μm . Since this study has found that the length of the mesaxonal intercellular pathways are reduced 12% by tubular lattice connections to these pathways, the tubular lattice may provide an additional element of efficiency for the removal of excess potassium. As a first attempt we may use the Einstein relation to calculate the effect of decreasing

the diffusion length (Berne & Levy, 1988):

$$\text{Equation 9.} \quad (\Delta x)^2 = 2Dt$$

where $(\Delta x)^2$ = average displacement squared, D = diffusion coefficient, and t = time elapsed since molecule started diffusing, a comparison can be made of the extracellular diffusion time in the adaxonal glial layer with tubular lattice and without tubular lattice by the following calculation.

$$\begin{aligned} t_{(\text{with T.L.})} / t_{(\text{without T.L.})} &= \\ &= (\Delta x)^2_{(\text{with T.L.})} / (\Delta x)^2_{(\text{without T.L.})} \\ &= (3.79)^2 / (4.32)^2 = .77 \end{aligned}$$

therefore, the tubular lattice decreases the diffusion time in the adaxonal glial layer by 23%. Since the time constant, measured by Shrager, et al. (1983), for the crayfish is 2.0 ms, a decrease of 23% by the presence of the tubular lattice suggest that the tubular lattice does not provide a major decrease in the time constant. The tubular lattice may be a component in the removal of excess potassium from the periaxonal space, however, most likely in combination with other pathways.

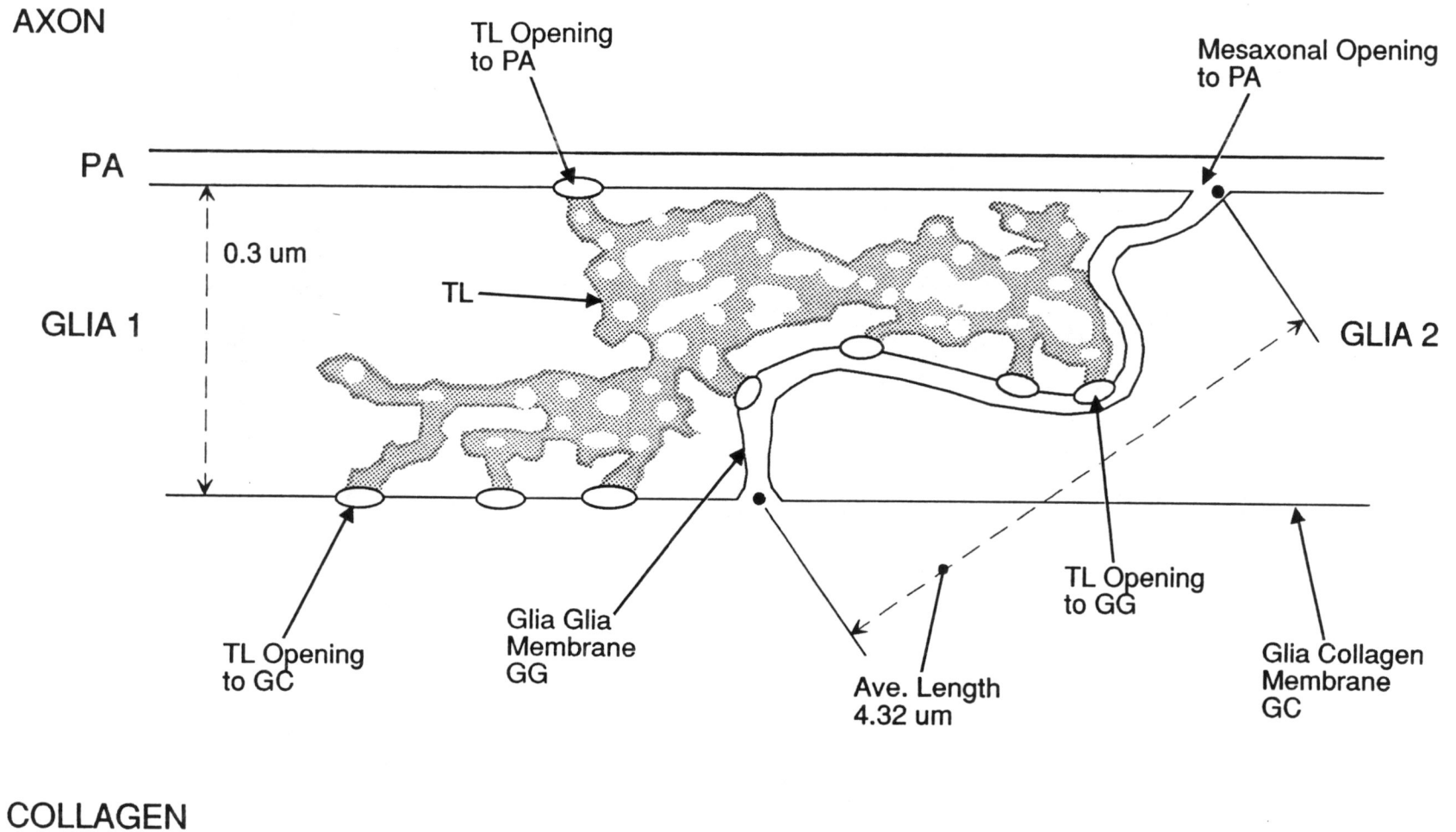
The morphometric data concerning the tubular lattice provides information to form a structural model of the

adaxonal glial layer (see model on following page) which may provide some insight into the function of this system in ionic homeostasis. The tubular lattice opens not only to the glia-glia interface in the adaxonal layer, but also directly to the periaxonal space and to the outer layer glia-collagen interface. Each of these tubular lattice accesses may provide the structural feasibility of ion movement. Direct access of the tubular lattice to the outer glia-collagen interface provides an immediate pathway through the glial layer between collagen matrix. Since the movement of ions from glial layer may occur by diffusion through the connective tissue, the direct opening of the tubular lattice to that space may aid this process. Potassium clearance from the periaxonal space may be facilitated through the glia by the tubular lattice.

From this data, it may be suggested:

1. The tubular lattice is accessible to small ions and molecules from the extracellular space.
2. The long tortuous intercellular diffusional length may be short circuited by the presence of the tubular lattice.

ADAXONAL GLIAL LAYER



In summary:

1. Lanthanum gains access to the tubular lattice during 1/2 hour incubation and subsequent precipitation. The lanthanum tracer stains the inner membrane of the tubular lattice indicating that it is bound to the membrane.
2. A 50 Hz stimulation pulse for two minutes showed evidence of tissue disruption in the adaxonal glial layer, areas of disruption of the periaxonal membrane and increased vacuole formation.
3. There was no evidence of lanthanum redistribution in the presence of carbachol or forskolin.
4. There is a structural difference between the adaxonal glial layer and the outer characterized by: greater glial volume density in the adaxonal layer; greater glia-glia interface density; greater volume density and numerical density of the tubular lattice in the adaxonal layer; greater volume density of adaxonal glial mitochondria.
5. The adaxonal tubular lattice short circuits the length of the long, tortuous glia-glia interface.
6. There is no difference in the number of openings of the adaxonal tubular lattice to the periaxonal space compared to mesaxonal openings to the periaxonal space.
7. When a tubular lattice patch spans both the inner and outer membrane of a glial layer there is no difference

in the number of openings to each membrane.

8. Estimates of the number of glial layers and glial cells correlate with previously reported data (Lieberman et al., 1981).

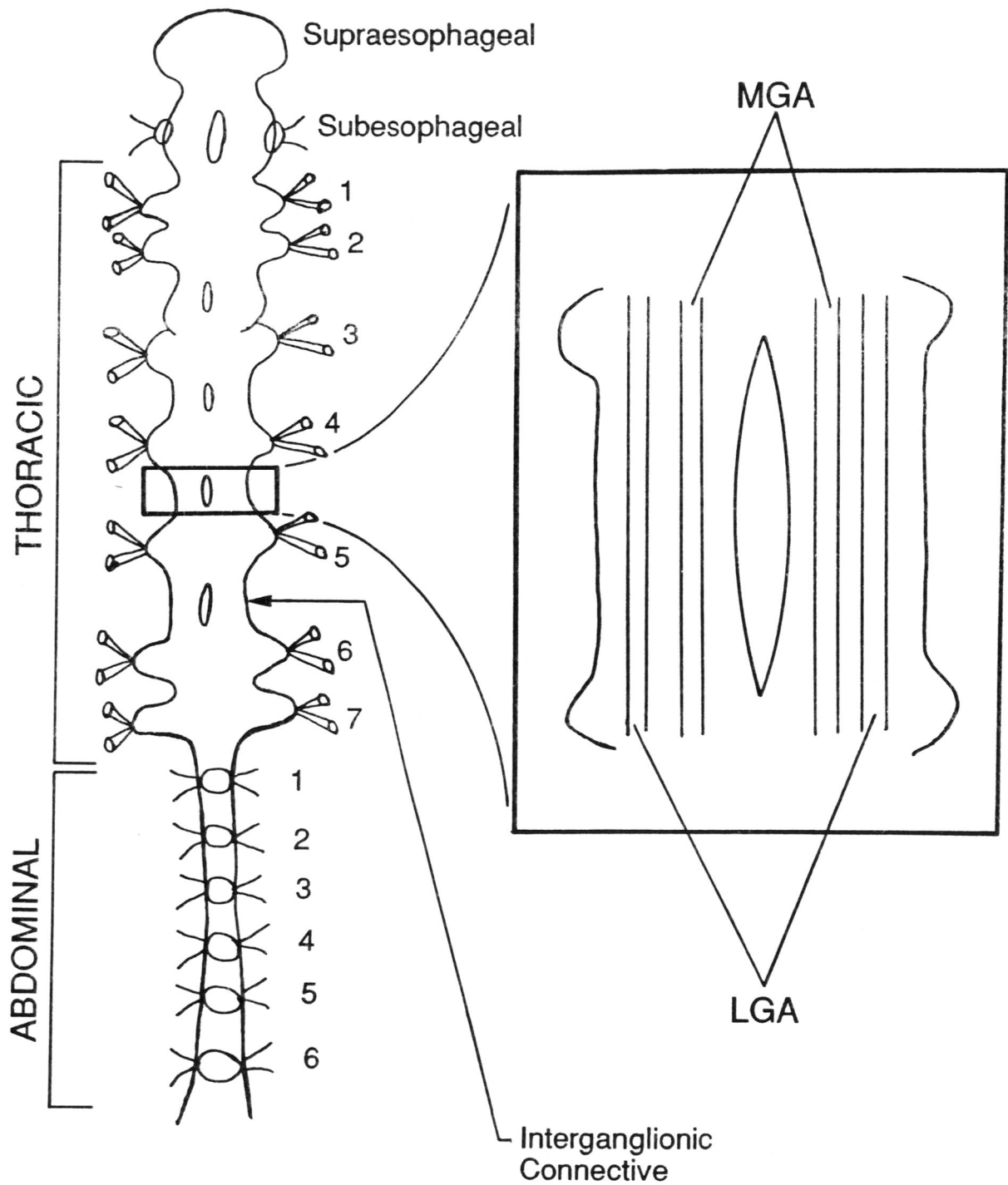
Recommendations for future research

1. Determine the optimal fixation solution for conditions in the excited tissue. With optimal fixation, stimulation experiments may be performed and observed for structural changes caused by action potential generation.
2. Determine the volume and surface area of the tubular lattice by morphometric methods. The volume of the tubular lattice would be important in relating the ionic dilutional potential of the lattice. Since it has been suggested that potassium may enter the glial cell cytoplasm from the tubular lattice it would be interesting to know the tubular lattice surface area available to that process.
3. Perform morphometric study of the excited tissue to determine if any changes in structure occur as compared to the resting condition.
4. Repeat lanthanum tracer experiments with high frequency stimulation experiments and current pulse experiments using the optimum fixative and examine the tissue for

changes in the lanthanum ionic space distribution.

5. Localize Na^+ - K^+ ATP-ase and determine relation to structures such as the tubular lattice. This may help in localizing active areas related to axonal transport across the membrane.
6. Perform high magnification electron microscopy analysis of the mesaxonal openings to the periaxonal space and observe for the existence of tight junctions at these sites. Tight junctions would increase the time required for the removal of potassium and necessitate the need for a structural component with a more rapid clearance ability. If tight junctions exist at the mesaxonal openings this may support the role of the tubular lattice in the rapid removal of potassium from the periaxonal space.

APPENDIX 1. Diagrammatic scheme of the ventral nerve cord of the
crayfish Procambarus clarkii



APPENDIX 2.

Composition of Normal crayfish solution

	Concentration mM
NaCl	190.0
KCl	5.4
CaCl ₂	13.5
MgCl ₂	2.6
TRIS	20.0
pH 7.4	430 mosmol

APPENDIX 3.

Composition of Fixative solution

	Concentration mM
NaCl	190.0
KCl	5.4
CaCl ₂	13.5
MgCl ₂	2.6
Veronal acetate	40.0
2% glutaraldehyde	
pH 7.4	430 mosmol

APPENDIX 4.

Post fixation protocol

-
1. Rinse with three changes of normal crayfish solution (NCS) over one hour
 2. Post fix in ice-cold 1% osmium tetroxide (OsO_4) in veronal acetate buffered NCS for one hour
 3. Two ten minute rinses in veronal acetate buffered NCS
 4. Two ten minute rinses in 0.05M maleic acid at pH 5.2
 5. Stain for one hour in 0.5% uranyl acetate in 0.05M maleic acid, pH at 5.2
 6. Two ten minute rinses in maleic acid buffer
 7. Dehydrate in ascending alcohols (alcohols were diluted in NCS: 50%, 70%, 80%, 90% ethanol (two ten minute rinses of each), absolute ethanol (three 10 minute rinses each)
 8. Two ten minute rinses in 1:1 propylene oxide to absolute ethanol
 9. Two ten minute rinses in propylene oxide
 10. Infiltrate in 1:1 propylene oxide to Embed 812 resin for one hour
 11. Infiltrate in Embed 812 resin for one hour before embedding
 12. Tissue was placed in flat embedding molds containing Embed 812 resin and allowed to polymerize in a 65°C oven for 48 hours. The formula for the Embed 812 is as follows: Embed 812, 6.5 gm; DDSA, 4.0 gm; NMA, 3.5 gm; DMP-30, 0.18 gm.
-

APPENDIX 5.

Composition of Fixatives

FIXATIVE	%	BUFFER	OSMOLALITY (mosmol)
Paraformaldehyde	5.0	cacodylate	1340
Glutaraldehyde	4.0		
Paraformaldehyde	2.5	cacodylate	1226
Glutaraldehyde	2.0		
Paraformaldehyde	1.25	phosphate	1350
Glutaraldehyde	1.0		
Glutaraldehyde	2.0	NCS (TRIS)	560
Glutaraldehyde	2.0	NCS (cacodylate)	750
Glutaraldehyde	2.0	NCS (veronal acetate)	570

* All above fixatives were used for the following fixation times: 2 hr. fixation (70% ETOH overnight); overnight fixation; 2 hr. fixation (buffer overnight after OsO_4)

APPENDIX 6.

Solution for LaCl_3 precipitation

	Concentration mM
NaSO_4	100.0
KCl	5.4
CaCl_2	1.0
MgCl_2	2.7
Veronal acetate	40.0

pH 7.4

430 mosmol

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