The Neuronal Endomembrane System

III. The Origins of the Axoplasmic Reticulum and Discrete Axonal Cisternae at the Axon Hillock[1]

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Abstract

The axoplasmic reticulum (AR) and the discrete element (e.g., vesicles, vesiculotubular bodies, multivesicular bodies, etc.) constitute the endomembrane system of the axon. It is reported here that the AR of bullfrog sciatic nerve readily fills with osmium deposits during osmium impregnation. In contrast, the discrete elements and mitochondria are highly resistant to impregnation. Hence this preparation is well suited to address the nature of possible interactions between AR and rough endoplasmic reticulum (RER) in the axon hillock. It is also ideal to study the origin of the axonal discrete elements within the cell body as well as their interaction with other somal endomembrane system components. Tissues used in the present study were spinal ganglia, sciatic nerve, and spinal roots from *Rana catesbeiana*. Thick sections (1 to 2 μm) of this material were studied by high voltage electron microscopy. In some cases, osmium impregnation was followed by *en bloc* staining with lead aspartate. This made visible membranous structures that had not filled with osmium deposits during impregnation. Serial 170-nm-thick sections of this latter material were prepared and serial stereo pair electron micrographs of axon hillocks were collected. These were used to reconstruct three-dimensionally the AR and to study its relationship with RER and with discrete elements. The impregnated AR within the axon hillock was found to terminate as many proximally pointing finger-like projections. A large portion of these projections were found to form connections with RER. Some, however, terminated as true blind endings. Single unimpregnated discrete cisternae were found throughout the cytoplasm of the cell body, axon hillock, and axon. Large clusters of unimpregnated

vesicles were usually found in close association with the trans face of the Golgi apparatus. These results indirectly support the hypothesis that vectors of fast axonal transport, namely the discrete elements, form directly at the trans face of the Golgi apparatus. From here they move toward and subsequently down the axon without any membrane fission-fusion events with either RER or AR. AR, although it forms continuities with RER, retains a distinctly different chemical composition from RER as evidenced by its much higher affinity for osmium. Thus, it should be considered as an endomembrane component separate from, although intimately related to the RER.

The axoplasmic reticulum (AR) is an extensive system of branched membranous tubules that extend in a continuous manner through axoplasm (Droz et al., 1975; Tsukita and Ishikawa, 1976). This reticulum can be subdivided into two categories: a central or core reticulum that pervades the main portions of axoplasmic space, and a subaxolemmal reticulum that forms networks of tubules adjacent to the inner surface of the axonal plasma membrane (Droz et al., 1975; Berthold, 1982). Connections between the core and subaxolemmal reticulum have been observed frequently. The observations in this study pertain mainly to elements associated with the core reticulum. In addition to at least two types of AR, several forms of discrete membrane-bound organelles are also found within axoplasm. These include multivesicular bodies, multilamellar bodies, and a variety of vesicles and vesiculotubular bodies (reviewed in Grafstein and Forman, 1980; Ellisman and Lindsey, 1983). The discrete nature of multivesicular bodies and multilamellar bodies has been unchallenged for over a decade (Grafstein and Forman, 1980). The discrete nature of these and many other axoplasmic organelles has been noted in reports using analysis of serial sections (LaVail et al., 1980) and stereoscopic viewing of thick sections (Ellisman and Porter, 1980; Ellisman and Lindsey, 1983). Together, the various discrete cisternae and the two forms of the AR constitute the endomembrane system of the axon. Within the cell body, the endomembrane system compartments include: the rough endoplasmic reticulum (RER), lysosomes, the Golgi apparatus and associated tubules, and small discrete elements such as vesicles and multivesicular bodies (Lindsey and Ellisman, 1985a, b).

Interest in the axonal endomembrane system originated from biochemical and autoradiographic evidence indicating that fast axonal transport is mediated by membranous components of the neuron (Barondes, 1967; Droz et al., 1973, 1975). Compelling evidence from several groups that discrete elements move relatively rapidly in both the orthograde and retrograde directions has recently

extended this concept. (Tsukita and Ishikawa, 1980; Smith, 1980; Brady et al., 1982; Ellisman and Lindsey, 1983). The AR, however, appears to be relatively stationary (Ellisman and Lindsey, 1983). Recent findings have also shown that most molecules destined for fast axonal transport pass through the Golgi apparatus (GA) (Hammerschlag et al., 1982). Collectively, these findings indicate that each of these systems may play distinct roles in the fast transport of new neuronal products.

Uncertainty remains, however, concerning the intimate anatomical interrelationships of these systems. Treatment of neurons with a double impregnation using heavy-metal salt solutions fills the lumen of all membrane compartments within the cell with an electron-dense deposit (Thierry and Rambourg, 1974). High voltage electron microscopic examination of treated neurons produced images that show AR joining to tubular profiles in the cell soma that look much like impregnated RER (Droz et al., 1975). This finding was recently confirmed using stereoscopic analysis (Rambourg et al., 1983) and, in the case of photoreceptors, by serial section reconstruction of conventionally fixed tissue (Mercurio and Holtzman, 1982). However, a histochemical study using silver proteinate (a technique that demonstrates the presence of sugars such as are found on glycoproteins) found that, although AR in the axon and tubular profiles near the GA in the cell body stain positive, there was no staining of RER (Quatacker, 1981). This latter finding at the very least argues against the presence of a simple continuity between RER and AR.

The origin of orthograde vectors and, in particular, their relationships with other endomembrane system components is not understood. Previous difficulties in resolving this relationship may be attributable to limitations in technologies available heretofore. For example, in conventionally prepared thin section images, it is often very difficult to determine whether a given vesicular profile corresponds to a true vesicle or to a cross-section of one of the various membranous tubule types found with the cell body (see Rambourg et al., 1974; Lindsey and Ellisman, 1985a). Even when the high voltage electron microscope (HVEM) can be used to examine the distribution and form of a complicated structure like the AR through the use of selective impregnation, it is difficult to determine relationships with unimpregnated (and, thereby, nearly invisible) structures in the same images. Therefore, in this study we combined both osmium impregnation (to delineate the AR) and en bloc lead aspartate staining (to delineate the RER and other membranous structures). Thus, all of the endomembrane components are readily visualized and identifiable in thin to moderately thick sections. Using this technique, it was possible to assess systematically whether AR forms direct connections with any other endomembrane component within the axon hillock or cell body. Also assessed was the relationship of discrete elements with RER, the GA, and AR within the hillock and cell body. Portions of these results have been published previously in abstract form (Lindsey and Ellisman, 1982, 1983).

Materials and Methods

Tissue preparation and microscopy. Spinal ganglia and 15-mm-long segments of sciatic nerve and spinal roots from *Rana catesbeiana* were fixed by immersion in 2% aqueous osmium tetroxide for 1 hr. Initial fixation temperatures of 0, 4, 22, and 37°C were tried. As described under "Results," each of the three higher temperatures had unique advantages. Subsequently, the tissue was impregnated, stained en bloc, and embedded as described by Lindsey and Ellisman (1985a). For comparison, other spinal ganglia were initially fixed in 1.0% glutaraldehyde, 1.0% paraformaldehyde in 0.12 M cacodylate buffer, rinsed in buffer, postfixed in buffered 1% OsO4 for 1 hr, and then embedded similarly.

Thin sections were grid stained with uranyl acetate and lead citrate before examination at 80 KeV on a JEM 100CX microscope equipped with a goniometric stage. Thicker sections up to 250 nm thick were sometimes grid stained and always carbon stabilized before examination at 100 KeV with the instrument mentioned above. Thick sections (1.0, 2.0, and 3.0 μm) were examined as 1 MeV with the JEM 1000 HVEM at Boulder, Colorado. Section thicknesses of sections thinner than 200 nm were estimated based on interference color reflections.

Analysis of serial sections. Serial series (170 nm thick) from tissue stained en bloc with Walton's lead aspartate (Walton, 1977) were collected on Formvar-coated slot grids, carbon stabilized, and examined at 100 KeV. Serial axon hillock images were collected at low magnification and at +5;° and −5° tilt (instrument magnification × 6600). These images were next enlarged to 20 × 25 cm prints. The prints were reproduced onto xerographic transparencies (Chartpak, AFX25) using a Minolta EP53OR copier at exactly 100% original magnification. (Most photocopiers were found not to reproduce at exactly 100%.)

To reconstruct the AR, stereoscopically, alignment marks were made at four separate points on the first xerographic film image from a given tilt series using a felt-tipped marking pen. On a light box, the next corresponding tilt film image of the series was aligned with the first based on ultrastructural landmarks such as lysosomes and mitochondria. The alignment marks on the first film image were then duplicated on the second film image with the felt pen. This was repeated in sequence for all of the film images of a given set and its matching tilt set (i.e., the other half of the stereo serial series). Next, a clear acetate transparency was laid over the first film image of one side of the stereo-tilt series. Outlines of the axolemma and AR were then drawn with a felt-tipped pen along with the alignment marks. The acetate transparency was then aligned over the next film image of the tilt series and outlines of this axolemma and AR were drawn. This was repeated for the remainder of the tilt series and also for the matching tilt series. The reconstructed drawings from each of the two sets were aligned in a stereoscopic viewing device so as to project one of the drawings to one eye and the other drawing to the other eye.

To study the relationship between AR and unimpregnated membrane systems of the axon, the xerographic film reproductions were overlaid onto prints from adjacent sections (all of the same tilt) so as to facilitate mapping the course of AR tubules as they traveled from section to section. Based on these maps, high magnification images of the AR at various tilts for stereoscopy were obtained from the regions of sections where the reticulum appeared to end and from the corresponding regions of the two adjacent sections. These images were then viewed in stereo to determine whether a continuity of the AR existed with any other structure.

Results

Myelinated and unmyelinated axons. The AR of moderately myelinated axons fills with an electron-dense deposit following 37°C primary fixation osmium impregnation (Fig. 1). In contrast, discrete membranous elements such as vesicles and vesiculotubular bodies, as well as mitochondria within these axons were never observed to impregnate. In the remaining axon types with different amounts of myelin, initial fixation temperature could be adjusted so that similar axons shared the impregnation pattern described above. In general, the more heavily myelinated fibers required higher initial fixation temperatures in order to fully impregnate the AR. In unmyelinated fibers, reliable AR impregnation occurred at lower (e.g., 4 to 22°C) primary fixation temperatures. At higher primary fixation temperatures (e.g., 37°C) occasional discrete elements of the unmyelinated fibers also became impregnated. Thus, the AR is much more readily impregnable than the discrete elements. Nevertheless, these findings underscore the importance of optimizing the primary fixation temperature for each nerve fiber type to be analyzed. The preservation of membrane macrostructure (i.e., the shape of membranous organelles) seen in osmium-impregnated axons (Fig. 1, a and b) is quite comparable to that seen in axons fixed by conventional aldehyde-osmium protocol (Fig. 1, c and d). This holds for AR, discrete elements, and the particularly fixation-sensitive mitochondria (see Busson-Mabillot, 1971). Cytoskeletal structures were not as well preserved by osmium impregnation as by conventional fixation. Neurofilaments and, occasionally, microtrabeculae were seen in impregnated axons, but they were less apparent than in conventionally fixed axons. Non-impregnated discrete elements occasionally appeared to touch the AR in images from 120-nm-thick sections. Stereoscopic viewing of such areas, or high angle tilting as is demonstrated in Figure 2, showed that these associations were mere overlaps of separate structures within the volume of the section.

The three-dimensional organization of the AR is readily appreciated in stereoscopic images of thick sections of impregnated axons
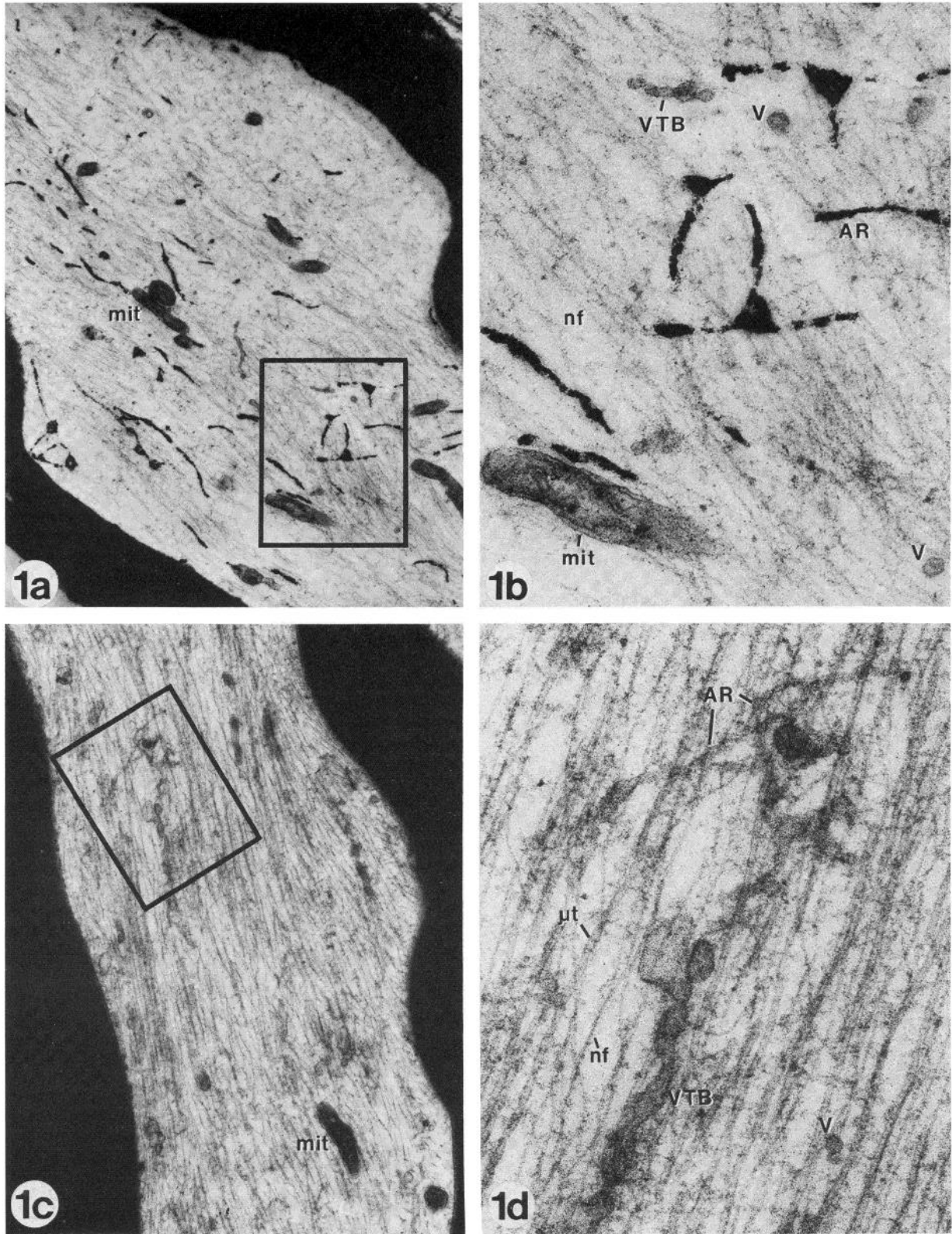
Figure 1. Comparison of endomembrane components within axons following fixation by osmium impregnation (*a* and *b*) and by conventional aldehyde fixation (*c* and *d*). *b* and *d* are enlargements of the *boxed areas* of *a* and *c*, respectively. Within osmium-impregnated axons, the axoplasmic reticulum (*AR*) is filled with an electron-dense deposit. Discrete elements such as vesicles (*V*) and vesiculotubular bodies (*VTB*) were much less impregnable than the AR. Note the similar morphology of discrete elements, AR, and mitochondria (*mit*) in the two preparations. Other labeled structures include: microtubules (*μt*), and neurofilaments (*nf*). Magnifications: *a*, × 13,000; *b*, × 45,000; *c*, × 15,000; *d*, × 58,000.
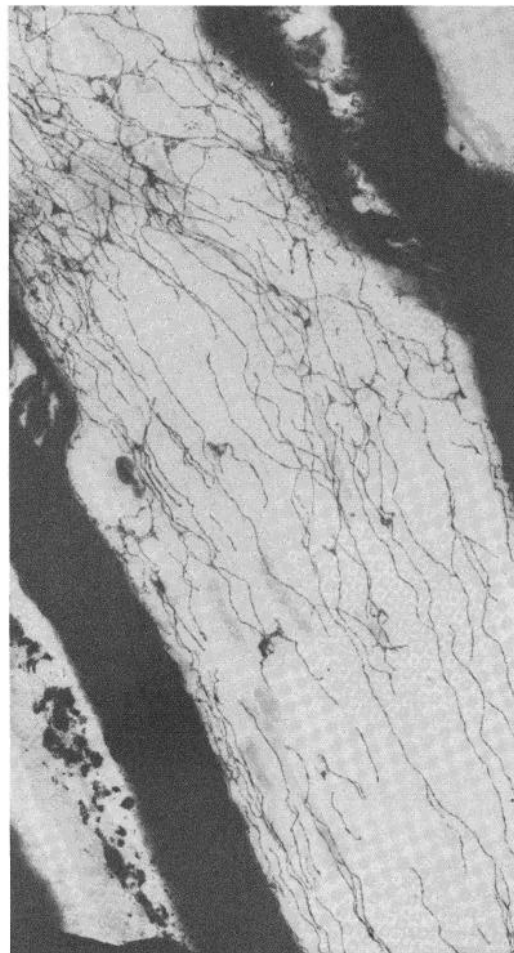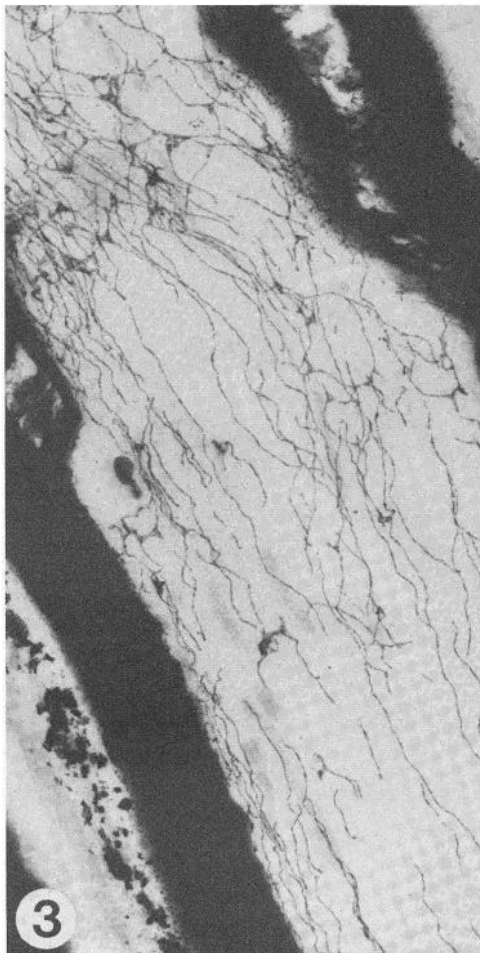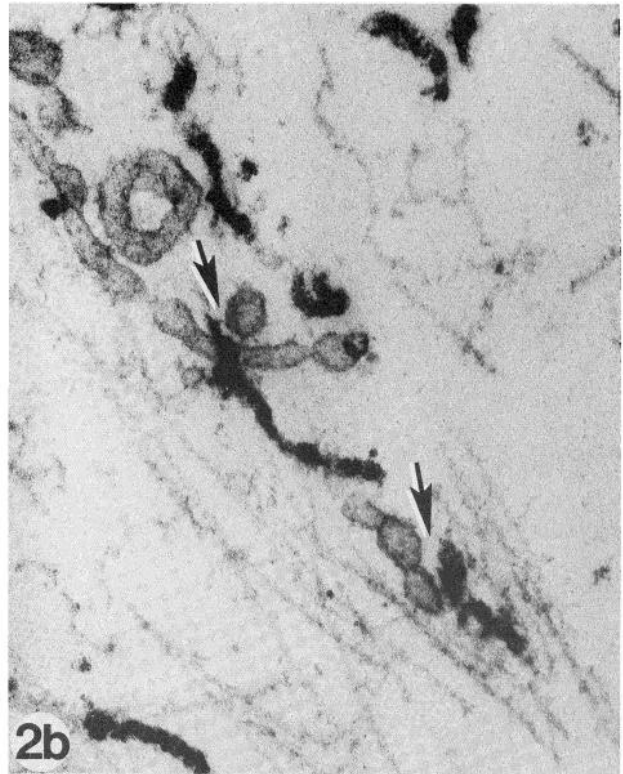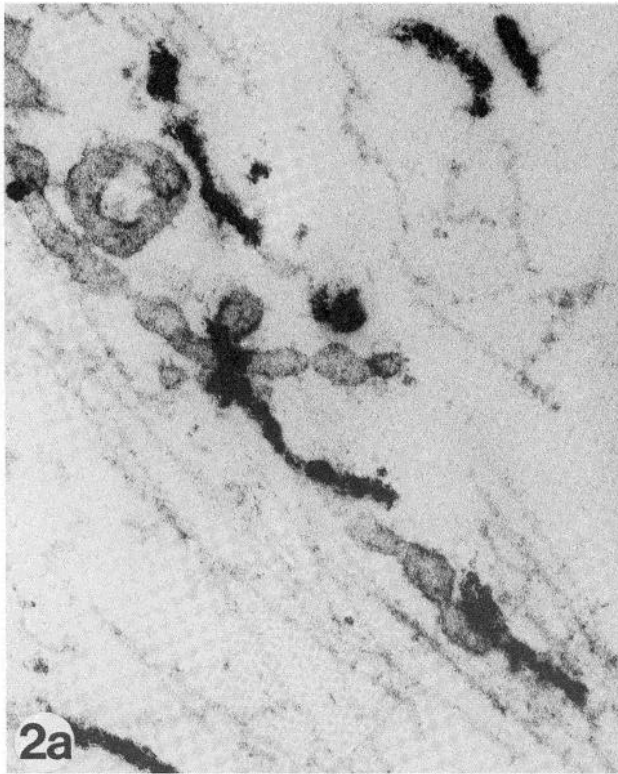
Figure 2. Noncontinuity of overlapping discrete elements and AR demonstrated by tilting the specimen on the goniometric stage of the electron microscope. *a* shows two discrete elements in partial overlap with AR. When the specimen was tilted 35°, these discrete elements were clearly seen to be separate from the reticulum (*arrows*). Magnification × 86,000.

Figure 3. Stereo pair of osmium-impregnated AR within a 1-μm-thick section of a myelinated axon viewed with the HVEM at 1000 KeV. Magnification × 8,100.
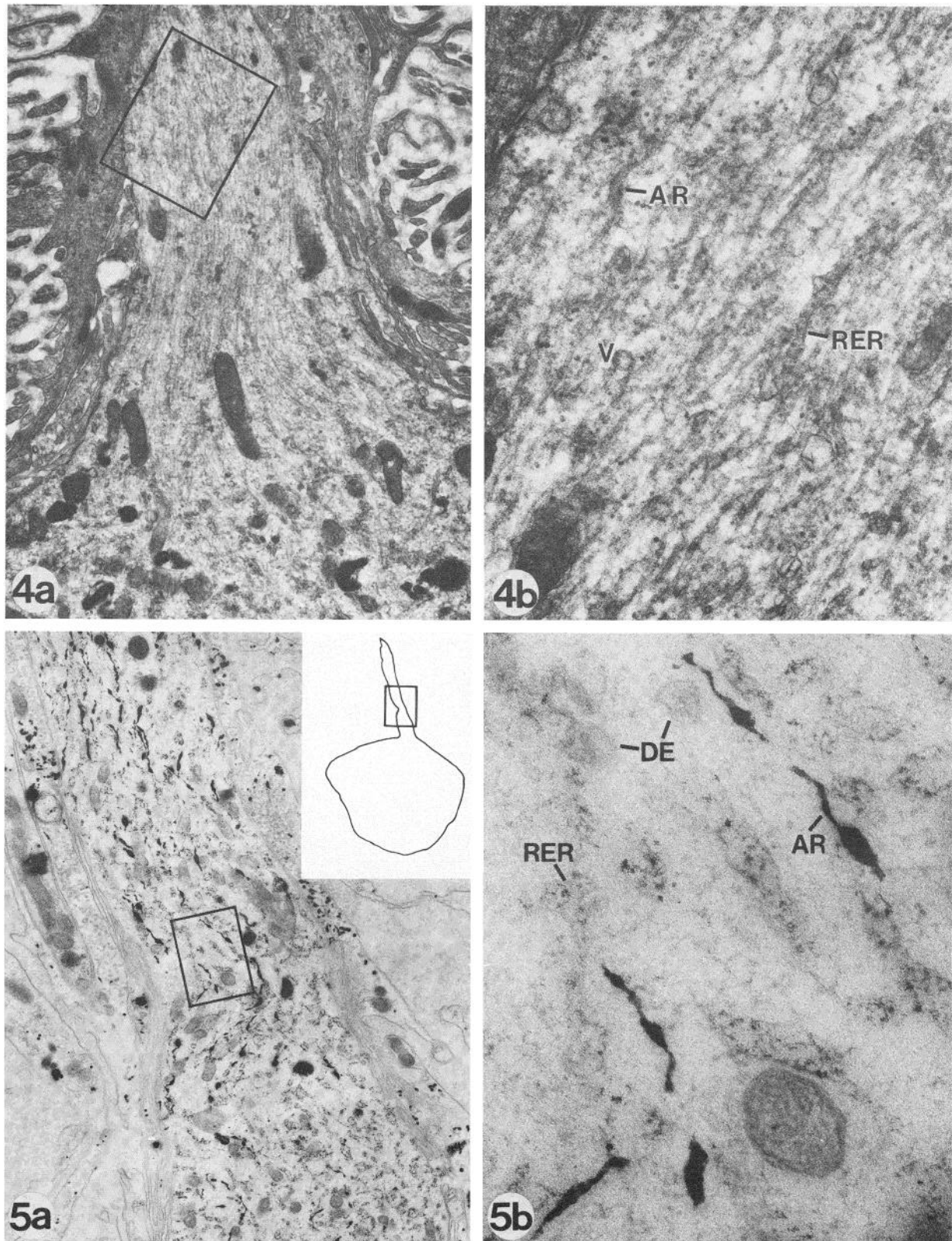
Figure 4. Axon hillock seen in a 170-nm-thick section of conventionally fixed spinal ganglion neuron. *b* is an enlargement of the *boxed area* in *a*. Note that rough endoplasmic reticulum (*RER*) extends well into the initial segment. Also labeled is a vesicle (*V*) and axoplasmic reticulum (*AR*). Magnifications: *a*, × 9,800; *b*, × 43,000.

Figure 5. Axon hillock and initial segment of osmium-impregnated spinal ganglion cell. *a* is taken from the proximalmost region of the initial segment as shown in the *inset*. It shows that impregnated axoplasmic reticulum and granular-appearing rough endoplasmic reticulum co-exist side by side in the hillock region. *b* details the relation between unimpregnated discrete elements (*DE*) and axoplasmic reticulum (*AR*) and between discrete elements and rough endoplasmic reticulum (*RER*). Magnifications: *a*, × 11,000; *inset*, × 580; *b*, × 72,000.
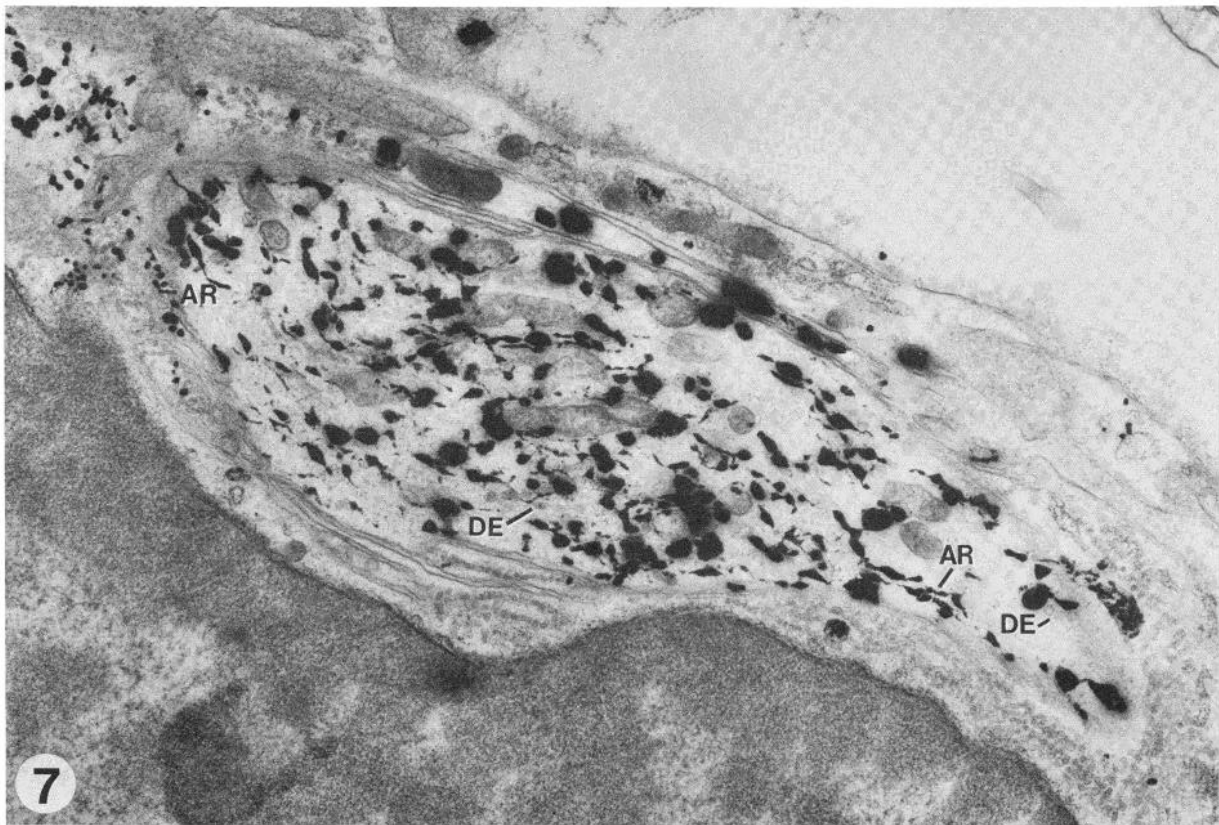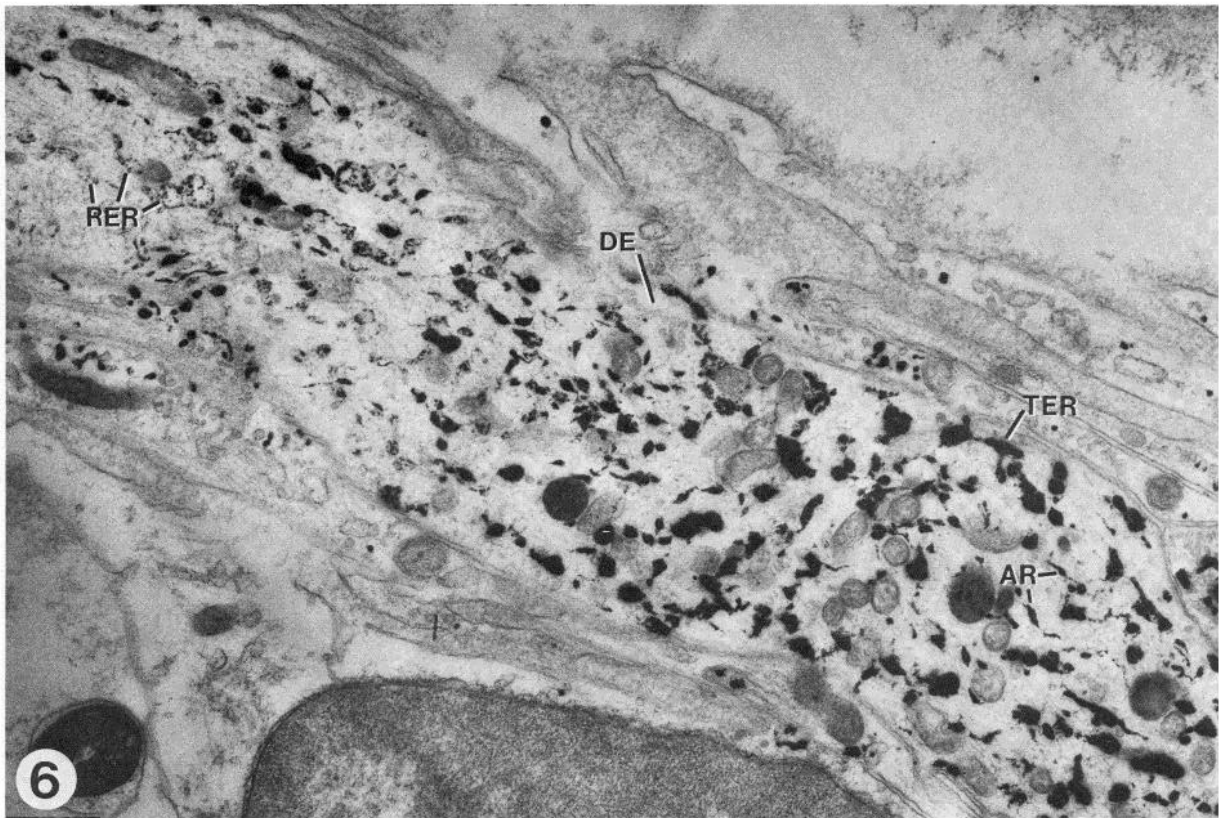
Figure 6. A more distal portion of the same initial segment shown in Figure 5. This image spans the region between 21 and 30 μm away from the base of the axon hillock lying toward the *left* of the image. Note the transformation from a dominance of RER to a dominance of AR. Also labeled are the unimpregnated discrete elements (*DE*) and the transitional endoplasmic reticulum (*TER*) that shares the morphology of RER with the impregnation characteristics of AR. Magnification × 16,000.

Figure 7. A yet more distal portion of the same initial segment shown in Figure 5. This image spans the region between 28 and 36 μm away from the base of the axon hillock lying toward the *left* of the image. Magnification × 16,000.
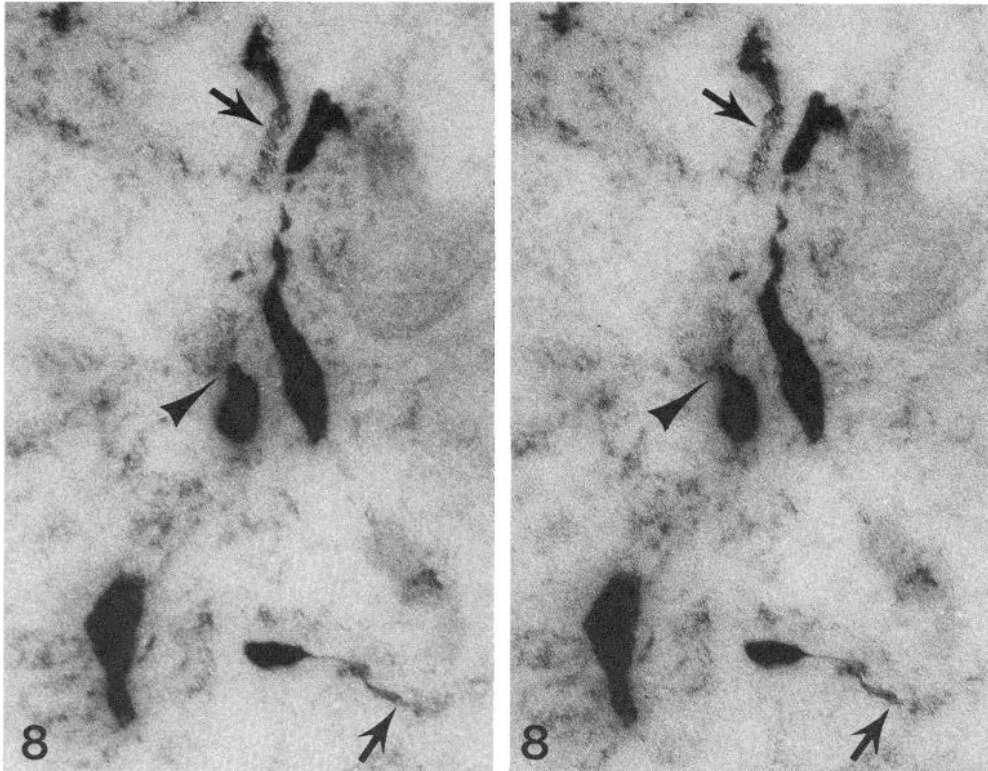
Figure 8. Stereo pair illustrating two continuities between RER and AR that are contained within the section (*arrows*). An overlap of RER and AR which stereoscopy reveals to be nonconfluent is indicated by the *arrowheads*. Magnification × 91,000.

viewed with the HVEM (Fig. 3). It consists of long membranous tubules that anastomose at small swellings and that are evenly distributed throughout the axoplasmic cylinder.

The relationship between AR and the endomembrane system of the cell body. To examine which endomembrane components occur normally within the region of the axon hillock, conventionally prepared sections of the axon hillock were stereoscopically studied. The components observed by the relatively nonselective staining were compared to the subset derived by examining similar serial sections of ganglia selectively stained by osmium impregnation. Three-dimensional reconstruction of the AR within an axon hillock facilitated conceptualizing the relative organizations of both membrane networks in the hillock. The "ends" of the reticulum tubules, identified during reconstruction, were analyzed by stereoscopy at high magnification stereoscopy in order to clarify the relationships between reticulum tubules and unimpregnated components, primarily those of the RER.

The RER can be seen in conventionally fixed preparations of this neuronal cell type to extend well into the axon hillock (Fig. 4). This finding is consistent with and extends previous observations on spinal ganglion neurons from a variety of vertebrates (reviewed in Zenker and Hogl, 1976). In addition to AR and RER, the hillock region contains membranous tubules without ribosomes and numerous discrete cisternae.

In osmium-impregnated neurons the AR impregnates more readily than does RER. Typically the AR is seen to be confluently filled with osmium deposits, whereas RER exhibits its typical slightly granular, but distinctly unimpregnated appearance (Fig. 5) (Lindsey and Ellisman, 1985a). At high magnification the AR, RER profiles, and discrete elements are all readily identified (Fig. 5b). The relative amounts of AR and RER gradually shift to the favor of AR as the axon progresses further away from the cell body (Figs. 6 and 7). Apparent continuities between AR and RER are also seen. At these continuities, the reticulum sometimes appeared highly kinked and branched like RER and yet were heavily impregnated like AR (Fig.

6). For the purpose of discussion, these regions are labeled *TER* in Figure 6 for transitional endoplasmic reticulum. The transition from RER to AR at most continuities, however, was characterized by a simultaneous change in both morphology and impregnation. This was confirmed by stereoscopic analysis as illustrated in Figure 8.

To investigate the organization of the hillock AR, a serial series of stereo pairs was collected from a serial section series containing an axon hillock. These were used to reconstruct the hillock AR in two drawings, one for each tilt series. When the two drawings were examined stereoscopically, the hillock AR was found to disperse as it approached the somal cytoplasm; i.e., the cytoplasmic AR density within the proximal hillock was much less than that of the distal hillock (Fig. 9a). Notably, not a single tubule of AR extended into the somal cytoplasm. Within the proximal hillock, short, osmiophilic, membranous tubules that were morphologically indistinguishable from varicose tubules (see Lindsey and Ellisman, 1985a) were frequently encountered.

The precise relationship of AR to RER in the axon hillock was assessed by tracing the path of the AR tubules as they progressed through the serial series and then examining all of the apparent "ends" of the AR tubules stereoscopically at high magnification, in order to determine whether continuities with non-impregnated membranous structures existed. The results are shown in Figure 9b. Many of the AR tubules formed direct continuities with membrane systems that had the characteristic granular appearance of RER. Some of the AR tubules did end blindly. Short tubules connected on one end with RER and either ending freely or connecting with a second element of RER were regularly found. These latter short tubules were more frequently encountered in the proximal hillock than in the distal hillock. These results were confirmed in stereoimages of heavily impregnated hillocks where both RER and AR were filled with osmium deposits (Fig. 10).

The relation of discrete elements with the somal GA, somal and hillock RER, and AR. The most heavily impregnated neurons displayed confluent osmium deposition in the cis and intermediate

9a

r RER continuity
p ends over RER
f free ending
v vesicle cluster
e exits series
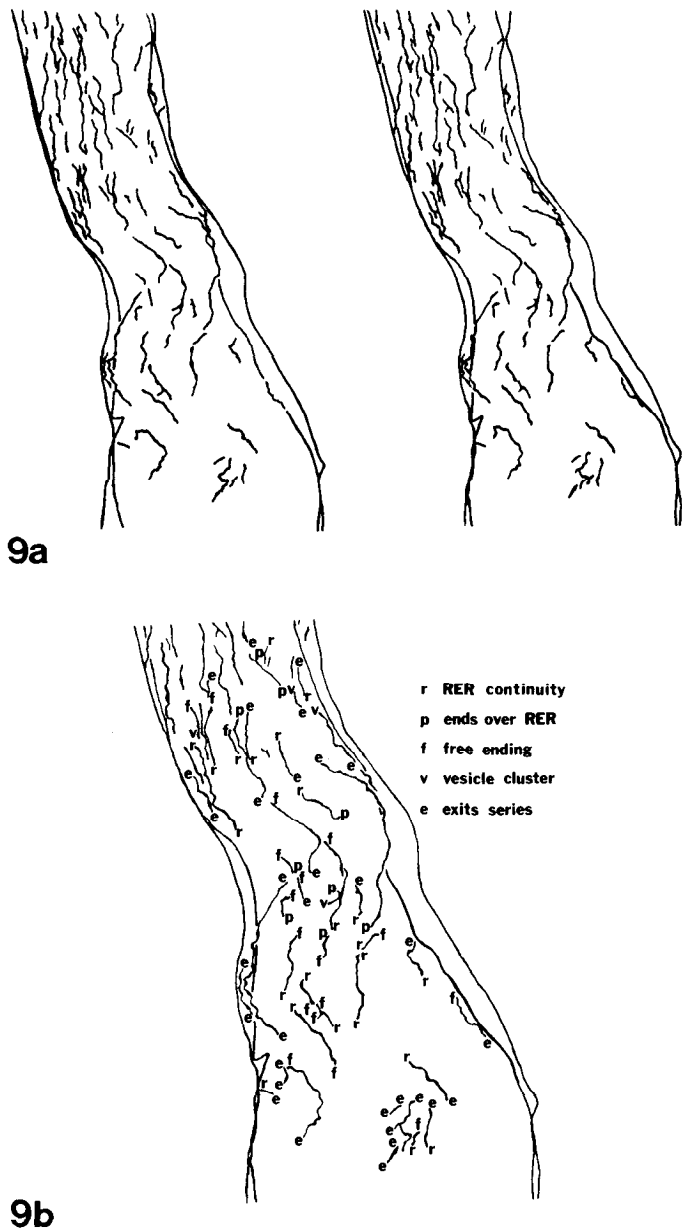
9b

Figure 9. Reconstruction of AR within five serial 0.17-μm-thick sections. *a* is a stereo pair illustrating the transition of the AR from a dense coherent organization in the distal axon hillock (*top*) to the sparser less coherent organization in the proximal hillock (*bottom*). *b* illustrates the relationship to the ends of AR segments shown with other endomembrane system components. *r* designates an RER-AR continuity totally contained within one of the sections of the series. *p* designates presumed RER-AR continuities where the AR ends near one of the surfaces of one of the sections and an apparent RER ending is seen at the appropriate adjacent surface of the adjacent section. *f* designates free AR endings either within or at the surface of one of the sections. *v* designates an AR ending that was immersed within a cluster of impregnated vesicles. *e* designates AR segments that were last observed at the exterior surface of one of the exterior sections in the serial series.

Golgi saccules, vesicles near the cis element, varicose and smooth tubule RER, and AR (Fig. 10). Not impregnated were the trans elements of the GA and vesicles that were found in the following locations: clustered near the trans element of the GA, between elements of RER in the cell body and in the proximal axon hillock, and free within the distal axon hillock, although often closely positioned nearby an AR tubule. This distribution of non-impregnated vesicles was found in the less heavily impregnated ganglia as well (Figs. 2 and 5 to 8).

Discussion

The present results show that AR forms direct continuities with RER. This confirms the AR-RER continuities reported by Droz et al. (1975) and Rambourg et al. (1983). Furthermore, it is consistent with observations on the agranular reticulum in the highly specialized neuron, the photoreceptor (Mercurio and Holtzman, 1982). The nature of the transition, however, suggests that AR is not just an extension of the RER membrane system. In the first case, AR and RER show markedly different affinities for osmium during osmium impregnation. This difference in affinity is further underscored by the often sharp transition in staining pattern seen at the AR-RER continuity sites. Although a transitional ER (labeled *TER* in Fig. 6) sharing the morphology of RER with the impregnation characteristics of AR was observed, it was only seen within a specific portion of the distal axon hillock. The different osmium affinities indicates that there is a difference in the chemical composition of RER and AR. This difference is consistent with the histochemical evidence of Quatacker (1981) which suggests that the carbohydrate composition of AR is different from that in RER. The second point supporting a complex relationship between RER and AR is the large number of AR "free endings" found within the axon hillock. This is in contrast to the continuous nature of the AR seen within the axons further away from the cell body (refer to Fig. 3). Thus, it appears that RER and ER should be viewed as two distinct components of cellular endomembrane system which forms intimate interactions within regions of the axon hillock.

The dramatic change in osmium impregnability seen at the continuities between RER and AR has important functional implications. As discussed by Mercurio and Holtman (1982), continuities between two membranous compartments within a cell could conceivably permit the exchange of molecules within the lumens as well as within the membrane bilayers of such compartments. The dramatic osmium impregnability change, however, suggests that whatever chemical component(s) gives rise to the impregnability of the AR cannot freely diffuse into RER at the RER-AR continuities. Thus, some mechanism may exist at these continuities that regulates the movement of material between RER and AR.

Osmium impregnation appears useful as a tool to help identify whether a given cytoplasmic vesicle is most closely related in composition to the cis or trans saccules of the GA, since the former are readily impregnable whereas the latter are highly resistant to impregnation. Although not conclusive, these results indirectly suggest that the osmiophilic vesicles are pre-GA in origin whereas the impregnation-resistant vesicles are post-GA in origin. It is notable that small, unimpregnated discrete elements (mostly vesicles) are seen clustered near the trans face of the GA and within the somal cytoplasm, the axon hillock, and axoplasm. Since there are no other impregnation-resistant membrane-bound components other than these just mentioned, it appears that the discrete elements arise from the trans face of the GA and, thereafter, as "transport vectors," travel directly through the hillock and on. Our results could also be interpreted to support observations indicating that retrogradely moving discrete elements move directly from the axon to lysosomes or the trans face of the GA without any intermediate membrane fission-fusion events with other endomembrane components. This concept was initially established on the basis of tracer uptake experiments that characterized the recycling of plasma membrane via an apparent shuttle between the trans element of the Golgi apparatus and the cell surface (reviewed in Mercurio and Holtzman, 1982).

In conclusion, a functional model based on these findings can be proposed. This model is diagrammed in Figure 11. Molecules destined for fast axonal transport are packaged into discrete elements at the trans face of the GA. This phase would correspond to the initiation phase of fast axonal transport, as operationally defined by Dravid and Hammerschlag (1975), and is supported by the finding that the structural integrity of the GA is essential for initiation to occur (Lindsey et al., 1981). Once packaging is complete, the discrete element then moves through the somal cytoplasm, the axon hillock,
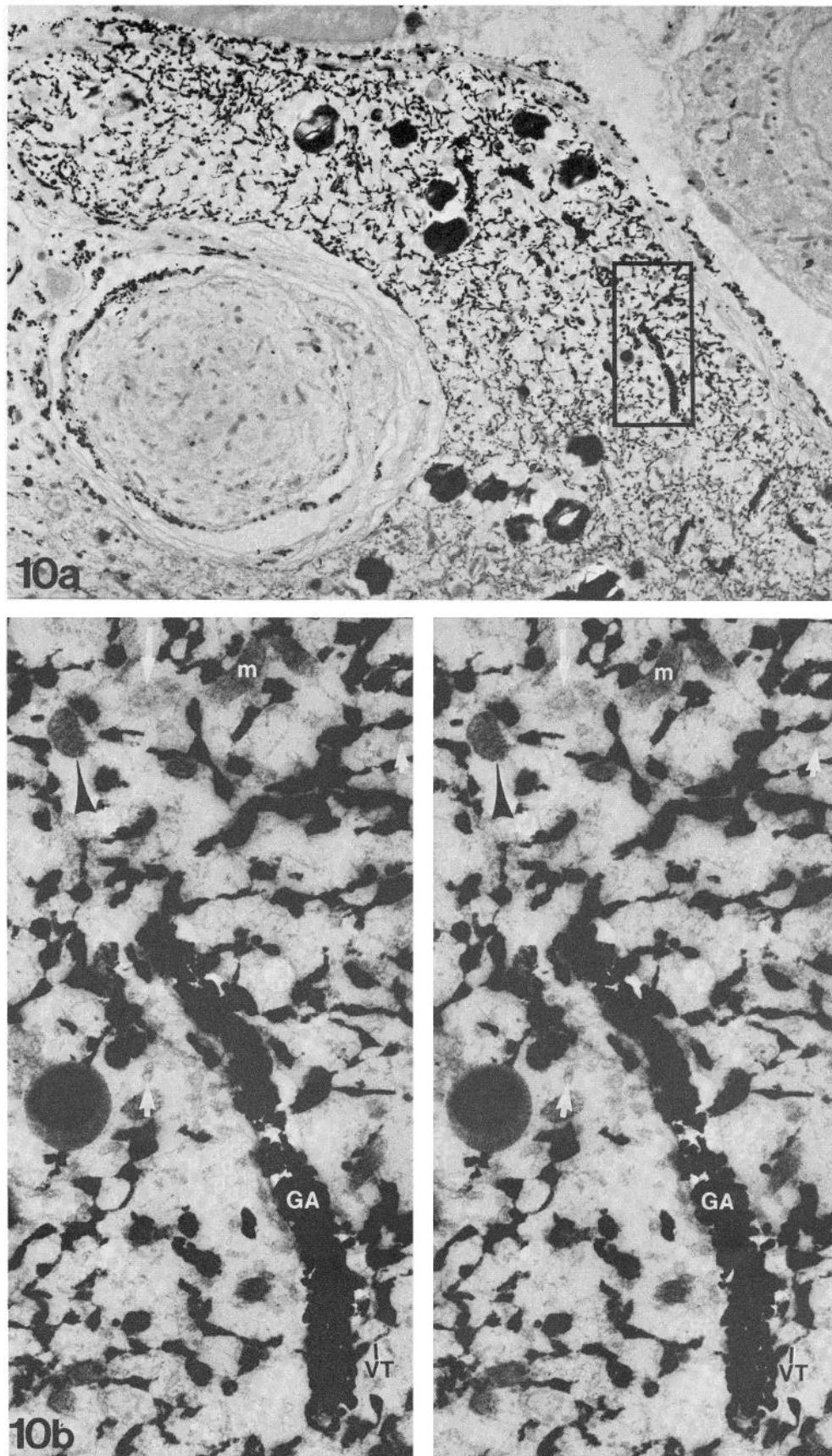
Figure 10. Axon hillock of an intensely impregnated neuron. *a* illustrates the gradual transition from RER dominance in the proximal hillock to AR dominance in the distal hillock. The *box* designates the region that is enlarged and shown as a stereo pair in *b. b* illustrates the unimpregnated discrete elements that are found among the continuous elements of the endomembrane system. These include vesicles (*white arrows*) *and a multivesicular body in the upper left corner (black arrowhead*). Also labeled are a mitochondrion (*m*) and a varicose tubule (*VT*) arising from the cis element of the Golgi apparatus (*GA*). Magnifications: *a,* × 4,500; *b,* × 28,000.
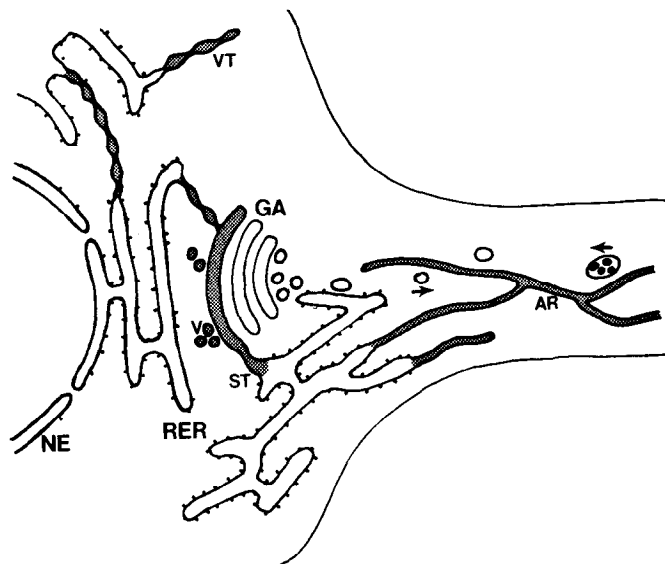
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Figure 11. Schematic diagram of the neuronal endomembrane system illustrating the relationships of the various components. Labeled structures include: the nuclear envelope *(NE)*, rough endoplasmic reticulum *(RER)*, Golgi apparatus *(GA)*, axoplasmic reticulum *(AR)*, varicose tubule *(VT)*, smooth tubule *(ST)*, and vesicles *(V)*. The *arrows* indicate the bidirectional movements of discrete elements within the axon. The *shading* indicates those structures readily filled with osmium during osmium impregnation.

and the axon proper at net rates suitable for fast axonal transport. Some molecules synthesized in the cell body in association with RER may be transferred to AR at the RER-AR continuities in the hillock. Since molecules within AR are not likely to diffuse at fast transport rates (Schwartz, 1979), and since AR does not move down the axon by bulk flow at fast transport rates (Ellisman and Lindsey, 1983), such AR-associated molecules would most likely move down the axon at rates comparable to those of slow axonal transport.

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