EFFECTS OF INTRACEREBRAL INJECTION OF OUABAIN ON THE FINE STRUCTURE OF RAT CEREBRAL CORTEX

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The occurrence of status spongiosus, swelling of glial nuclei, and perinuclear vacuolation of neurons in rat brains following the intracerebral injection of ouabain, a potent inhibitor of Na^+-K^+-activated ATPase, has been described with conventional histologic methods by Bignami and Palladini.[1,2] In the hope of increasing the scope and precision of these observations, we have undertaken an ultrastructural study of the ouabain-induced lesion in the rat brain, directing our attention especially to such unanswered questions as the intra- or extracellular localization of the lesion, the cellular elements involved, and the effects of ouabain on cellular fine structure. The interest of this study initially derived from the similarity of the light microscopic changes produced by ouabain to the status spongiosus seen in Jakob-Creutzfeldt disease,[1,2] but as the study progressed, it was recognized that the ouabain-induced lesion had no close relationship to this entity. At the same time, the action of ouabain raised questions concerning fluid and electrolyte distribution in the central nervous system, the role of glia in this process, and the importance of electrolyte balance in the function of the synapse.

Materals and Methods

Forty adult Wistar rats of varying weights and ages were used in the course of this study. Thirty of these animals were given intracerebral injections, under ether anesthesia, of solutions of ouabain in physiologic saline; 10 controls received injections of equal volumes of physiologic saline (PSS) without ouabain. All injections were made through a cranial burr hole into the frontoparietal region of the brain at a depth of 3 mm. and at a point 1 mm. posterior to the transverse suture and 1 mm. lateral to the midline. Of the total number of experimental animals, 6 given ouabain injections and 1 given PSS form the basis of the ultrastructural study, the remaining animals being used for preliminary studies by light microscopy. Of the 6 animals receiving ouabain, one (Rat 1) received a calculated dose of 0.15 mg. in 0.05 ml. of

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PSS (4×10^{-3} M ouabain) and the others (Rats 2–6) received doses of 0.005 mg. in 0.025 ml. of PSS (3×10^{-4} M ouabain). Because of unavoidable efflux of the solutions injected and because of additional loss through error in Rat 1, the doses actually received by the animals were often considerably below those calculated. The controls received 0.025–0.05 ml. of PSS, corresponding to the volume injected into the experimental animal with which the control was paired. Ouabain, U.S.P. (City Chemical Co., New York) and NaCl Injection, U.S.P. (Abbott) were used in the preparation of solutions for injection. The osmolarity of the PSS was 292 mosm.; of 4×10^{-3} M ouabain in PSS, 297 mosm.; and of 3×10^{-4} M ouabain in PSS, 292 mosm. (Advanced Osmometer). The pH of the unbuffered PSS and of all ouabain solutions varied from 5.3 to 5.4. Gentle heat was often required to achieve solution of the crystalline ouabain in PSS.

Ether-anesthetized rats were sacrificed by intracardiac perfusion with 5% glutaraldehyde in 0.2 M cacodylate buffer at varying intervals of time after injection with ouabain. Rats 1–4 were sacrificed at 2 hr.; Rat 5 at 5 hr.; and Rat 6 at 24 hr. after injection. After perfusion, cortical tissue for electron microscopy was obtained from the same side of the brain as the injection at a point on the lip of the central sulcus 4 mm. anterior to the injection site. Facing tissue blocks were processed for light microscopy. After tissues were minced, they were fixed for an additional 2–2.5 hr. in 5% glutaraldehyde in 0.2 M cacodylate buffer and then washed during the next hour in 3 changes of 0.1 M cacodylate buffer with 10% sucrose.[3] Tissue was then postfixed in Millonig's fixative containing 1% OsO_4 for 1 hr., dehydrated in graded alcohols and propylene oxide, and embedded in Araldite. Sections stained with lead citrate and uranyl acetate[4,5] were examined with a Siemens Elmiskop I.

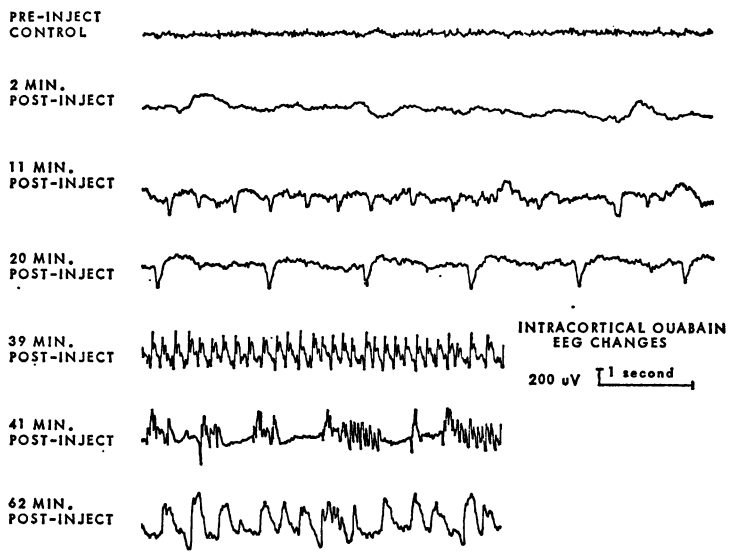
Four rats whose brains were examined by gross inspection had been given intraperitoneal injections of a 1% solution of trypan blue before receiving intracerebral injections of ouabain or physiologic saline. All 4 rats received 2 ml. of 1% trypan blue 12 hr. before the ouabain injection, and 2 of these had the same dose of trypan blue repeated immediately before intracerebral injection.

Five rats for electroencephalographic studies were prepared under ether anesthesia by tracheal cannulation, mid-dorsal cranial skin incision, and a left parietal cranial burr hole. The rats then received succinylcholine (0.5 mg., I.P., as needed), were given artificial respiration, and then allowed to recover from the ether anesthesia. The EEG's were obtained on an ink-writing electroencephalograph using small cotton wick electrodes connected through physiologic salt solution to silver-silver chloride lead wires. The cotton wick electrodes were placed directly on the previously exposed cranial periosteum. At least 45 min. were allowed after the ether anesthesia was discontinued before any EEG's were recorded. After suitable preinjection EEG recordings were obtained, either ouabain (in 3 rats) or an equal volume of PSS (in 2 control rats) was injected intracerebrally through the left parietal burr hole and the postinjection EEG's were recorded for 1 hr. The brains of these animals were examined by light microscopy.

Results

In about 1–5 min. after injection, the rats began to display short bursts of extreme motor activity, running and jumping wildly about their cages. Such bursts of activity were sometimes preceded or followed by focal or, less often, by generalized convulsive behavior. Periods of activity were followed by intervals of relative immobility lasting for 10–15 min. before the next period of activity. Many rats receiving the 0.005-mg. dose of ouabain died in generalized convulsions in slightly over 2 hr. In our experiments all rats (except Rat 1) died prior to 2 hr. when doses of

ouabain greater than 0.005 mg. were given, thus limiting the dose range which we were able to use in these experiments. A small number of rats which received 0.005 mg. of ouabain survived the critical 2-hr. period,



TEXT-FIG. 1. Progression of EEG wave forms with time following intracortical injection of ouabain (0.009 mg. in 0.025 ml. of physiologic saline) into rat given succinylcholine and artificial respiration. Ouabain was given 1 hr. after rat's recovery from ether anesthesia. Monopolar recording electrode over injection site; electropositive is down.

and showed a gradual return to completely normal behavior and function in the following 2–4 hr. Although no rat given ouabain injections was maintained beyond 24 hr., such rats would presumably have survived indefinitely. No behavioral abnormalities were noted in control rats that received intracerebral injections of physiologic saline.

Electroencephalographic Study

Neither of the control rats given PSS showed any postinjection EEG changes. In all 3 rats given ouabain, EEG changes were recorded within 1 min. after the injection. Throughout the hour of recording, various paroxysmal high-voltage wave forms were recorded synchronously in right and left frontal and right and left parietal areas. All wave forms showed a voltage accentuation in the left parietal area over the injection site. Text-figure 1 shows the progression of the wave forms in the left parietal area over the injection site during the course of the hour following injection of ouabain.

Gross and Light Microscopic Observations

Gross findings in the brains of both experimental and control rats were limited to focal hemorrhage into and around the needle track and slight congestion of meningeal vessels in the rats which were not sacrificed by perfusion. In the rats which received intraperitoneal trypan blue before intracerebral injection of ouabain, there was no spread of the trypan blue beyond the needle track in the brain, either in the control rats or in those given ouabain injections.

In the rats given ouabain injections, including those receiving succinylcholine, light microscopic examination of brain tissue away from the injection site revealed diffuse vacuolation of the cortical neuropil, giving a characteristic picture of status spongiosus. At times, groups of large vacuoles occurred in well-defined bands or laminae, which were often prominent at the junction of the gray and white matter. The latter often showed separation of fiber tracts, suggesting edema. In addition, small vacuoles were noted in or near glial nuclei and around the perikarya of neurons (Fig. 1), but even with oil immersion it was impossible to be certain whether these vacuoles were inside or outside the cells with which they were associated. Small foci of hemorrhage were occasionally encountered near the needle tracks created by injection. Except for such hemorrhagic foci, no significant histologic abnormalities were found in the brains of the control rats.

Electron Microscopic Observations

Saline-Injected Controls. No significant changes from the normal ultrastructural anatomy of the cortex were found. Especially to be noted (Fig. 2) in contrast to the findings in the experimental animals were the compactness of the mass of cell processes composing the neuropil, the normal granularity of nuclear chromatin, and the typical cytoplasmic organelles found in neurons and other cell types. Numerous axodendritic and axosomatic synapses were present, with presynaptic endings measuring in the range of 500–600 Å, and containing normally distributed synaptic vesicles. The ribosomes of neurons showed a distinct polysomal configuration.

Animals Receiving Intracerebral Ouabain. Numerous swollen cell processes could be seen throughout the neuropil of the ouabain-injected cortex (Fig. 3), corresponding to the status spongiosus seen with the light microscope. Although the origin of many such processes could not be determined, occasional fibrils identified some of them as glial processes. Rupture of plasma membranes occasionally created an irregular and artifactual enlargement of extracellular space, but true extracellular space was not increased. Astrocytes showed swelling and clearing of the

cytoplasm, which often contained abnormal membranous profiles (Fig. 3 and 4), and occasional swollen mitochondria with either light or dense matrices (Fig. 8 and 9). There was often peripheral condensation of nuclear chromatin in these cells. Clear zones noted about cortical capillaries actually consisted of swollen astrocytic processes. The perineuronal vacuolation observed with the light microscope could be seen to consist of swollen cell processes which completely surrounded the perikarya of many neurons (Fig. 5). Even the most extreme degree of perineuronal vacuolation could usually be shown to be intracellular by the presence of double plasma membranes (Fig. 6). A few perineuronal cell processes contained glial fibrils, and others contained clustered vesicles which identified them as the presynaptic endings of axosomatic synapses (Fig. 5 and 7). Six or more of these swollen presynaptic endings were seen about a single neuronal perikaryon, with as many as 3 adjacent to each other. Such swollen presynaptic processes often achieved diameters of 2 μ or more, approximately 4 times the normal size, and contrasted with the normal synaptic terminals of the neuropil, which often occurred immediately adjacent to those which were swollen (Fig. 7). Similar swelling could often be noted in the presynaptic endings of axodendritic synapses (Fig. 8), and the swollen endings often exhibited abnormal clustering of synaptic vesicles with some reduction in vesicle number. The ratio of swollen to normal presynaptic endings varied within wide limits from field to field. Occasional swelling of dendrites was also present (Fig. 10), but was a rare finding in comparison with the frequent swelling of presynaptic terminals.

In contrast to the swelling of astrocytes and presynaptic endings were the virtually normal size and appearance of oligodendroglia, neuronal perikarya and axons. Some neurons surrounded by swollen cell processes exhibited increased density of the cytoplasm, suggesting compression (Fig. 3). These neurons also showed loss of the normal polysomal configuration of the ribosomes and slight pallor of the mitochondrial matrices (Fig. 6). A few showed a slight apparent dilatation of the cisternae of the rough endoplasmic reticulum.

Although the electron micrographs (Fig. 3–10) are from the cortex of Rat 1, which received the highest dose of ouabain, qualitatively similar though less extensive changes were found in all other rats. The rats (Rats 5 and 6) which survived beyond 2 hr. showed lesser degrees of the lesion than the rats sacrificed at 2 hr., with a gradual return toward normal with the passage of time. Rat 6, which survived for 24 hr., showed an increased number of myelin figures in the residually dilated cell processes of the cortex, but this was the only qualitative change which was not present at the earlier times.

Discussion

The behavior of the experimental rats following the intracerebral injection of ouabain was similar to that reported by Bignami and Palladini,[1,2] as were the EEG changes. The much larger doses of ouabain used by these authors were usually instantly fatal under the conditions of our experiments, but this discrepancy may be explained by the use of different ouabain preparations or different injection sites or techniques. The EEG recordings from the ouabain-injected rats are the electrical manifestations of convulsions which do not differ specifically from those associated with other experimentally induced convulsions. The pattern of behavior of the rats closely resembles the running fit of dogs or mice following ingestion of methionine sulfoximine.[6] That the spongiosus seen after ouabain injection is not the result, rather than the cause, of the abnormal motor activity is shown by the occurrence of the same lesion by light microscopy in the brains of rats immobilized by succinylcholine before ouabain injection.

The generalized swelling of astrocytes and the lack of neuronal swelling in the ouabain lesion is in contrast to the focal cytoplasmic vacuolation of astroglia and neurons reported in Jakob-Creutzfeldt disease by Gonatas, Terry, and Weiss,[7] so that at the ultrastructural level there is no close correlation between these 2 types of status spongiosus. The swelling of the astroglia seen in the ouabain lesion is similar to that observed in other experimental edemas of the cortex,[8] especially the cytotoxic type,[9] but differs from the most of these by its marked involvement of presynaptic endings. Methionine sulfoximine (MS), a substance which also produces convulsions and spongiosus of the cortex,[6] has recently been shown to cause glial swelling[10] and swelling of presynaptic endings, with clustering and loss of synaptic vesicles.[11] It has been postulated[11] that MS may act by inhibition of glutamine synthetase, a membrane-bound enzyme primarily localized in noncholinergic nerve endings. Since the Na^+-K^+-activated ATPase, which is inhibited by ouabain, has recently been localized primarily to cholinergic nerve endings,[12,13] the similarity of the behavioral response and morphological lesions produced by ouabain and MS is remarkable. As yet, it is not clear whether the 2 enzymes have any functional similarities in relation to the synapse which would explain the similar effects resulting from enzyme inhibition.

In general our observations are in good agreement with the ultrastructural studies of Zadunaisky, Wald, and de Robertis,[14,15] obtained with isolated frog brains incubated for 2 hr. in 10^{-5} M ouabain in Ringer-Conway solution. With this distantly related species and under the con-

ditions of an in-vitro as opposed to an in-vivo experiment, they also noted swelling confined to ependymoglial processes and presynaptic endings with virtual normality of neuronal perikarya, axons, and dendrites. Further support for the selective action of ouabain on glial cells with sparing of neurons comes from the work of Stefanelli, Palladini, and Ieradi,[16] who found that in trypsinized cultures of chick spinal ganglia exposed to 10^{-4} M ouabain, the glial cells showed cytoplasmic vacuolation whereas the neurons were unaffected. The direct exposure of both cell types in this in-vitro system to the action of ouabain suggests that the differential sensitivity of neurons and glia to the drug is an inherent property of the cells themselves rather than a barrier effect of perineuronal glia, which might shield neurons from direct exposure to ouabain in the in-vivo experiments. In partial contrast to these findings are the ultrastructural studies of Birks,[17] who noted swelling of neuronal perikarya and cells processes—including axons, dendrites, and presynaptic endings—with lack of swelling of Schwann cells in cat cervical ganglia perfused with plasma containing digoxin. However, it must be pointed out that Birks did find cell processes more sensitive to swelling than neuronal cell bodies, and that peripheral Schwann cells may be more comparable to the ouabain-resistant oligodendroglia of the CNS than to the ouabain-sensitive astrocytes.

The role of a Na^+-K^+-activated membrane-bound ATPase inhibited by ouabain in the active transport of these ions in many sites has been established beyond reasonable doubt by the work of Skou[18] and others. Impressive evidence exists that inhibition of ATPase by ouabain is accompanied in the CNS, as elsewhere, by intracellular accumulation of sodium and loss of potassium.[19,20] Since the concentrations of ouabain used in most of these experiments are in the range known to inhibit ATPase but well below those causing direct cytotoxicity,[21] the changes of the ouabain lesion are most likely due to its enzyme-inhibiting action, with resulting intracellular accumulation of sodium and water in those cellular elements which exhibit swelling. If this is so, the sensitivity of astroglia and certain presynaptic endings to ouabain action and the resistance of other cellular elements may perhaps be explained by differences of enzyme content or activity in these sites, by differences of membrane permeability to water and sodium, or possibly by differences of electrolyte balance in the cell types concerned. The localization, by Cummins and Hydén,[22] of membrane-bound ATPase on the inner surface of neuronal cell membranes and the outer surface of glial membranes might explain the differential sensitivity of these cells to ouabain, but the lack of Na^+ activation of the glial enzyme found by these authors and its pH optimum (pH 8.0) raise doubts as to whether it is the same as

the neuronal enzyme. Unfortunately, the ouabain sensitivity of the glial enzyme was not tested.

As suggested by Birks,[20] the greater resistance of cell bodies than of cell processes might also be due to surface-volume relationships which would permit more rapid increases in sodium concentration in the processes than in the cell bodies. This would not serve, however, to explain the sensitivity of presynaptic endings to ouabain action as opposed to dendrites or axons which have similar surface-volume ratios. The marked concentration of ouabain-sensitive ATPase in cholinergic nerve endings of rat brains which has recently been demonstrated by Albers *et al.*[12,13] provides what seems an easier way of understanding the swelling of a portion of the presynaptic terminals of the cortex in response to ouabain. The numerous presynaptic endings which are not sensitive to ouabain may be noncholinergic; may belong to different functional units from the affected endings and be separated from them by enveloping glia; [23] or, by random differences of drug diffusion, may have received lower doses of ouabain than the affected endings. The increased release of acetylcholine from cholinergic nerve endings noted by Birks [20] in cat cervical ganglia perfused with digoxin or ouabain is a possible explanation of the hyperkinetic and seizure activity noted in our rats after ouabain injection.

The finding of astroglial swelling in response to ouabain must be considered in the light of recent evidence of Hartmann,[24] Katzman,[25] and others that astrocytes may be high-sodium cells, since it is known that in general the amount of membrane-bound ATPase is inversely proportional to the sodium content of the cell.[18] As pointed out by Davson,[26] however, high-sodium cells would still presumably require the active removal of sodium to meet the requirements of the Donnan equilibrium, and might even be expected to be unusually sensitive to inhibition of the sodium pump because any influx of sodium in such cells would not be even partly balanced by efflux of potassium, as is the case with the high-potassium cell. Obviously, these experiments alone cannot answer the still-disputed [27] question of the electrolyte content of the glia. Although presumably the result of enzyme-inhibiting action, the precise pathogenetic mechanisms by which ouabain produces its highly selective effects in the CNS must await further studies of the localization and content of ATPase and electrolytes in the affected cellular elements.

Summary

1. Ultrastructural study of rat cortex following intracerebral injection of ouabain revealed marked swelling of astroglia and of certain presynaptic terminals. Swelling of glial and presynaptic cell processes in the

neuropil corresponds to the status spongiosus seen with the light microscope and also accounts for the perineuronal vacuolation. Neuronal perikarya, axons, dendrites, and oligodendroglia are relatively unaffected by ouabain action under the experimental conditions utilized.

2. The effects of ouabain presumably result from its inhibitory action on Na^+-K^+-activated membrane-bound ATPase, and its selection action in the CNS may relate to local differences in enzyme content, to differences in membrane permeability to water and electrolytes, or to cellular differences in electrolyte balance.

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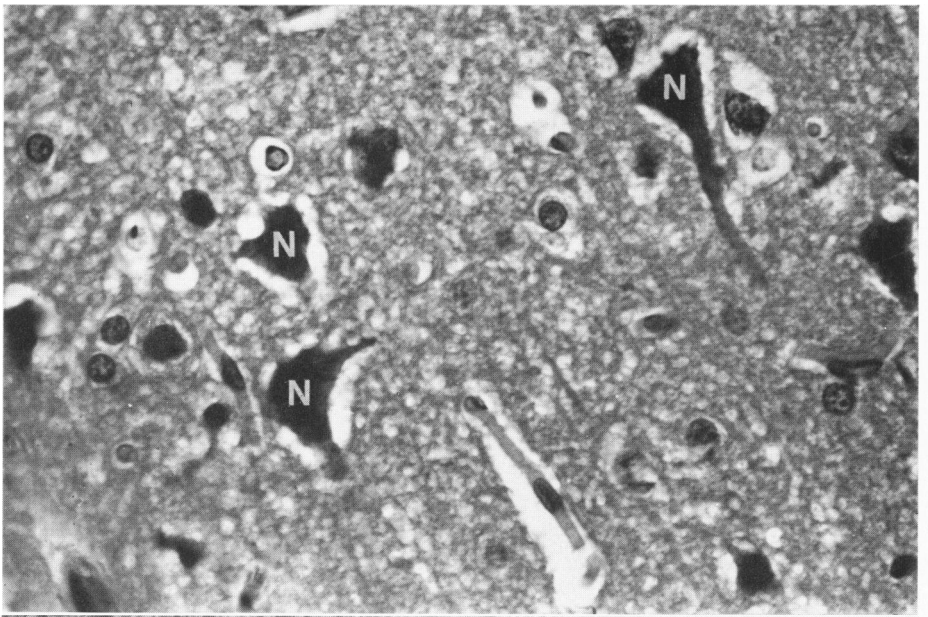
LEGENDS FOR FIGURES

FIG. 1. Cortical spongiosus following ouabain injection. Pyknotic neurons (N) are surrounded by clear vacuoles. There is clearing and swelling of cytoplasm of some glial cells and fine, diffuse vacuolation of neuropil. Pericapillary clear zones are also prominent. × 570.

FIG. 2. Cerebral cortex of rat given physiologic saline injection and sacrificed after 2 hr. Neuron (N) is surrounded by compact mass of neuronal and glial cell processes. M indicates myelinated axons; arrows, axodendritic and axosomatic synapses. × 7300.



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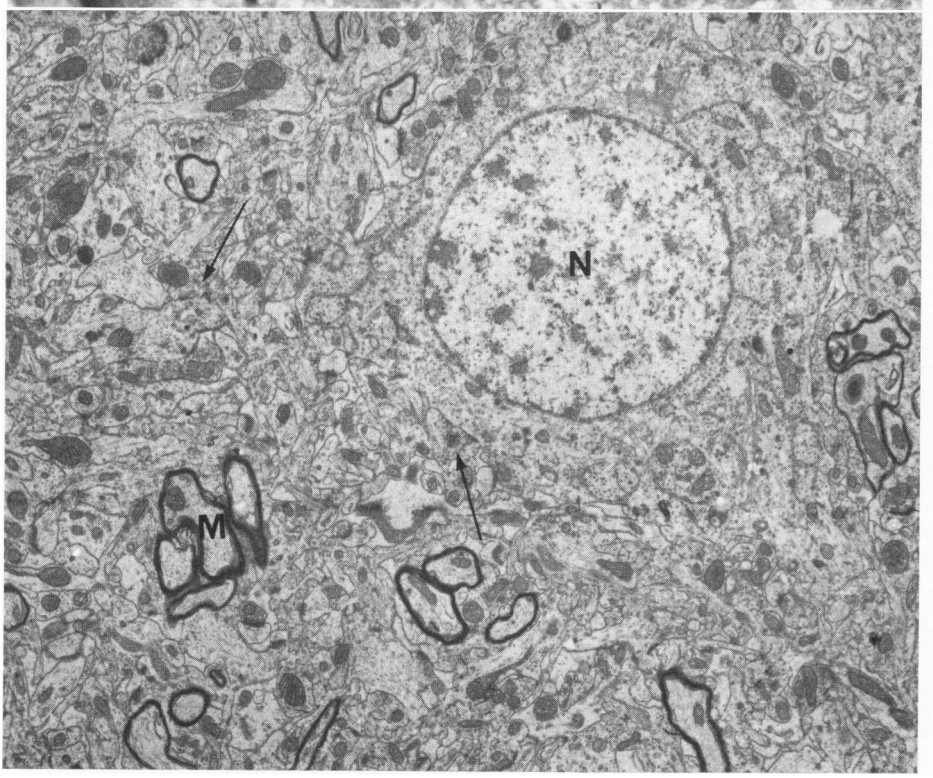

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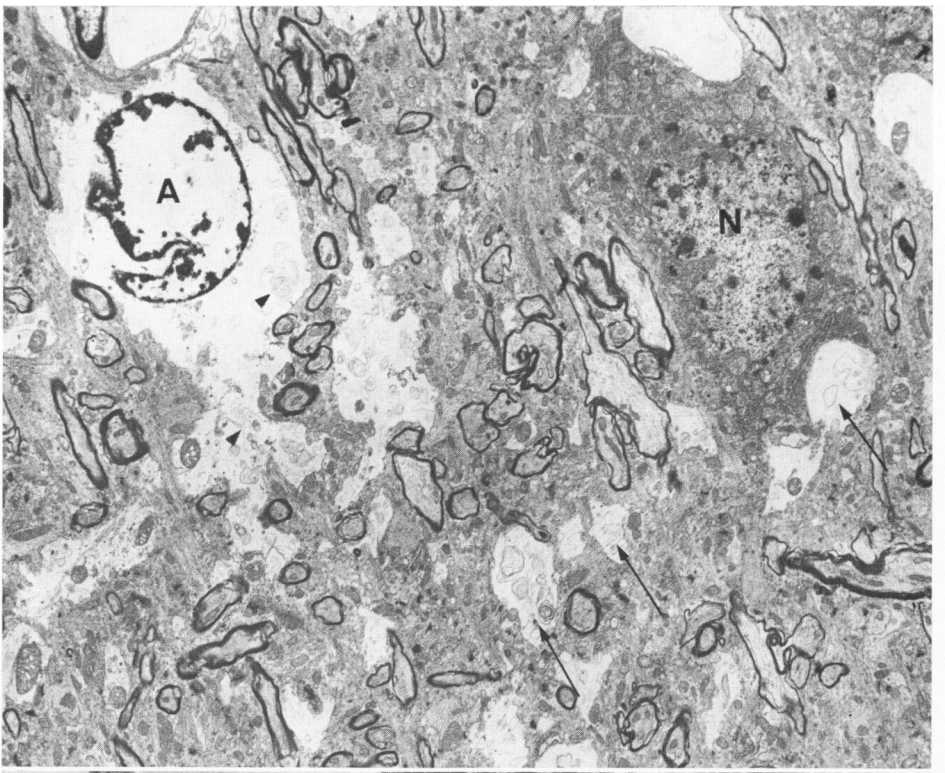
Figures 3–10 are from cortex of Rat 1, given ouabain injection and sacrificed after 2 hr.

FIG. 3. Perivascular astrocyte (A) shows peripheral condensation of nuclear chromatin and swollen, clear cytoplasm containing abnormal membranous profiles (arrowheads). Neuron (N) is partly surrounded by swollen but unidentifiable cell processes, many of which are also present in neuropil (arrows). × 4300.

FIG. 4. Cytoplasm (Cy) of perivascular astrocyte (A) is swollen. Note peripheral condensation of nuclear chromatin. C indicates capillary. × 7700.



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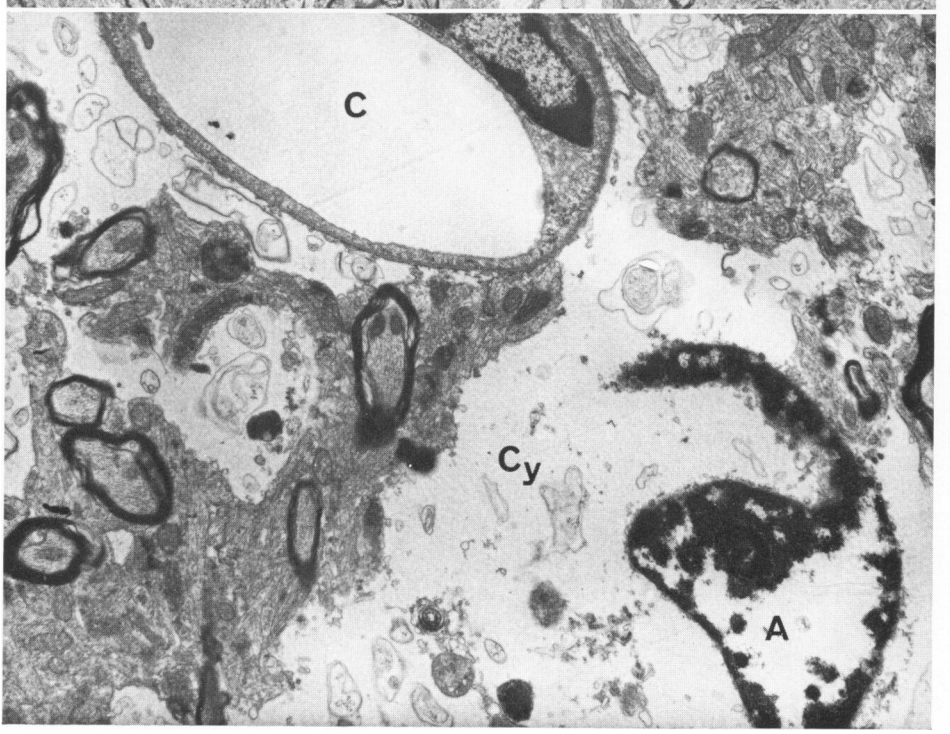

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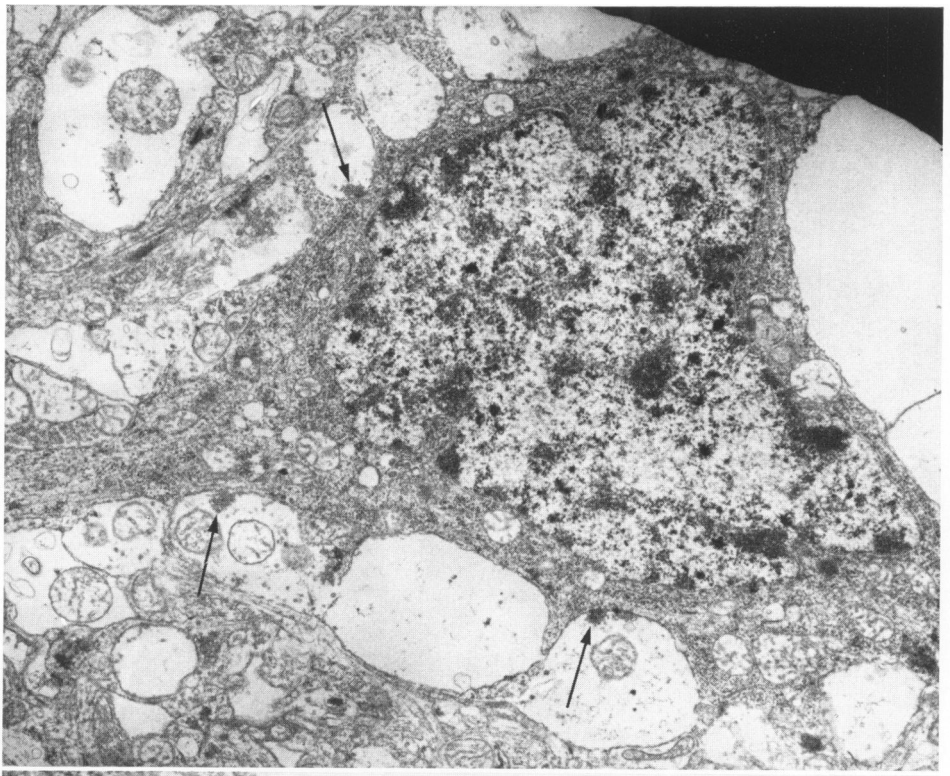
FIG. 5. Neuron with perikaryon surrounded by swollen cell processes. Presence of clustered vesicles (arrows) identifies some of these processes as presynaptic terminals of axosomatic synapses. × 9500.

FIG. 6. High power view of border between neuronal perikaryon and large perineuronal vacuole seen in light microscope. Presence of double plasma membranes (arrows) shows that even this extreme degree of perineuronal swelling is actually intracellular. Note here and in Fig. 5 slight swelling of neuronal mitochrondia (M) and loss of distinct polysomal configuration of ribosomes. × 44,000.



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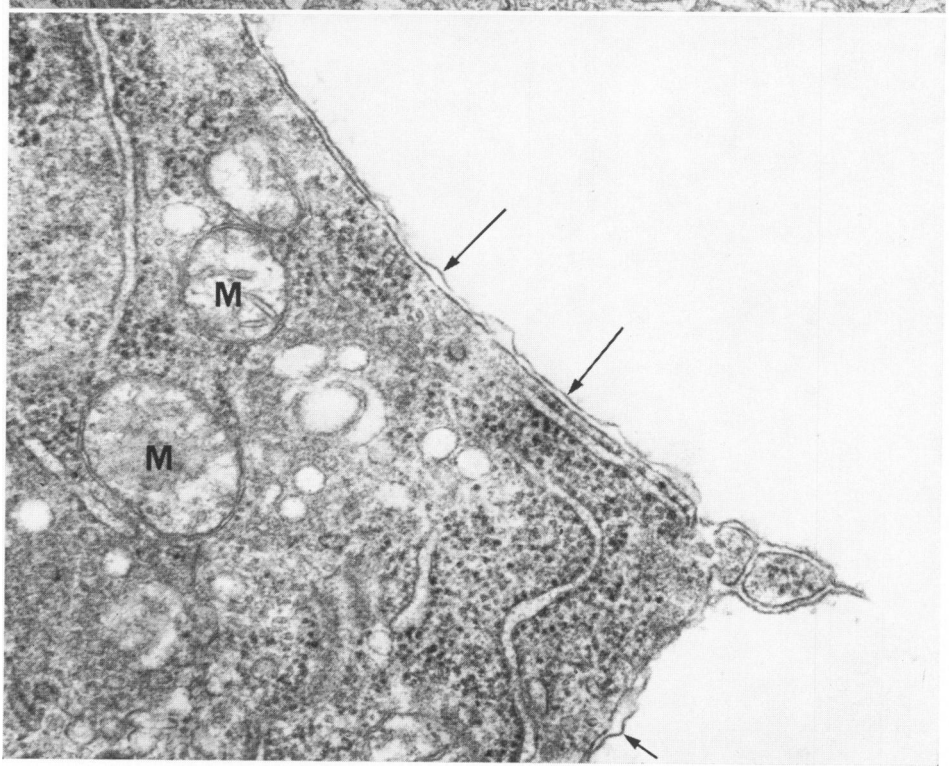

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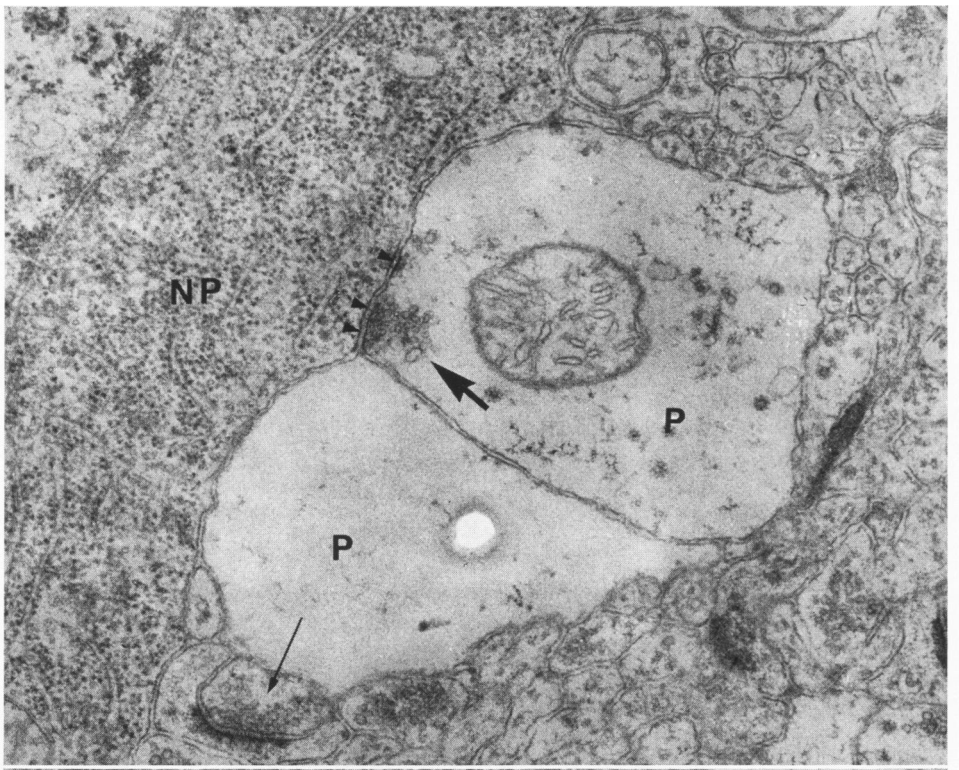
FIG. 7. Swollen cell processes (P) adjacent to neuronal perikaryon (NP). Presence of clustered vesicles (large arrow) and interstitial osmiophilia (small arrowheads) identifies one of these cell processes as presynaptic terminal of axosomatic synapse. Degree of swelling can be judged by comparison of swollen terminal with adjacent normal presynaptic ending (small arrow). × 24,000.

FIG. 8. Dendrite (D) from ouabain-injected cortex is surrounded by swollen cell processes, some of which (arrow) are presynaptic terminals. Mitochondrial swelling and pallor may be seen. × 12,700.



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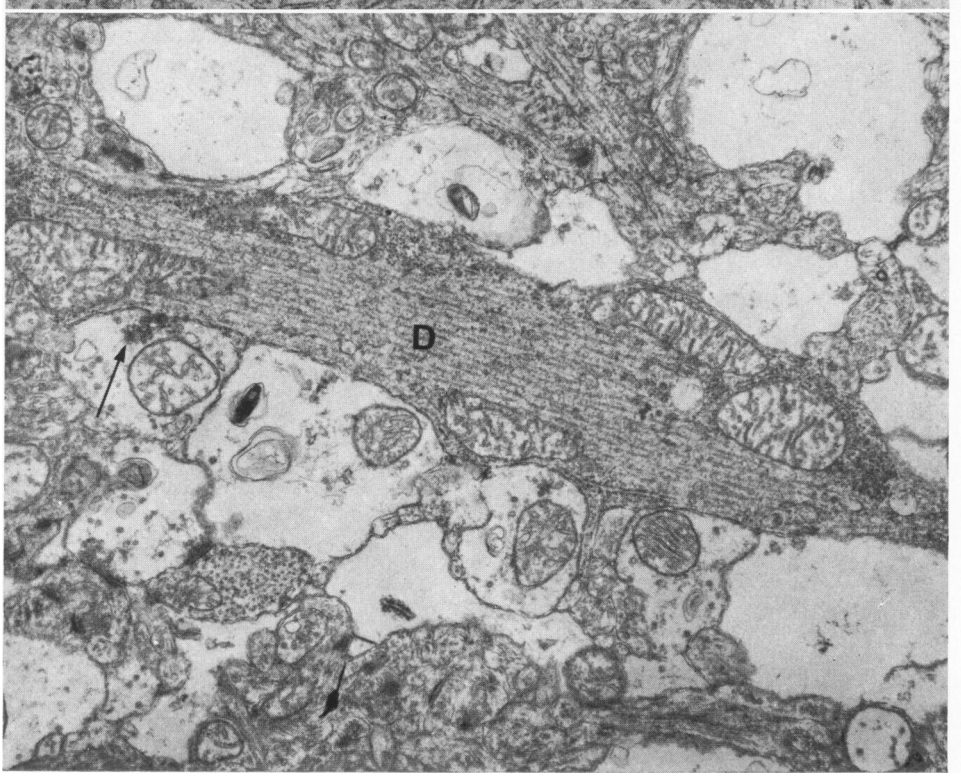

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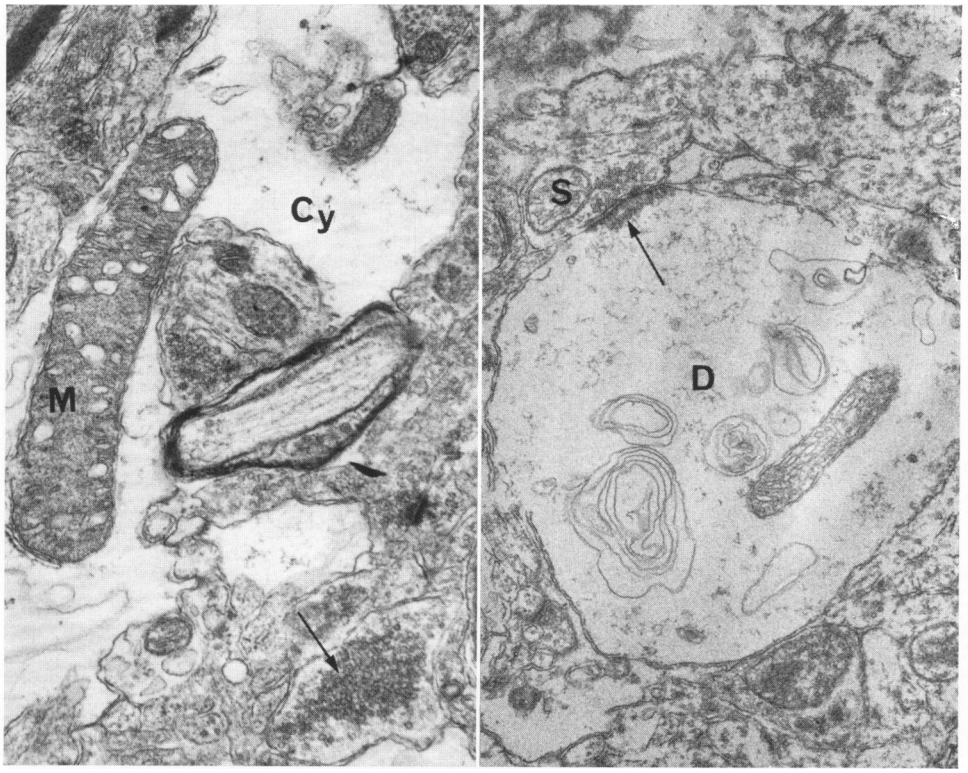

9 10

FIG. 9. Swelling of cristae and increased density of matrix is evident in mitochondrion (M) in cytoplasm (Cy) of swollen astrocyte. Clustering of synaptic vesicles (arrow) is frequently observed. × 19,600.

FIG. 10. Rare occurrence of dendritic swelling in response to ouabain, where dendrite (D) is identified by presence of subsynaptic web (arrow) and presynaptic terminal (S). × 16,800.