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Neurons containing retrogradely transported Fluoro-Gold exhibit a variety of lysosomal profiles: a combined brightfield, fluorescence, and electron microscopic study

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Summary

The advantages of axonally transported Fluoro-Gold as a retrograde fluorescent marker are numerous. The objective of the present study was to determine whether transported Fluoro-Gold is visible in either semi-thin sections for light microscopy or thin sections for electron microscopy. Rats received injections of Fluoro-Gold into either the striatum or thoracic spinal cord. After appropriate survival times, labelled neurons were observed with the fluorescence microscope in brain regions that are known to project to the injected areas.

Sections that contained labelled cells were embedded in plastic and examined with a fluorescence microscope. Semi-thin sections of unosmicated tissue displayed high-resolution fluorescent labelling of somata and dendrites. In contrast, osmicated tissue did not fluoresce, but numerous dark granules were observed in the dendritic and perikaryal cytoplasm of labelled neurons in toluidine blue stained sections that were examined with brightfield optics. The unosmicated tissue did not display these granules, and this finding suggested that the granules are composed of membranes. Neurons in other brain regions that are known not to project to the injection sites did not contain these dark granules.

Adjacent thin sections examined with the electron microscope displayed numerous electron-dense, lysosome-like organelles in the cytoplasm of labelled neurons. The electron density of these organelles was greater than that of lysosomes in unlabelled neurons. Three types of distinctive organelles were observed in these preparations: (1) relatively dense concentric lamellar bodies of various sizes; (2) heterogeneous or lipofuscin-like lysosomes; and (3) coarse grained lysosomes. Control sections and unlabelled neurons did not display these organelles. Therefore, these organelles appear to correlate with Fluoro-Gold localized within the somata and dendrites of retrogradely labelled neurons. It is not known if they are the Fluoro-Gold itself, or represent a physiological effect on membranes. The results of this study indicate that Fluoro-Gold may be useful for tract tracing at the electron microscopic level.

Introduction

During the past two decades, neuroanatomists have exploited the property of neurons to sequester certain exogenous substances from their terminals and retrogradely transport the substance to the cell body of origin for the purpose of tracing connections in the brain. The enzyme horseradish peroxidase (HRP; LaVail & LaVail, 1972) was the first substance to be widely employed. When fluorescent, intra-axonally, retrogradely transported dyes were introduced (Kuypers *et al.*, 1978; Bentivoglio *et al.*, 1980; Schmued & Swanson, 1982; Schmued & Fallon, 1986), they were embraced by many researchers for a variety of

reasons. These included increased sensitivity, compatibility with other neuroanatomical techniques and simplicity of use when compared with HRP histochemistry. A recently introduced retrograde tracer, Fluoro-Gold (Schmued & Fallon, 1986), has been shown to be highly sensitive and retained permanently in retrogradely labelled neurons. Additionally, the tracer extensively fills cells and dendrites, requires no histochemical processing, and is not taken up by intact fibres of passage.

One limitation of fluorescent retrograde tracers, however, is the inability to visualize these dyes at the

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electron microscopic level. In contrast, HRP, which is not directly visible with the electron microscope, will catalyse the oxidation of benzidine salts to produce an electron-dense reaction product (*see* Broadwell & Brightman, 1979). The goal of this study was, therefore, to determine whether retrogradely transported Fluoro-Gold that is visualized with the fluorescent microscope can be correlated with the presence of specialized organelles within the same cell when examined with the electron microscope.

Materials and methods

A 2.5% solution of Fluoro-Gold (Fluorochrome Inc.) was dissolved in 0.9% saline, and either 1.0 μ l was pressure injected into the upper thoracic spinal cord or 0.2 μ l into the striatum of young adult female albino rats. Survival intervals ranged from 5 to 10 days. Following this interval the rats were anaesthetized with Nembutal and perfused via the ascending aorta with 50 ml saline followed by 500 ml of 2% paraformaldehyde and 1% glutaraldehyde dissolved in 0.1 M neutral phosphate buffer. The brains were post-fixed overnight in the same fixative solution. Brain regions that were expected to contain retrogradely labelled cells were then cut at 50–100 μ m thickness on a Vibratome. These thick sections were then examined with a Zeiss fluorescence microscope using wide band ultraviolet excitation (emission max: 408 nm, excitation max: 323 nm). Sections with cells containing the fluorescent dye were plotted and photographed. These sections were then osmicated with 1% osmium tetroxide for 1 h. One series was left unsmicated. Both series of sections were then dehydrated and infiltrated with Spur plastic polymer. Semi-thin sections were cut from the embedded blocks and stained with toluidine blue. Lastly, thin sections were cut on an ultramicrotome and stained with 1% uranyl acetate. After rinsing with distilled water, these grid-mounted sections were examined and photographed with a Philips CM-10 electron microscope.

Controls involved the examination of non-fluorescent cells adjacent to labelled cells. Also, in cases involving a predominantly unilateral projection (e.g. nigro-striatal pathway), the unlabelled contralateral structure was used as a control. In addition, several age-matched, *non*-injected rat brains were used as controls. Injections into the spinal cord

of another fluorescent dye, rhodamine isothiocyanate (50 nl, 2.5% solution), yielded no distinctive labelling in electron microscopic preparations.

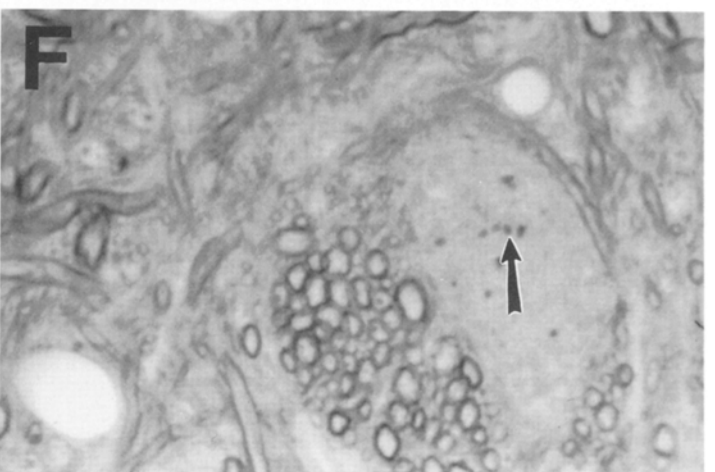
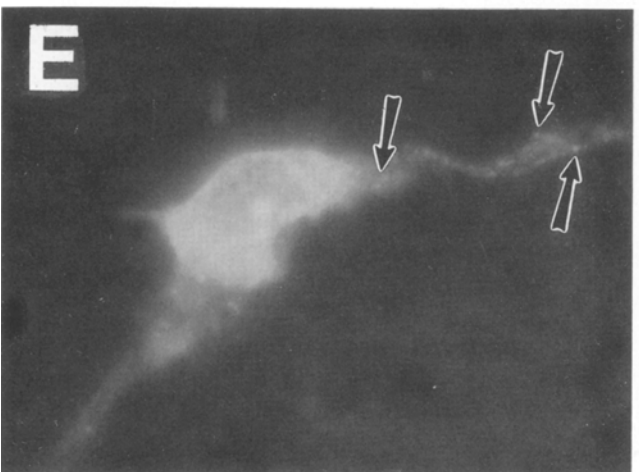
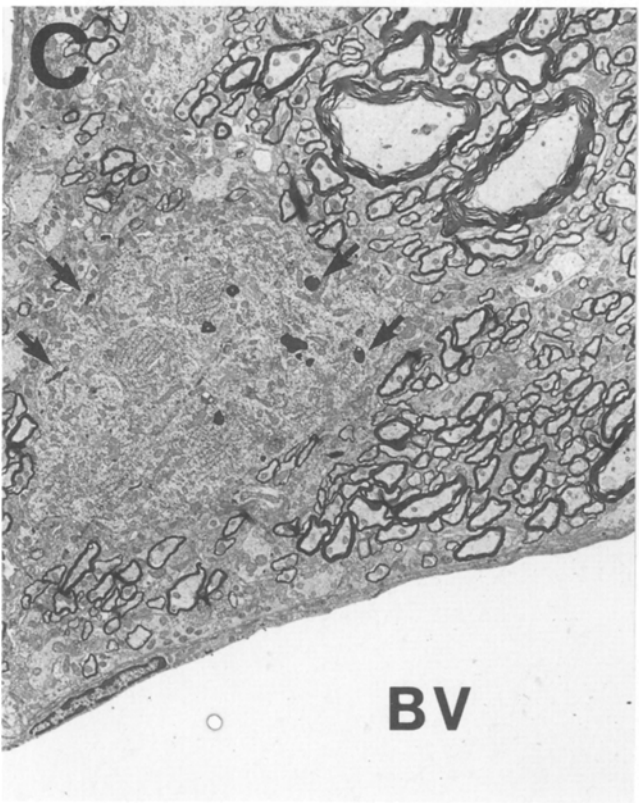
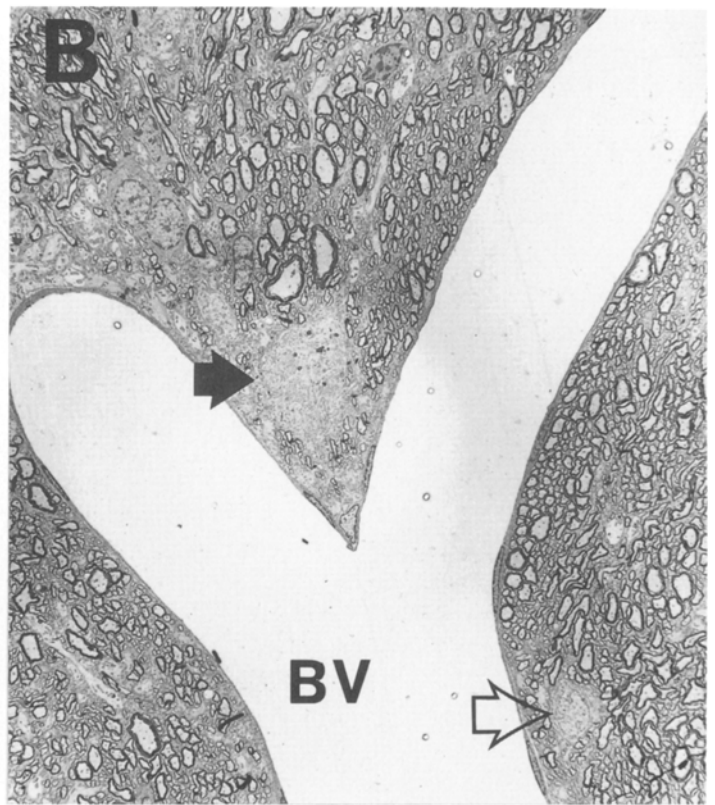
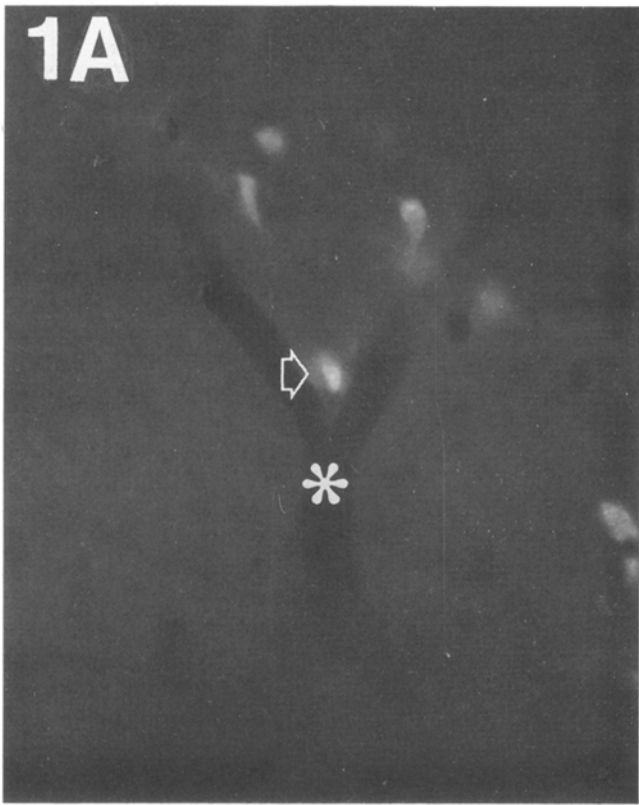
Results

Following the injection of Fluoro-Gold into the spinal cord, the retrogradely transported Fluoro-Gold was observed in neurons in all appropriate afferent sites including the brainstem reticular formation, the red nucleus, the paraventricular nucleus of the hypothalamus, and layer V pyramidal cells in sensori-motor cortex. Striatal injections resulted in large numbers of labelled cells within the substantia nigra, pars compacta – ventral tegmental area, the dorsal raphe, the intralaminar nuclei of the thalamus, and layer V pyramidal cells in the neocortex.

The fluorescence microscope revealed gold-coloured fluorescent granules in both thick Vibratome sections (Fig. 1A) and unsmicated plastic embedded semi-thin sections (Fig. 1E). The semi-thin sections displayed high-resolution fluorescent labelling of somata and dendrites. In the brightfield light microscope, dark granules could be observed in osmicated, toluidine blue stained semi-thin sections (Fig. 1F). However, when these sections were examined with the fluorescent microscope, they did not display any fluorescence. This finding suggested that osmication of sections quenches the fluorescence or dissolves the Fluoro-Gold. Conversely, it is interesting to note that the unsmicated tissue did not display any dark granules in labelled cells, and this finding suggested that some of these granules are composed of membranes.

To determine whether the same cells labelled in the fluorescence microscope were labelled in the electron microscope, sections that displayed labelled cells adjacent to tissue landmarks (e.g. blood vessels) were plotted, photographed (Fig. 1A) and then osmicated and embedded in plastic. These cells displayed dark granules in their somata, and dendrites in semi-thin sections. More importantly, they displayed a number

Fig. 1. (A) Vibratome section under ultraviolet light illumination reveals a number of Fluoro-Gold labelled cells within the mesencephalic reticular formation following an injection of the spinal cord. One of these cells (arrow) is located at the bifurcation of a blood vessel (asterisk). $\times 300$. (B) Electron micrograph of the labelled cell adjacent to the bifurcating blood vessel (BV) seen in A. This cell (solid arrow) contains a number of dark lysosomes. In contrast, an unlabelled cell (open arrow) lacks these structures. $\times 1750$. (C) Enlargement of the labelled soma and blood vessel (BV) in (B). Dense heterogeneous lysosomes (arrows) are distributed throughout the cytoplasm. $\times 3300$. (D) Enlargement of an area that shows the two indicated lysosomes on the left in (C) to show their dense lamellar structures (open arrows). Also shown in this labelled cell are heterogeneous lysosomes (solid arrows). $\times 12\ 200$. (E) A neuron in the lateral sector of the ipsilateral substantia nigra, pars compacta that is retrogradely labelled with Fluoro-Gold following an injection of the tracer into the lateral striatum. This unsmicated, semi-thin plastic section provides improved resolution over thick sections (*see* (A)) and shows individual fluorescent granules (arrows) that are observed within dendrites. $\times 720$. (F) Example of a neuron within the red nucleus that exhibits dark granules (arrow) within its perikaryal cytoplasm following injection of Fluoro-Gold into the spinal cord. This semi-thin section was osmicated and it lacked fluorescence. $\times 1200$.



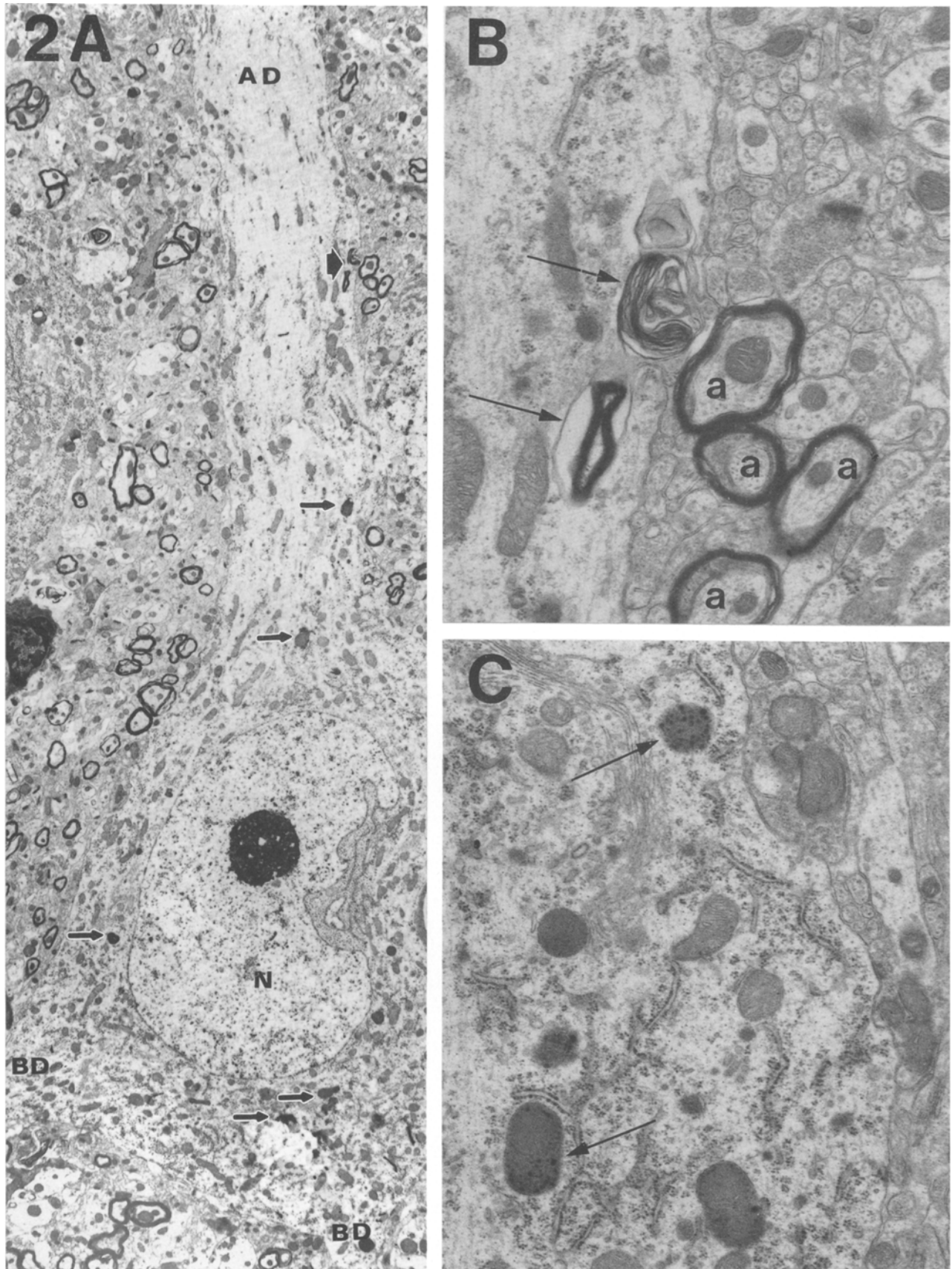


Fig. 2. (A) Electron micrograph of a pyramidal cell in layer V of sensory-motor cortex. A variety of dense, heterogeneous lysosome-like structures are indicated by arrows in the cell body and apical (AD) and basal (BD) dendrites. Two lamellar bodies in the apical dendrite are indicated by the solid arrowhead. The nucleus (N) lacks these structures. $\times 3100$. (B) Higher magnification of the two lamellar bodies (arrows) in the apical dendrite in (A). They lie within a portion of the dendrite adjacent to the neuropil that contains four myelinated axons (a). $\times 27\,000$. (C) Higher magnification of coarse grained lysosomes (arrows) with patchy electron densities from the soma of the cell in (A). $\times 18\,700$.

of interesting lysosome-like structures that are not typically found in normal brain tissue (Fig. 1C, D). These electron-dense lysosomes facilitated the identification of the labelled cell body at low magnification (Fig. 1B) and the widespread distribution of these organelles in the perikaryal cytoplasm was confirmed at higher magnifications (Fig. 1C, D). It is interesting to note that the electron-dense lysosome-like structures did not disrupt the normal pattern of organelles in the labelled cell because the Golgi complex and Nissl bodies appeared normal (Fig. 1D).

As described above, both injections of Fluoro-Gold into the spinal cord and into the striatum resulted in the labelling of neurons in many brain regions. A thorough analysis of each of these brain regions was made at the electron microscope level to determine whether the same staining pattern of lysosomal structures was observed. Since some differences were found in the labelling pattern of organelles between different brain regions, each examined region will be presented separately.

Cerebral Cortex

Following injections of Fluoro-Gold into either the thoracic spinal cord or the striatum, pyramidal cells in layer V of sensory motor cortex displayed fluorescent labelling. Neurons in other cortical layers as well as non-pyramidal cells were unlabelled. The semi-thin sections of osmicated cerebral cortex displayed a similar pattern of labelled neurons. For example, only layer V pyramidal cells were labelled and they showed numerous dark granules in the perikaryal cytoplasm and also in the apical dendrite. The dark granules in the apical dendrite were found along the full extent but it was not possible to follow any into spines. The distribution of these granules was similar to the distribution of fluorescent granules observed in the semi-thin sections from unosmicated brain sections of cortex. It is important to note that in cerebral cortex from non-injected animals the apical dendrites are lucid and lack such dark granules. Also, somata and apical dendrites of layer III pyramidal cells from injected animals lacked this staining pattern.

Electron micrographs of layer V pyramidal cells

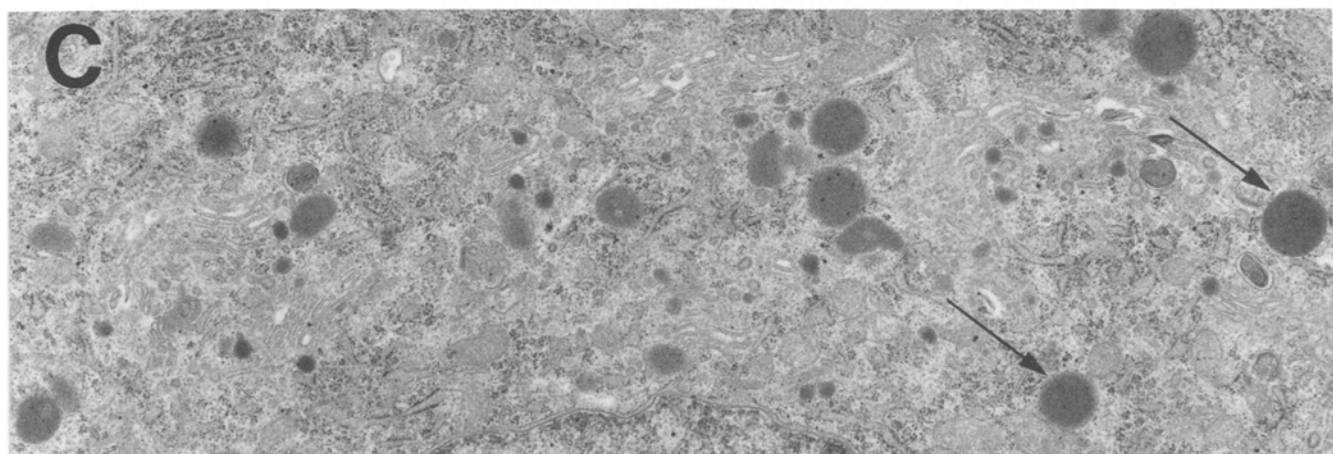
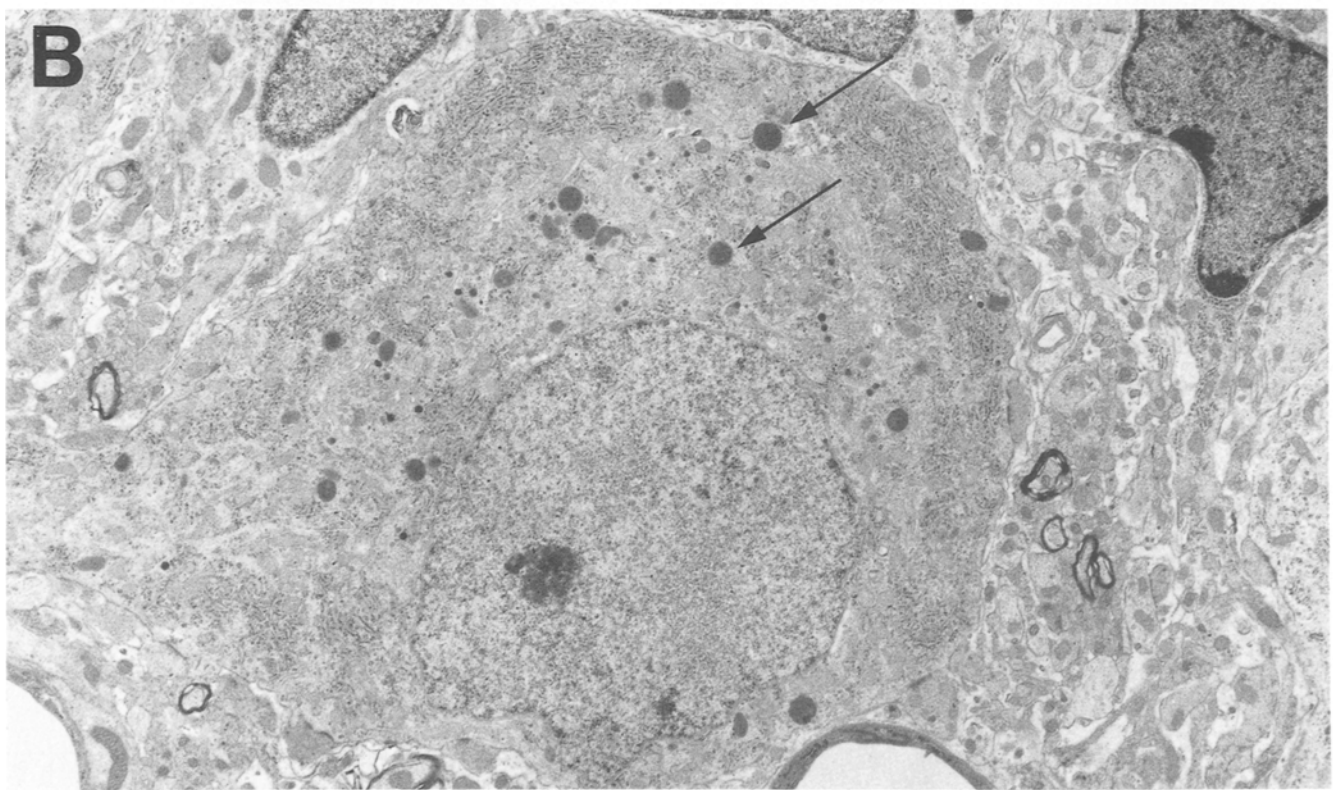
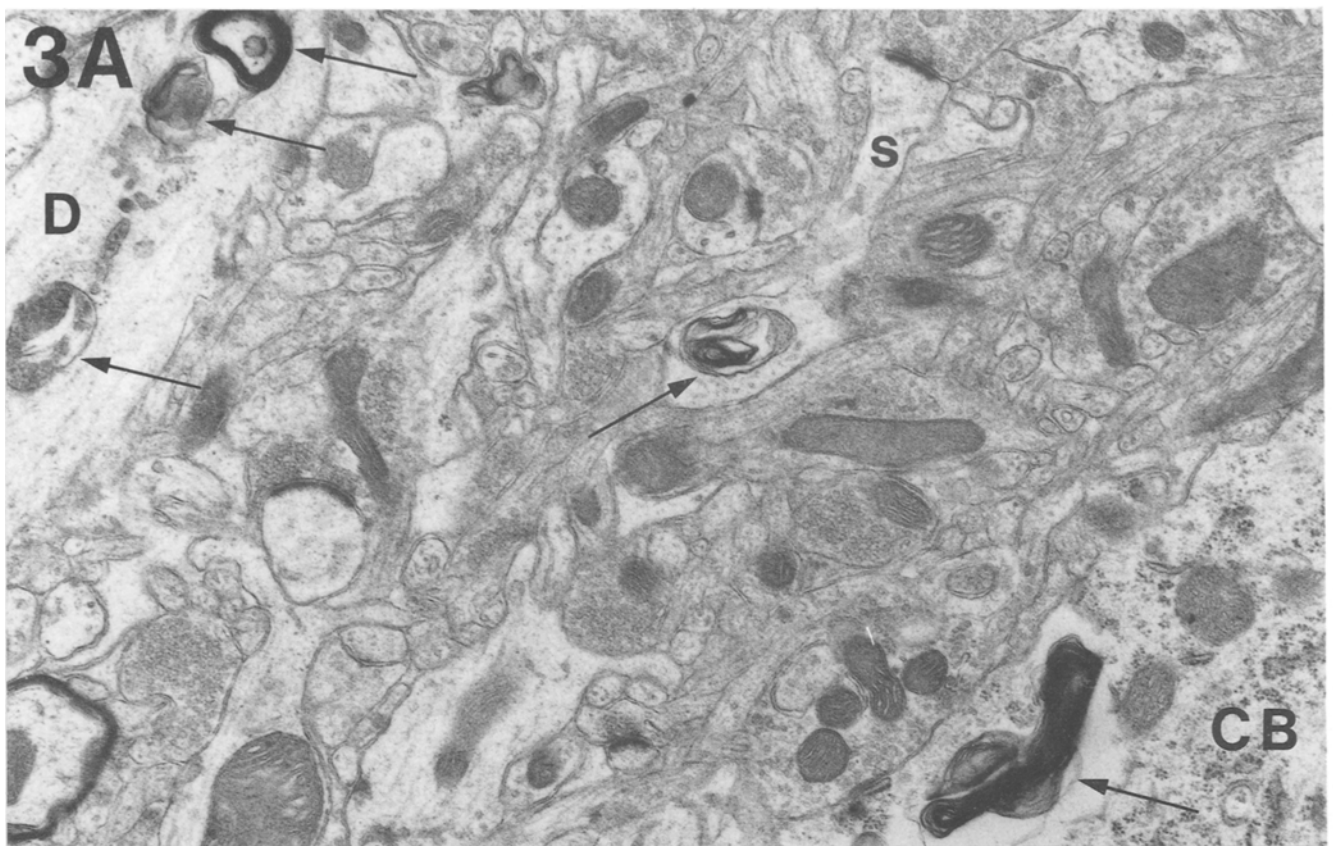
showed numerous electron-dense, lysosome-like structures in their cell bodies and dendrites (Fig. 2A). The distribution and types of lysosomes were not observed in pyramidal cells from control preparations. The lysosome-like structures found in the apical dendrites were virtually all of the lamellar body type (Figs 2B, 3A). They appeared as concentric lamellae of membrane coiled into a spiral. Often, they appeared as collapsed concentric rings. They were most commonly found in the periphery of the dendritic cytoplasm. However, spines adjacent to labelled dendrites did not display these lamellar bodies (Fig. 3A). This finding is consistent with the fluorescent distribution of Fluoro-Gold which does not include the labelling of spines of pyramidal cells. Rarely, these lamellar bodies were continuous with the extracellular space. This finding and the finding that they may occur outside the labelled structure indicate that they may be exocytosed from labelled cells. The somata of pyramidal cells displayed some lamellar bodies (Fig. 3A) but more frequently showed the dense heterogeneous lysosomes (Fig. 2C). Similar to the results shown for the mesencephalic reticular formation (Fig. 1B–D), the other organelles in the perikaryal cytoplasm were not affected by these lysosome-like structures (Fig. 2C). In addition, such lysosomes were also found in axons.

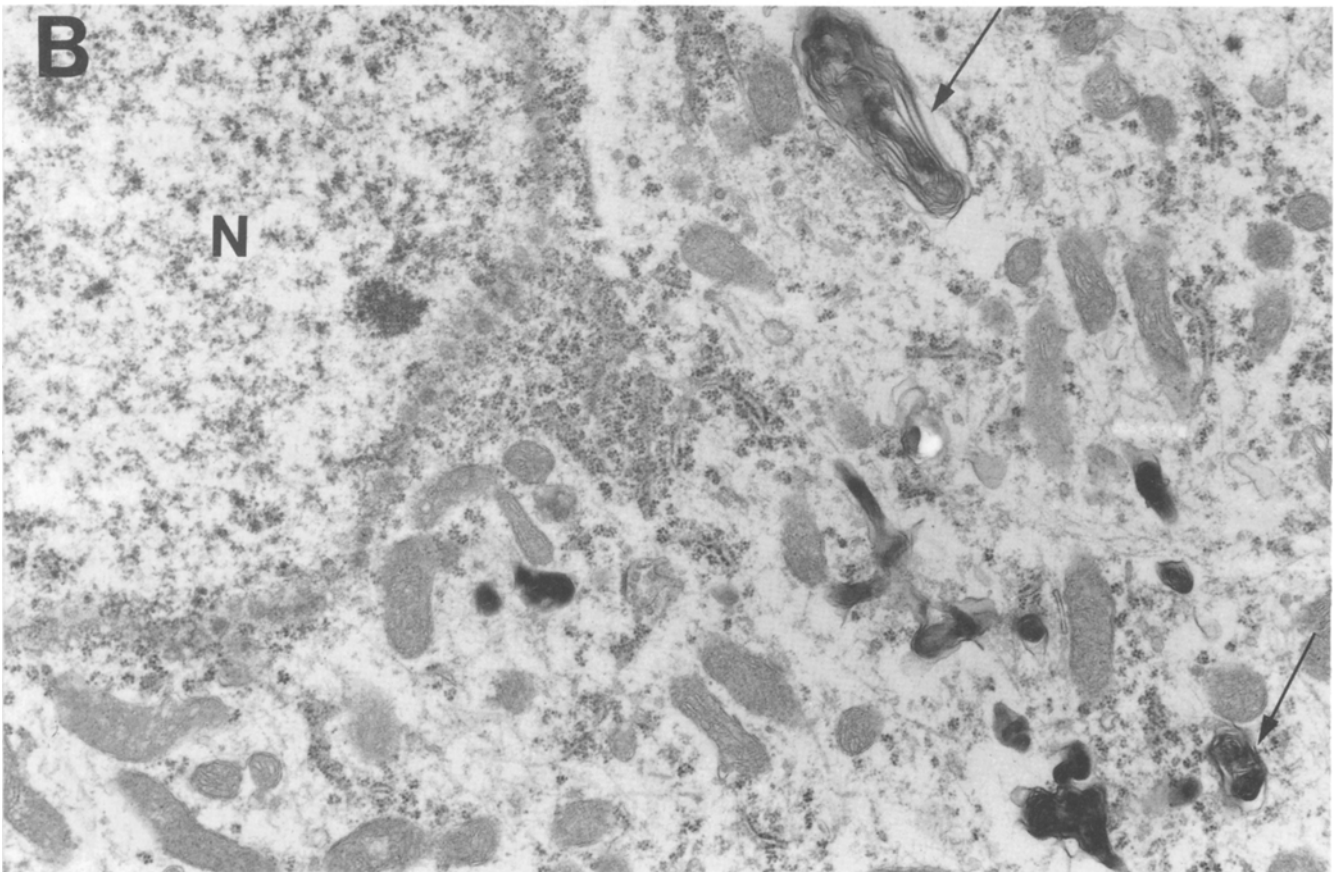
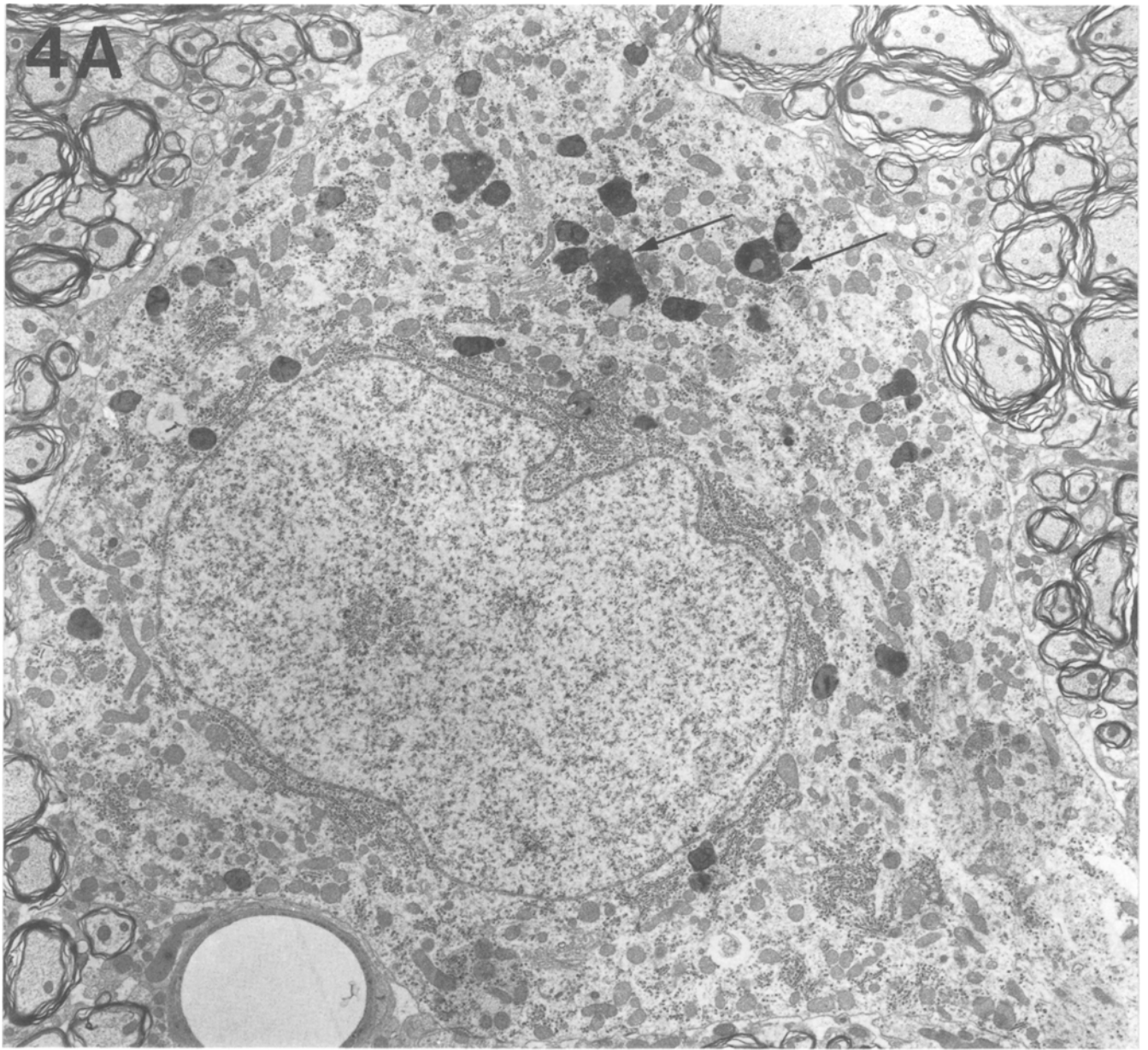
Paraventricular nucleus of the hypothalamus

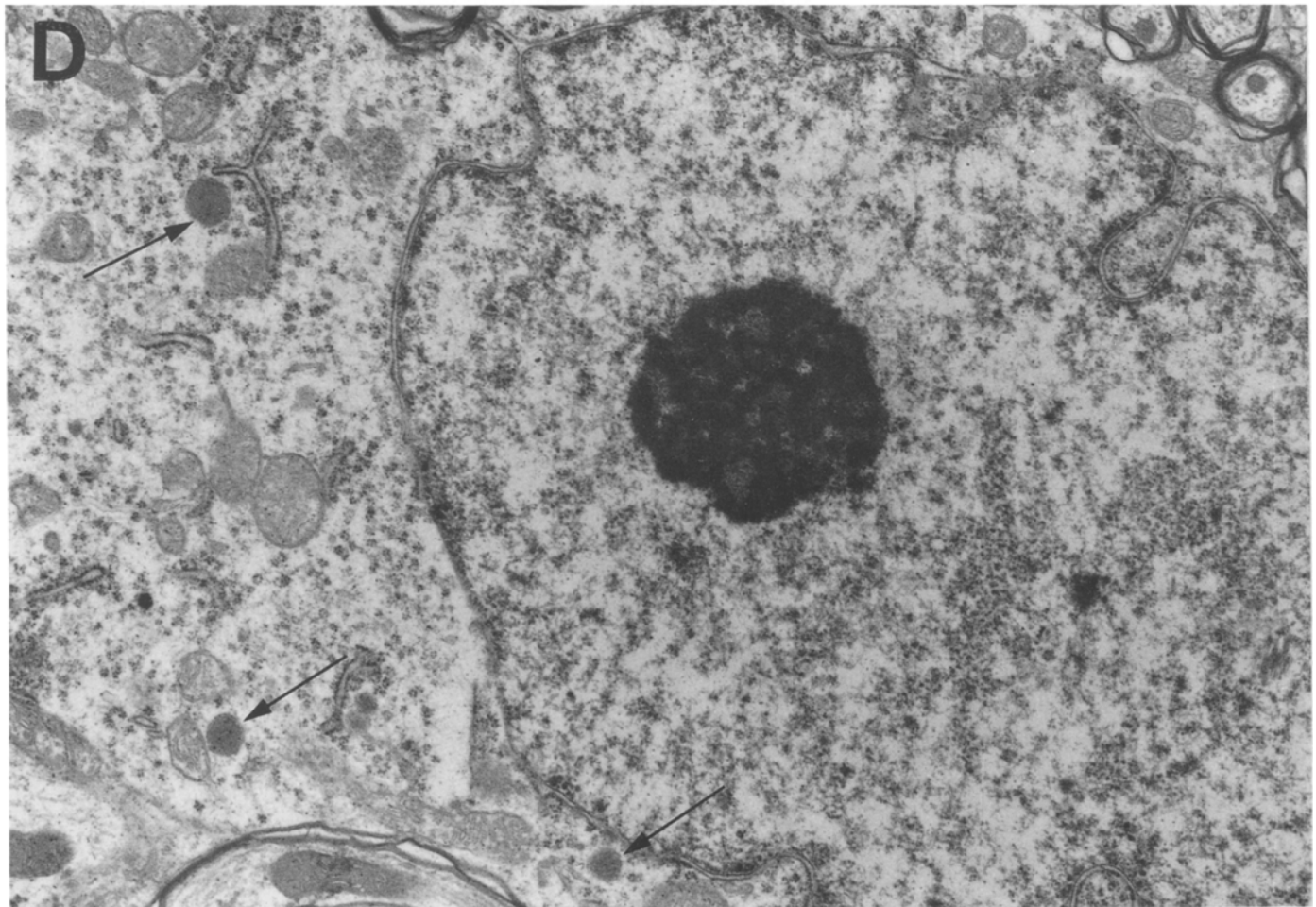
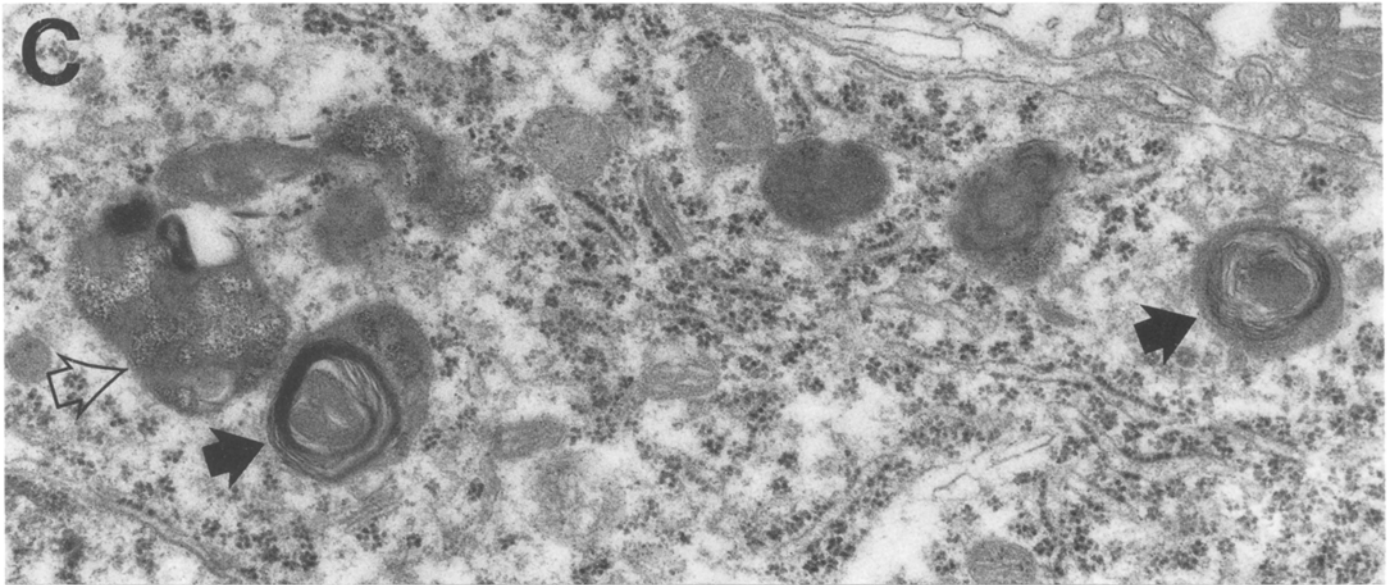
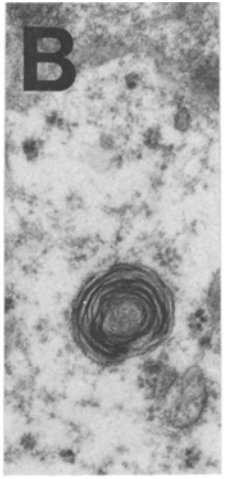
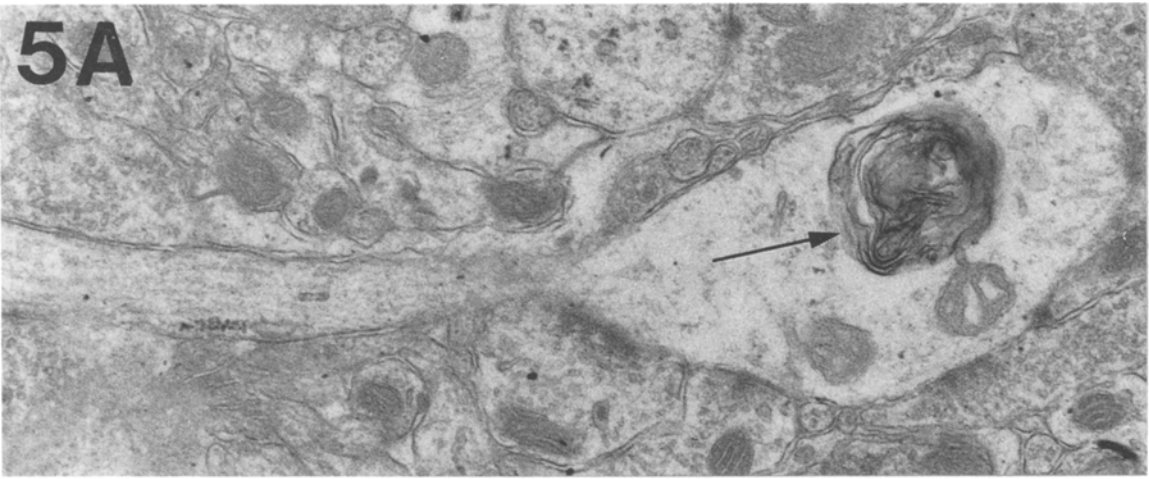
The neurons in the paraventricular nucleus that were labelled in the fluorescent microscope displayed dark granules in their perikaryal cytoplasm in semi-thin sections from osmicated tissue. The labelled neurons were distributed in the lateral parvocellular portion of the nucleus that is known to give rise to projections to the spinal cord. In electron-microscopic preparations, virtually all of the neurons in this region displayed large numbers of electron-dense lysosomes of various sizes (Fig. 3B,C). The number of these dense lysosomes was greater than that found in the labelled layer V pyramidal cells in the cerebral cortex. However, the paraventricular neurons rarely displayed any of the other two types of lysosome-like structures, i.e. they lacked the lamellar bodies and the complex lysosome-like structures that resembled lipofuscin

Fig. 3. (A) A portion of a pyramidal cell body (CB) from layer V of somatosensory cortex and the adjacent neuropil. A variety of lysosomal-like structures (arrows) are observed within the cell body, an apical dendrite (D), and a dendrite from which a spine (s) appears to arise. Lamellar bodies were not found in spines. $\times 25\ 000$. (B) Low magnification and (C) high magnification of a labelled neuronal cell body in the paraventricular nucleus of the hypothalamus. This cell exhibits a large number of round, various-sized, relatively homogeneous granular lysosomes (arrows indicate the same two lysosomes). Lamellar bodies were not observed in this labelled cell. $\times 6600$ and $\times 12\ 000$, respectively.

Fig. 4 (A) Low-power electron micrograph of a cell body in the red nucleus following a Fluoro-Gold injection into the spinal cord. This labelled cell body displays large, irregular, heterogeneous lipofuscin-like structures (arrows). $\times 6000$. (B) Enlargement of a portion of a cell body from another labelled rubral neuron showing the details of electron-dense lamellar bodies (arrows) adjacent to the nucleus (N). $\times 22\ 000$.







granules. The dense lysosomes of various sizes that were found in somata were also observed in the dendrites of the labelled paraventricular neurons.

Red nucleus

Neurons in the red nucleus that were labelled following injections of Fluoro-Gold into the thoracic spinal cord were of the magnocellular type. These neurons were found to display numerous dark granules in their perikaryal cytoplasm in semi-thin sections from osmicated and plastic embedded tissue (Fig. 1F). These labelled cells were observed in the electron microscope to contain an array of lysosome-like structures in their somata and dendrites (Figs 4A, B, 5A). The red nucleus displayed neuronal somata that contained the highest proportion of lamellar bodies (Fig. 4A). Similar to the labelled cells in the cerebral cortex, the dendrites of rubral neurons displayed mainly the lamellar bodies in their dendrites (Figs 5A, B). The concentric lamellae of these lamellar bodies were either tightly wrapped with a granular core (Fig. 5B) or loosely spiralled with an amorphous core (Fig. 5A). It is interesting to note that the sizes of these lamellar bodies varied but were never less than 0.1 μm in diameter. This may explain their absence in small calibre dendrites in this nucleus (Fig. 5A) and in dendritic spines in the cerebral cortex (Fig. 3A).

The labelled rubral neurons displayed a mixed type of lysosome that was not found as commonly in other brain regions. This type displayed an irregular shaped lysosome that contained lamellar inclusions. These inclusions were always extremely electron dense. This type of lysosome was found adjacent to the lipofuscin-like type that displayed pale inclusions. Although the fixation of the numerous myelinated axons that are found around the somata of rubral neurons was not optimum in these experiments (Fig. 4A), the preservation of the internal structures of the rubral neurons was of good quality (see granular endoplasmic reticulum in Fig. 4A, B). Therefore, these lysosome-like structures are not caused by inadequate fixation but are most likely due to the labelling of the neuron with retrogradely transported Fluoro-Gold. Further proof for this notion was obtained by analysing the unlabelled small neurons in this nucleus (Fig. 5D). Such neurons displayed a thin rim of perikaryal cytoplasm that contained a few organelles but lacked the ly-

some-like structures found in labelled cells. The lysosomes that were found in these neurons were less electron dense than those found in the labelled rubral neurons (cf. Figs 4A, 5D). Furthermore, the type of lysosome observed in the small neurons in the red nucleus lacked any complexity and they appeared as regular round structures.

Substantia nigra

Neurons in the pars compacta of the substantia nigra were labelled with Fluoro-Gold following injections into the ipsilateral striatum. These neurons were also found to contain dark granules in their perikaryal cytoplasm in semi-thin sections from osmicated tissue. The pattern of labelling observed in the electron microscope for these neurons was similar to the pattern found in the red nucleus. Thus these neurons displayed numerous lysosome-like structures of all three varieties – lamellar bodies, dense lysosomes and lipofuscin-like lysosomes (Fig. 5C). The number and variety of lysosome-like structures in labelled neurons were much greater than those found in nigral neurons from control preparations.

Summary of lysosome-like structures in labelled neurons

Electron microscopy revealed three populations of lysosomes typically observed in the cell bodies and dendrites of neurons which exhibited fluorescence at the light microscopic level. The three morphological profiles were as follows:

1. Lamellar bodies. These lysosome-like structures are characterized by dense concentric lamellae that resemble endoplasmic reticulum without any associated ribosomes and that often surround a fine grained central region (Figs 1D, 2B, 3A, 4B, 5A–C).
2. Heterogeneous or lipofuscin-like lysosomes. This population of profiles is characterized by large, dark, irregularly shaped structures which appear to contain a variety of both light and dark structures (Figs 4A, 5C).
3. Coarse grained lysosomes. These large lysosomes typically exhibit an irregular coarse grained appearance over most of the structure.

Other inclusions may also be observed (Figs 2C, 3C). Although the majority of lysosomes observed could be

Fig. 5 (A) An example of a main dendrite in the red nucleus that contains a lamellar body (arrow). Note that lysosomal-like structures are not found in the small dendritic branch on the left. $\times 38\,000$. (B) Enlargement of a lamellar body with regular concentric rings of membrane found in a dendrite that was continuous with the cell body shown in (B). $\times 31\,000$. (C) A variety of lysosomal profiles are found in a labelled soma from a neuron in the substantia nigra following an injection in the striatum. They include lamellar bodies (solid arrows) and lysosomes containing heterogeneous granular material (open arrow). $\times 33\,000$. (D) A small unlabelled neuron from the red nucleus that does not project to the injection site in the spinal cord. A few small, round, pale, homogeneous lysosomes are observed within the cytoplasm (arrows). Note the absence of lamellar bodies and electron-dense heterogeneous lysosomes. $\times 21\,000$.

classified into one of the aforementioned categories, examples were found in which a lysosome may exhibit mixed morphologies or those common to a few of the groups (Fig. 5C).

Discussion

This study presents data from a number of brain regions to show that the presence of the retrogradely transported fluorescent tracer, Fluoro-Gold, as observed in the fluorescence microscope, is correlated with an abundance of several types of lysosomes at the electron microscopic level. It is not yet known whether any of the lysosomal profiles observed actually represent a direct visualization of Fluoro-Gold, or rather a physiological reaction to the dye. Simple damage by injection is unlikely to explain this phenomenon since HRP injections (Broadwell & Brightman, 1979) have not been reported to induce the lamellar bodies or heterogeneous lysosome profiles described in this study. This does not, however, rule out the possibility that the Fluoro-Gold may induce a more specific type of damage.

Several other interpretations of the three types of lysosomal profiles observed in Fluoro-Gold containing neurons are possible. One is that each profile represents a distinct and separate process. Thus, for example, the lamellar bodies may represent portions of axonal membrane and myelin, which were endocytosed as the result of axonal damage. Similarly, the lysosomes containing coarse grained material may represent an accumulation of Fluoro-Gold that is combined with osmium tetroxide to form a complex. Furthermore, the heterogeneous profiles may represent a fusion of either of the above two forms with a primary digestive lysosome to form a secondary, or lipofuscin granule.

A second interpretation of the three types of lysosomal profiles is that they are all simply different phases of the same process. Thus, for example, the lamellar bodies may represent the first phase consisting of endocytosed plasma membrane surrounding a Fluoro-Gold and osmium tetroxide complex. The second phase may be represented by the heterogeneous lysosomes in which the membranes are partially degraded. As the process continues, the membranous inclusions are degraded, leaving mainly a coarse grained lysosome.

A third interpretation is that the three lysosomal profiles represent three independent processes. If this is true, only one profile would be characteristic of the dye itself, although it might be associated with other lysosomal profiles resulting from degenerative reactions at the injection site. The fact that different distributions of lysosome-like structures were found in brain regions following the same injection site adds

support for this notion. For example, neurons in the paraventricular nucleus rarely displayed lamellar bodies whereas those in the red nucleus commonly exhibited this type of lysosome-like structure.

Although the controls did not exhibit the aforementioned types of lysosomal profiles, one cannot rule out the possibility that such profiles might vary in their quantity and distribution in other brain regions containing the tracer. This analysis of multiple neuronal systems suggests that the reported phenomenon is not system specific. However, the quantity and variety of lysosome-like structures can vary for neurons between brain regions. This variation may relate to the metabolism of neurons found in different brain regions. The use of young adult animals is important, as only homogeneously dense lysosomes are typically found in such animals (Peters *et al.*, 1976). These authors report that this is in contrast to older animals whose lysosomes may contain membranes in stacks, whorls, granules of various sizes and densities, and lucent vacuoles. It should also be pointed out that older animals also frequently exhibit lipofuscin granules (Toth, 1968) reminiscent of the large irregular heterogeneous structures found in this study.

Other researchers have reported the induction of irregular lysosomal profiles as the result of various aetiologies. Lamellar bodies have been reported within neurons of the hypothalamic-hypophyseal system as a response to salt stress (Broadwell & Cataldo, 1983; 1984). Injection of leupeptin or chloroquine into the lateral ventricle has been reported to produce lysosomes or lipofuscin granules containing granular substance, heterogeneous material, or swirls (Ivy *et al.*, 1984). Intracellular storage diseases such as Tay-Sachs disease are characterized by neurons containing lysosomes with laminated swirls (Terry, 1971; Holtzman, 1976).

Since the induction of irregular lysosomal profiles may result from a number of causes, the presence of dense irregular lysosomes cannot unequivocally be attributed to the presence of Fluoro-Gold. This does not, however, preclude the exploitation of this phenomenon as an anatomical technique when applied to animals such as normal young rats which do not typically possess a significant number of large, dense, irregular lysosomes. In such animals it is thus possible to determine a neuron's projection first at the fluorescence light microscopic level, and then at the ultrastructural level. The fact that these structures are found in most dendrites except for smaller calibre ones and dendritic spines may help with the demonstration of afferents to these labelled neurons. As Fluoro-Gold has been demonstrated to be compatible with virtually all commonly used neuroanatomical techniques, it should also be possible to combine these techniques at the electron microscopic level.

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