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# Use of an Amino-Cupric-Silver Technique for the Detection of Early and Semiacute Neuronal Degeneration Caused by Neurotoxicants, Hypoxia, and Physical Trauma

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DE OLMOS, J. S., C. A. BELTRAMINO AND S. D. OLMOS DE LORENZO. *Use of an amino-cupric-silver technique for the detection of early and semiacute neuronal degeneration caused by neurotoxicants, hypoxia, and physical trauma.* NEUROTOXICOL TERATOL **16**(6) 545–561, 1994.—A new amino-cupric silver protocol is described for detection of neuronal degeneration. We describe its selectivity in visualizing both early and semiacute degeneration after intracerebral or systemic administration of a variety of neurotoxicants in rats, and after transient ischemic episodes in gerbils. As early as 5 min after physical trauma, or 15 min following either intrastriatal injections of glutamate analogs or exposure to ischemic episodes, neuronal silver staining was evident at primary sites of trauma (i.g. injection sites) and at hodologically related secondary sites. With intoxication by peripheral injections of trimethyltin (IP) or intracerebral injections of Doxorubicin, reproducible patterns of degeneration are demonstrable after 24 h or after 9–13 days, respectively. The amino-cupric silver method permits simultaneous detection of all neuronal compartments against a clear background. Degeneration in the neuronal cell bodies, dendrites, axons and terminals, as well as the recruitment of new structures in a progressive pathologic process, could be accurately followed. The inclusion of new reagents increased the sensitivity vis-à-vis previous versions of the cupric-silver method. The advantages and disadvantages of the current method in comparison with other means of neurotoxic assessment are discussed in detail, with special emphasis on its unique ability to discriminate irreversible degenerative phenomena and degeneration of axonal components in cases where the cell body remains apparently intact. The amino-cupric silver method is an especially useful tool for surveying neuronal damage in basic neuroscience investigations and in neuropathologic and neurotoxic assessment.

Brain    Silver method    Neuronal degeneration    Excitotoxicity    Ischemia    Physical trauma
Neuropathology    Neuroscience    Neurotoxicology    NMDA    Glutamate analogs

THE USE OF silver impregnation procedures for the study of degenerative changes in the nervous system has increased significantly during the past few years, as investigators have rediscovered the unique value of these procedures in identifying damaged neuronal elements. Recent studies include the demonstration of axonal projections arising from neurons undergoing transneuronal anterograde degeneration (17,41), the identification of cells undergoing developmental neuronal death (50,66), or the detection of neuronal damage by neurotoxins including colchicine, 6-hydroxydopamine, puromycin,

tetanus toxin, capsaicin, and organometallic compounds (4–5,26,35,57,61). The silver methods have also been used to detect the excitotoxic effects of glutamate and its analogues, the action of 3-acetylpyridine and diazepam, as well as the effects of ischemia, drugs of abuse, epilepsy, hypoglycemia, proton irradiation, and other forms of trauma (3,9,20,26,28, 30,35,43,44,46,51,54,56,57,62,63). Many of the above investigations relied on the use of the Cupric-silver method.

Compared to the earlier versions, the new protocol is less elaborate and can be applied with greater latitude in some of

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the more sensitive steps (16,23,25,64). Reproducible results, therefore, can be obtained even by relatively inexperienced technical staff after a reasonable training period. The signal to noise ratio has also improved, both as a result of increased signal intensity and by a reduction of the background staining. In fact, the clear background obtained allows for efficient scanning with darkfield optics, and could potentially lead to automated evaluation of degeneration in certain instances.

## METHOD

### Normal Material

Brains from normal young adult rats, guinea pig, armadillos (Chaetophractus vellerosus), rabbits, and monkeys (macaque, squirrel monkey, and marmoset) were examined for the distribution of normally occurring granular argyrophilic neurons.

### Experimental Material

Rats of both sexes weighing from 200 to 300 g were used for experiments involving: (a) physical trauma (introduction of a pin in the cerebral tissue), (b) electrocoagulation (electrochemical trauma), (c) excitotoxic damage (intracranial delivery of different glutamate analogues), (d) injections of doxorubicin (suicidal retrograde transport), and systemic injections of (e) trimethyl tin (intoxication with a trialkyltin compound). All animals were operated under chloral hydrate anaesthesia (6 mg/kg IP). Experiments b to d were carried out using a stereotaxic approach.

### Direct Physical Trauma

In 2 rats a stainless steel insect pin was lowered into the cerebral cortex after drilling a small hole in the skull that caused minimal damage to the meninges. Survival time: 5 min and 15 min. In 10 rats the olfactory bulb was aspirated unilaterally under direct observation. Following bulbectomy the cavity was filled with gelfoam and the wound sutured. Survival time raised from 2 to 30 days.

### Electrochemical Trauma

In two cases, a stainless steel electrode less than 0.25 mm in diameter and insulated except at the tip was inserted stereotaxically in the nucleus accumbens and an electrolytic lesion produced by passing a DC anodal current of 0.8 to 1.2 mA for 15 s. Survival time: 2 days.

### Excitotoxic Damage

In 100 female rats the histological changes that follow injections of quinolinic acid into the striatum or the cerebral cortex were examined after survival times that ranged between 15 min and 21 days. Different concentrations (15 to 240 nmoles/0.5 $\mu$l) of this excitotoxic aminoacid were delivered by hydraulic pressure through stereotaxically guided 10–20 $\mu$m diameter micropipettes. In four additional cases, microinjections of quisqualic acid (60 nmoles/0.5 $\mu$l) ($n$ = 2), ibotenic acid and kainic acid (0.4 nmoles/0.5 $\mu$l) ($n$ = 2) were delivered into striatum and the animals allowed to survive for 2 days.

### Suicidal Retrograde Transport

In 30 female rats, 20–50 nl of a 4 to 5% Doxorubicin (sold commercially as Adriamycin) solution was injected sterotaxically in the main olfactory bulb, hippocampal formation, cerebral cortex, striatum, parabrachial area, and cerebellum using glass micropipettes 10–30 $\mu$m in diameter. Survival time ranged from 7 to 23 days.

### Intoxication With a Trialkyltin Compound

Seven adult male Long Evans rats (250–300 g body weight) were given a single IP injection of trimelhyltin hydroxide (8 mg/kg) or chloride (7.6 mg/kg) and housed in separated containers for survival times of 1 ($n$ = 2), 2 ($n$ = 2), 4 ($n$ = 2), and 7 ($n$ = 1) days.

### Ischemia

The pattern and time course of postischemic neuronal regression and degeneration were studied in 75 adult female Mongolian gerbils, which underwent 10 min reversible bilateral common carotid occlusion performed by clamping. Survival times ranged from 5 min to 72 h following recirculation.

All animals were fixed by perfusion under chloral hydrate (rats, gerbils, armadillos), ether (guinea pigs, rabbits) or pentobarbital (rats, monkeys) anesthesia. Usually this was accomplished by a brief transcardial perfusion of a blood-washing solution consisting of 0.8% sucrose, 0.8% NaCl and 0.4% glucose followed by 4% paraformaldehyde in 0.1M cacodylate buffer (pH 7.4). The brains were left in the skull for 24 h at 4°C. After removal from the skull, the brains were postfixed in cold fixative containing 30% sucrose until they sank to the bottom of the container. The brains were cut at 30 to 35 $\mu$m either on a freezing microtome, a vibratome or a cryostat, and the sections collected serially in compartmented plastic boxes containing the same type of fixative used in the perfusion procedure. The sections were kept at 4°C for variable times ranging from 1 day to 6 months, or in 30% sucrose in 0.07 M cacodylate buffer (pH 7.2) when staining procedures other than the Aminoacidic-Cu-Ag were used.

## PROCEDURE

(Note: a complete flow chart of the procedure is shown in Fig. 10).

### Chemicals and Method

### I. Fixation and Sectioning

A. Rinse
   0.4% glucose, 0.8% sucrose, 0.8% NaCl
B. Fixation
   Alternative Fixatives
   1. 4% paraformaldehyde, 0.1M cacodylate buffer, pH 7.2–7.4 (adjust with 50% HCl)
   2. 4% paraformaldehyde, with 50 mg of sodium sulfite/liter. Adjust pH to 7.6–7.8 with 0.2 M borate buffer, pH 8.5.[2]
   3. 4% paraformaldehyde, with 50 mg of sodium sulfite/liter. Adjust pH to 7.6–7.8 with 0.1 M sodium phosphate buffer.

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[2] The use of sodium sulfite was inspired by Richardson's (60) fixative.

Fixative 1 is preferable to fixative 2 and 3, but 2 and 3 may be used with satisfactory results if cacodylate buffer is not available.

## C. Sectioning

Frozen or vibratome sections were cut at 30–35 μm, and stored in fixative for 2–3 days. Good results, however, have been obtained in sections that have been postfixed for only 25 h or at the other extreme for 2–3 months in a refrigerator (4°C). The postfixation eventually suppresses normal fiber staining; at 24 h some normal fibers will be stained, while with extended postfixation, impregnation of degenerating neuronal cell bodies, dendrites, axons, and their terminals may be reduced.

The age of the animal and the thickness of the section will affect the results to varying degrees. The present protocol is based on rats weighing 250–300 g. In general, brain sections from younger animals show more nuclear staining, which can be diminished by reducing the amount of pyridine in the pre-impregnation step (e.g., from 2 to 1 ml as a first approximation). Similarly, with thinner sections more staining of nuclei may occur, whereas with thicker sections less complete impregnation of degenerating elements may be seen.

Some variability may be observed with different species; for example, mouse and armadillo brains stain similarly to young rats, whereas young guinea pig (250 g) brains stain similarly to adult rats.

## II. Preimpregnation

### A. General

Sections should be briefly (15–20 s) washed in distilled water before preimpregnation. The water should be scrupulously clean and it may be necessary to use doubly distilled water or deionized and polished water (18 mega ohm pyrogen free). Because any contamination may lead to artifactual deposits, acid-cleaned glassware should be used. Nitric acid is preferred; mixtures such as *Chromerge* are not recommended. Glassware and water should be absolutely free from any trace of detergent, which is likely to produce patchy staining, artifactual deposits, and failure to impregnate degenerating fibers.

### B. Preparation of the Preimpregnating Solution

Make fresh for each group of sections (to make approximately 108 ml; will stain approximately 60 sections). The solution should be made in the following order:

| | |
|---|---|
| AgNO₃ | 100 mg |
| distilled water | 100 ml |
| dl-α-amino-n-butyric acid | 53 mg |
| dl-alanine | 46 mg |
| 0.5% Cu(NO₃)₂ | 2 ml |
| 0.5% Cd(NO₃)₂ | 0.2 ml |
| 0.5% La(NO₃)₃ | 1.5 ml |
| 0.5% Neutral Red | 0.5 ml |
| pyridine | 1.0 ml |
| triethanolamine | 1.0 ml |
| isopropanol | 2.0 ml |

Before being used, this pinkish-to-orange preimpregnating solution should be irradiated in a 700W microwave oven using 60% of its maximal power until the temperature of the solution reaches 50°C (i.e., 115°F). The solution is then cooled to room temperature (22–25°C) for 1 or more hour or even overnight. During this storage time and depending on the commercial brand of the neutral red, an orange to brown precipitate may develop; if this happens, the solution should be filtered, after which it acquires a light and transparent orange color.

## C. Treatment of the Sections

Pre-impregnate sections in a conventional laboratory oven at 50°C for 45–50 min. This may vary depending on the quality of fixation. Shorter incubation times result in staining of cell nuclei, capillaries, and reticular fibers of blood vessels, while excessive incubations suppress staining of the finest products of degeneration. Following incubation sections should be allowed to cool to room temperature (22–25°C) while still in the preimpregnating solution. Normally, this is accomplished by allowing sections to sit for 2–3 h. After that time, sections routinely show a slight color change from grey-brown to brown. If sections remain grey, the staining procedure is likely to be unsuccessful.

As a strongly recommended alternative, the preimpregnation process can be carried out in a 700 W microwave oven. The preimpregnating solution containing sections of brain tissue, is irradiated using 60% of the maximal power of the microwave oven until 50°C (115°F) is reached as measured by the (stainless steel) temperature probe usually provided with such ovens.

Even after the microwave oven has been turned off, the temperature of the pre-impregnating bath will continue to rise; it is therefore vitally important to initially calibrate the irradiation internal so that the final temperature of the bath will not exceed 50°C. The total process takes only a few seconds; the length of the irradiation depends on the volume of solution used and the microwave-oven power applied. Keep the tissue in the impregnation solution until the solution reaches room temperature (2–3 h).

## III. Silver Impregnation

Transfer sections through two changes of acetone while agitating continuously (approximately 30–60 s total) and from there transfer directly into the diamine-silver solution that is kept in a covered container to limit exessive evaporation of ammonia which is detrimental for the staining. Sections should remain for 45–50 min in the diamine silver solution at room temperature and under constant stirring with a rotating platform. Shorter incubations result in the staining of normal cell nuclei, whereas longer incubations reduce impregnation of degenerating neuronal somata and processes while increasing the impregnation of nondegenerating argyrophilic areas (e.g., central amygdala and bed nucleus of the stria terminalis; see technical notes; see Fig. 9b later).

*Diamine-silver solution.* Make a diamine-silver solution fresh for each group of sections (10 ml is enough for 15 sections). The chemicals should be added in the order listed. Before adding the alcohol, the silver nitrate should be completely dissolved. A brown precipitate should normally form after adding the LiOH. After addition of the NH4OH, the solution becomes transparent after continuous stirring with a glass rod. Usually only a slight granular precipitate remains. If the solution clears too rapidly or if no precipitate remains, it is possible that the NH₄OH is too concentrated. If there is excessive precipitate remaining, the solution should be filtered. This may indicate that the NH₄OH is not fresh and therefore not concentrated enough. In general, plastic containers and stirring rods made of plastic should be avoided.

| | | |
|---|---|---|
| AgNO₃ | 412 mg | |
| distilled water | 5.00 ml | |
| 100% ethanol | 4.00 ml | |
| acetone | 0.05 ml | |
| 0.4% LiOH | 3.00 ml | |
| NH₄OH | 0.65 ml | (Fisher) or 0.70 ml |
| | | (EM Sciences) |

## IV. Reduction

With a glass rod, transfer sections without washing directly to reducer solution (at 32°C) for 25 min. Gently agitate the solution for the first 1-2 min and then for a short time every 5 min.

*Reducer solution.* Stock solution, approximately 910 ml, can be stored up to 1 month. Approximately 50 ml is used for 15 sections.

| | |
|---|---|
| 10% formalin | 11 ml |
| 1% citric acid monohydrate | 6.3–6.5 ml[3] |
| 100% ethanol | 90 ml |
| distilled water | 800 ml |

Note: After the sections have remained in the reducer for 5 min under continuous agitation, add for every 100 ml of reducer a total of 1.2 ml of the same diamine solution used for the silver impregnation of sections, delivered in 4 samples of 0.3 ml each at 5-min intervals. The sections gradually acquire a dark brown color during this treatment. Once this occurs, the sections are transferred immediately to distilled water for 1-2 min.

Following treatment in distilled water, transfer sections immediately to a 0.5% glacial acetic acid solution (1-2 min) to stop any further reduction. Rinse in at least two changes of water, (approximately 1 h total, with 15 min in the first rinse). Following this, the sections may be stored overnight in distilled water. Although this is a rather long washing time, it appears to allow the silver impregnation to stabilize before the subsequent bleaching procedure.

## V. Bleaching

Bleaching is done in two steps: First the sections are bleached in an acidic ferricyanide solution at room temperature until they become relatively transparent; then they are washed with distilled water and transferred to a second bleaching solution made of acidic permanganate, where the sections acquire a pinkish-yellow and rather opaque appearance after a short time.

1. First bleaching solution (~ 11 ml solution)

| 6% potassium ferricyanide | |
|---|---|
| in 4% potassium chlorate solution | 10.0 ml |
| undiluted lactic acid | 0.2 ml |

Carry out bleaching (usually for 20-60 s) at room temperature. To control bleaching, it is important to monitor the color of the sections against a white background and under a bright white light. The loss of silver is rapid and can eliminate the signal if allowed to proceed too far. Rapid bleaching facilitates the removal of background silver deposits without removing fine products of neuronal degeneration. Wash very thoroughly in distilled water after this step.

Sections are then bleached in the following solution to remove any remaining nonspecific precipitate.

2. Second bleaching

| 0.06% potassium | |
|---|---|
| permanganate | 30.0 ml |
| 5% sulfuric acid | 1.0 ml |

In this solution (usually 15-20 s) the sections acquire a yellowish color. The bleaching is terminated by transferring the sections to distilled water. The sections are rinsed in at least two changes of water (room temperature), 1-5 min before proceeding to the next stabilizing step.

## VI. Stabilization

1. Transfer sections to an aqueous 2% sodium thiosulfate solution (with agitation) for 1-3 min, and rinse again in distilled water (2-5 min).
2. Transfer sections to a Rapid Fixer solution (Kodak concentrated Rapid Fixer solution A + B, diluted 1 : 6 in distilled water). The sections become transparent after approximately 1 min, and may remain in the solution for longer intervals (5 min max.).

Wash thoroughly in distilled water. Afterward, sections may be mounted, dehydrated, cleared, and coverslipped.

*Gold toning (optional).* Some of the slightly silver-impregnated structures may be difficult to visualize against a neutral red or other Nissl counterstain. In this case, additional stabilization of the degeneration products may be attempted by gold toning before the counterstaining procedures.

Gold toning solution contains the following:

| | |
|---|---|
| Gold Chloride 0.2% | 30 ml |
| 0.1 N HCl | 30 ml |
| Distilled water | 30 ml |

Prepare this solution immediately before use. Sections are incubated at room temperature until the silver stained structures become blue-black (overnight), then washed in distilled water (2 × 5 min). Stabilize in 2% thiosulfate (100 ml) to which 2 drops of 5% sodium carbonate has been added (1 min) and wash thoroughly (3 × 5 min).

*Mounting and counterstaining.* Any light counterstain may be used on the mounted sections; we have commonly used the following procedure. Mount prior to dehydration from a solution composed of equal parts of 0.5% gelatin and 80% alcohol (1). Hydrate and immerse in a 0.5% neutral red solution in water, 15-20 min, rinse briefly twice in distilled water and dehydrate in 50%, 70%, 95%, and 100% alcohol, 15-20 s each. Differentiate and clear in alcohol-xylene (1 : 1 and then 1 : 6) and twice in pure xylene. Coverslip using DPX as the mounting medium. (Note: a complete flow chart of the procedure is shown in Fig. 10 later).

### RESULTS

The new amino-cupric silver protocol allows for remarkably clear-cut impregnation of degenerating neuronal perikarya, dendrites, axons, and the finest terminal arborizations. The neuronal components are impregnated black against a grayish-white background with hardly any impregnation of normal fibers, an occasional exception to this are the forebrain granular argyrophilic neuropil and neurons that have a very specific distribution and appearance in the hypothalamus, bed nucleus of the stria terminalis, and central amygdala (16,23-25,27). The morphology of degenerating perikarya and dendrites, including their finest branches and spines, can usually be easily appreciated if appropriate survival times and planes of sectioning are used. Degenerating axons can also be traced back to their parent cell bodies or toward their terminal fields.

In general, only a relatively small amount of nonspecific silver is bound to the tissue, which may appear as a diffuse,

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[3] 6.5 ml is preferable, however, in some cases 6.3 ml will give a more stable result with respect to the following bleaching step. On the other hand, this less acidic solution generally allows more staining of nuclei and more nonspecific precipitate.
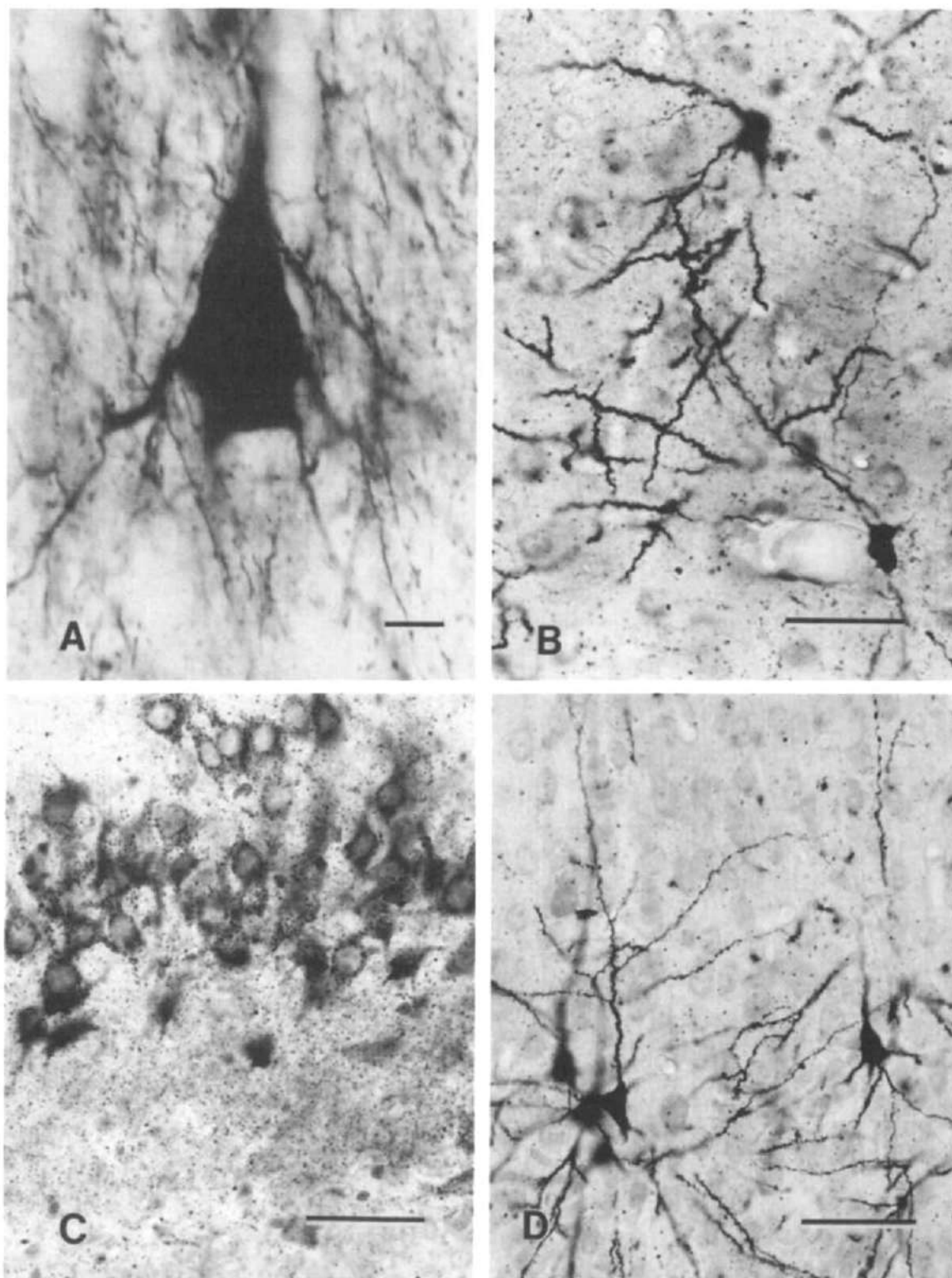
FIG. 1. (A) Pyramidal neuron in layer II of the frontal cortex, 5 min after a lesion produced by a stainless steel insect pin. The cell shows a dark, smoothly contoured profile of soma and processes (Scale bar = 10 μm). (B) Staining pattern of striatal neurons with spiny dendrites 15 min after an intrastriatal injection of quinolinic acid. Scale bar = 100 μm. (C) Swollen neurons in piriform cortex 24 h after an IP injection of trimethyltin. The neurons marked by coarse granular deposits on soma and processes are surrounded by granular terminal-like deposits. Scale bar = 100 μm. (D) Degenerating neurons in somatosensory cortex (layers 2–3) of a ischemic gerbil brain 15 min after recirculation. The section was counterstained with neutral red. Scale bar = 100 μm.

finely dusted background against which the outline of healthy, unstained perikarya can be appreciated. Neuronal nucleoli, and nuclei of glial or capillary endothelial cells are sometimes stained but this is usually due to (nonfatal) technical flaws. Erratic staining of apparently normal cells occurs occasionally. In brain areas subjected to contusion, electrocoagulation or other forms of local trauma, the background tends to show a paler staining.

The staining of traumatized cell bodies and dendrites may begin shortly after the brain tissue has been damaged, depending on the nature of the insult. For example, mechanical trauma to the rat cerebral cortex, caused by superficial penetration of a stainless steel pin, evokes somatodendritic argyrophilia as early as 5 min following the injury (Fig. 1A). Moreover, intrastriatal microinjections of excitotoxicants like quinolinic acid or systemic administration of an organometallic compound like trimethyltin, induce the appearance of somatodendritic argyrophilia as early as 15 min and 24 h after their delivery, respectively (Figs. 1B and C). In gerbils, transient ischemic episodes (10 min) elicit similar argyrophilic reaction in neurons of the somatosensory cortex 15 min after recirculation (Fig. 1D). The importance of the survival time is clearly illustrated by the following examples: 6 h after an intrastriatal injection of quinolinic acid, dust-like terminal degeneration is already visible in specific layers of some cortical fields. On the other hand, after only 11–12 days survival time

following an olfactory bulb injection of doxorubicin, clear-cut terminal and axon degeneration can be observed in the olfactory peduncle.

Neurons exposed to toxic substances display a variety of appearances, from the typical "dark neuron" of Cammermeyer (Figs. 2A, 3A) to those showing fine, often dense granular silver deposits in their perikarya and processes, including their main axon and axon collaterals (Fig. 3B). Other neurons, especially those exposed to doxorubicin or trimethytin, instead show gross deformities of their perikarya and main dendrites, and are filled with coarse granular deposits (Fig. 1C). Sometimes these different staining patterns coexist with one or the other predominating (Fig. 3C). A more detailed description of the diverse effects observed in the six experimental models tested with the present procedure is given next.

### Physical Trauma

Cortical lesions caused either by electrocoagulation or by the introduction of a pin show a very characteristic pattern of neuronal staining. As early as 5 min after the insertion of a stainless steel pin into the frontal cortex of a rat increased argyrophilia is observed including neurons displaying a Golgi-like appearance (Figs. 1A and 9A), which indicates the extent of the damaged area. The Golgi-like neurons show smooth contours and homogeneous impregnation of the cell bodies
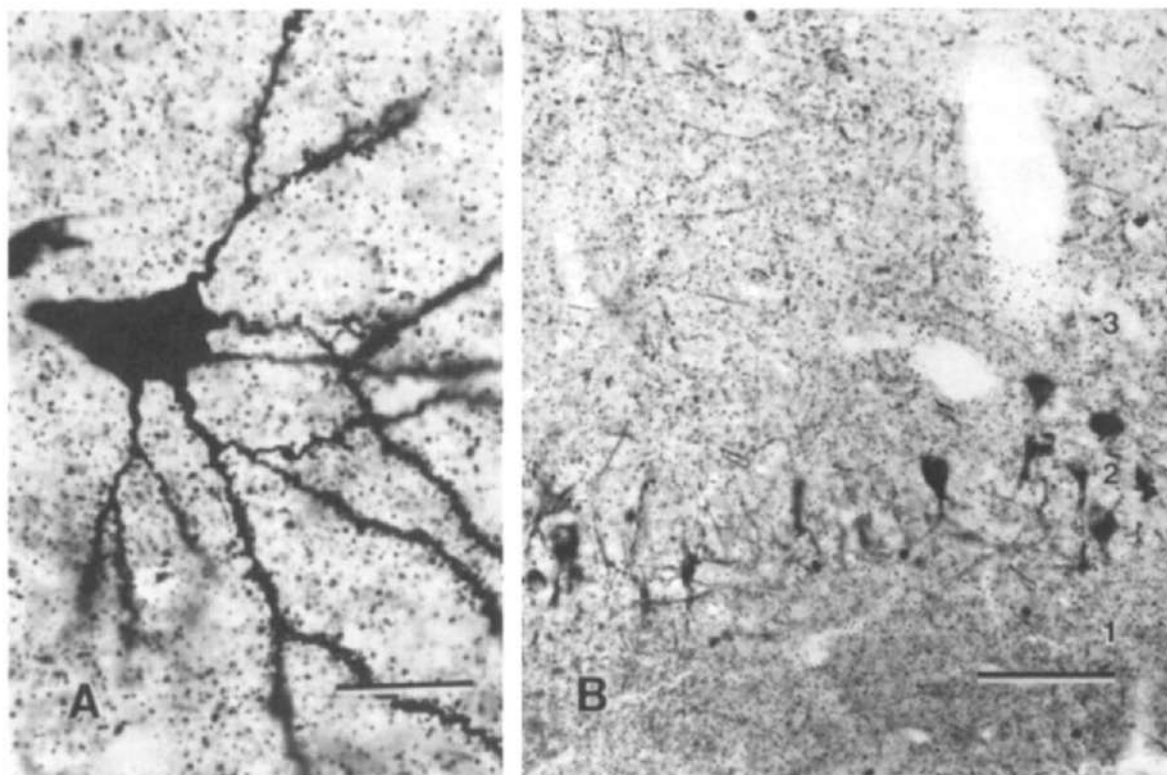


FIG. 2. (A) Argyrophilic dark neuron in layer III of the insular cortex of an animal subjected to an intrastriatal injection of quinolinate. The neuron is surrounded by terminal degeneration (6 h survival). Scale bar = 40 μm. (B) Argyrophilic neurons in layer II of the primary olfactory cortex following a doxorubicin injection in the main olfactory bulb. Note massive terminal degeneration both in the molecular and polymorph layers, 13 days survival. Scale bar = 200 μm.
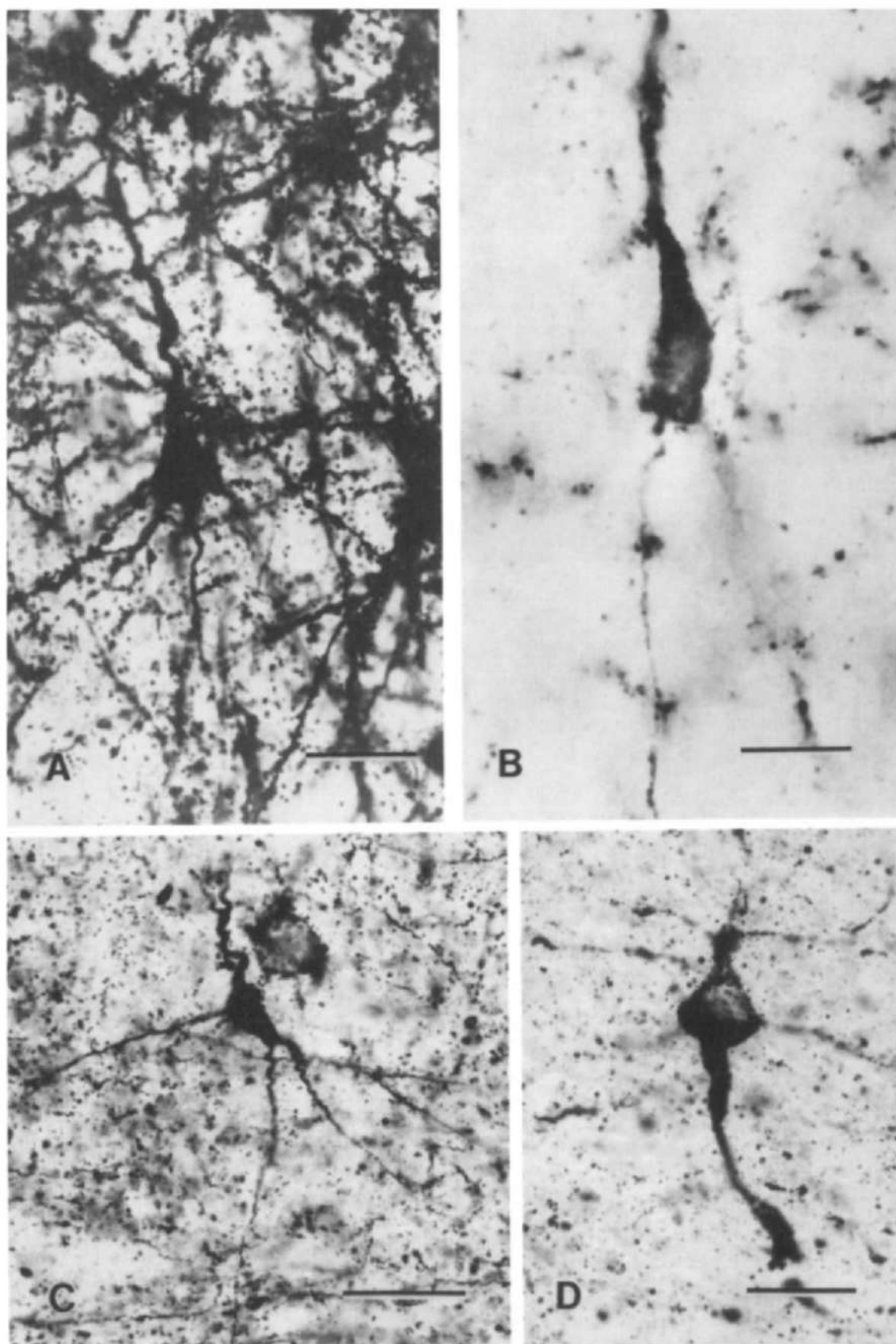
FIG. 3. (A) Argyrophilic dark pyramidal neuron in the somatosensory cortex following a striatal injection of quinolinate. Note the characteristic corkscrew appearance of its apical dendrite. The axon is shown leaving the cell body. Twelve h survival. Scale bar = 50 μm. (B) Cortical pyramidal neuron affected by an injection of quinolinate in the striatum. Survival, 48 h. Scale bar = 25 μm. (C) Neurons in pyriform cortex 13 days after an injection of doxorubicin in the main olfactory bulb. Both granular background (debris) of neuropil and terminal degeneration can be noted. Scale bar = 60 μm. (D) Neuron in the posteromedial cortical amygdaloid nucleus 30 days after a doxorubicin injection in the caudal hippocampus. Coarse granulation on the cell body, dendritic fragmentation, and axonal ballooning can be observed. Scale bar = 60 μm.

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and dendritic arbors. Although axons can also be seen in the damaged area, they can be followed only for short distances.

*Excitotoxic Lesions*

Excitotoxically induced lesions caused by the injection of glutamate analogues, acting on different subsets of glutamate receptors, either locally (ibotenate) or both locally and at a distance (quinolinate, quisqualate, kainate), show as a common feature the presence of sizable numbers of argyrophilic neurons resembling those subjected to postmortem trauma or in vivo mechanical damage (Fig. 4 A,B). Thus, in those experiments in which *dark neuron* or *collapsed neuron* argyrophylic profiles were observed at a distance from the injection site, their topographical distribution varied with the type of excitotoxic aminoacid used. For example, in the case of intrastriatal injections of quinolinate, neuronal degeneration is seen predominantly in the supragranular cortical layers (Fig. 4A), whereas quisqualate and kainate injections affected cells in the infragranular cortical layers (Fig. 4B). Furthermore, in cases with survival times ranging between 36 to 48 h, the distribution of the concomitant axonal and terminal degeneration is consistent with the location of their degenerating presumed parent perikarya. By testing other neuronal systems with well known connectivity, we established that at least a sizeable number of the amino-cupric silver stained neurons were undergoing irreversible degenerative changes.

*Ischemia*

Transient (10 min) arrest of the cerebral circulation in gerbils caused neuronal argyrophilia with staining characteristics very similar to those following local administration of amino acidic excitotoxins (Figs. 5 A,B,C). In this instance, the amino-cupric silver technique uncovers argyrophilia after a strikingly short survival time of 15 min following recirculation (Fig. 1D), and a very extensive distribution of the traumatized neuronal pools (29) is evident. As in the excitotoxic experiments, the capability of the present protocol to reveal indirect Wallerian axon degeneration may contribute to the identification of the neuronal systems that have been irreversibly compromised.

*"Suicidal" transport of doxorubicin.* The semiacute pathologic model provided by the retrograde transport of doxorubicin was used to test the capability of the amino-cupric silver procedure to reveal changes of semiacute nature in neurons intoxicated by this anthracycline compound. Our experimental series, which included local doxorubicin injections in several brain structures (see Method section), show that while fast retrograde axonal transport (as detected by fluorescent
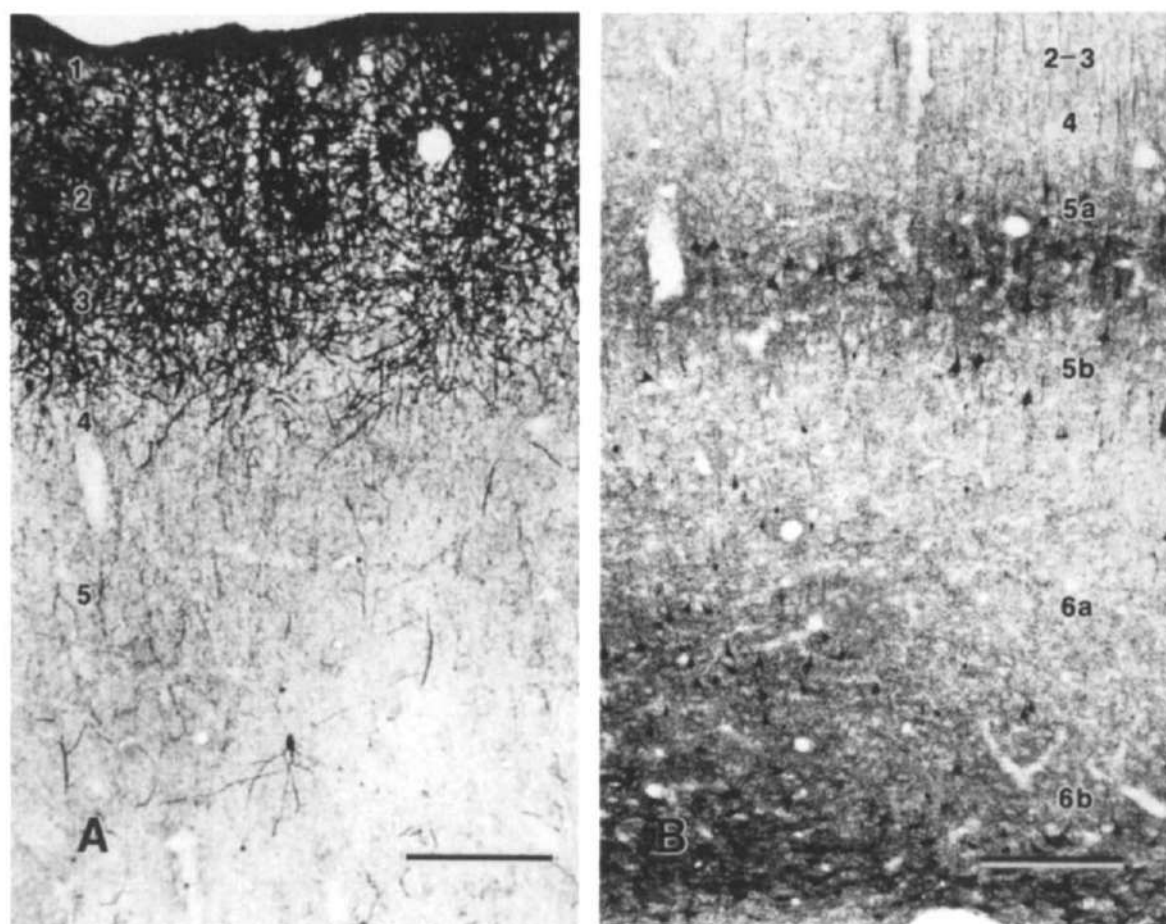


FIG. 4. (A) Dense neuronal degeneration in the supragranular layers of the frontal cortex 6 h after an intrastriatal injection of quinolinate. Scale bar = 300 μm. (B) Pattern of neuronal degeneration in the infragranular layers of the temporal cortex after an intrastriatal injection of kainate. Scale bar = 300 μm.
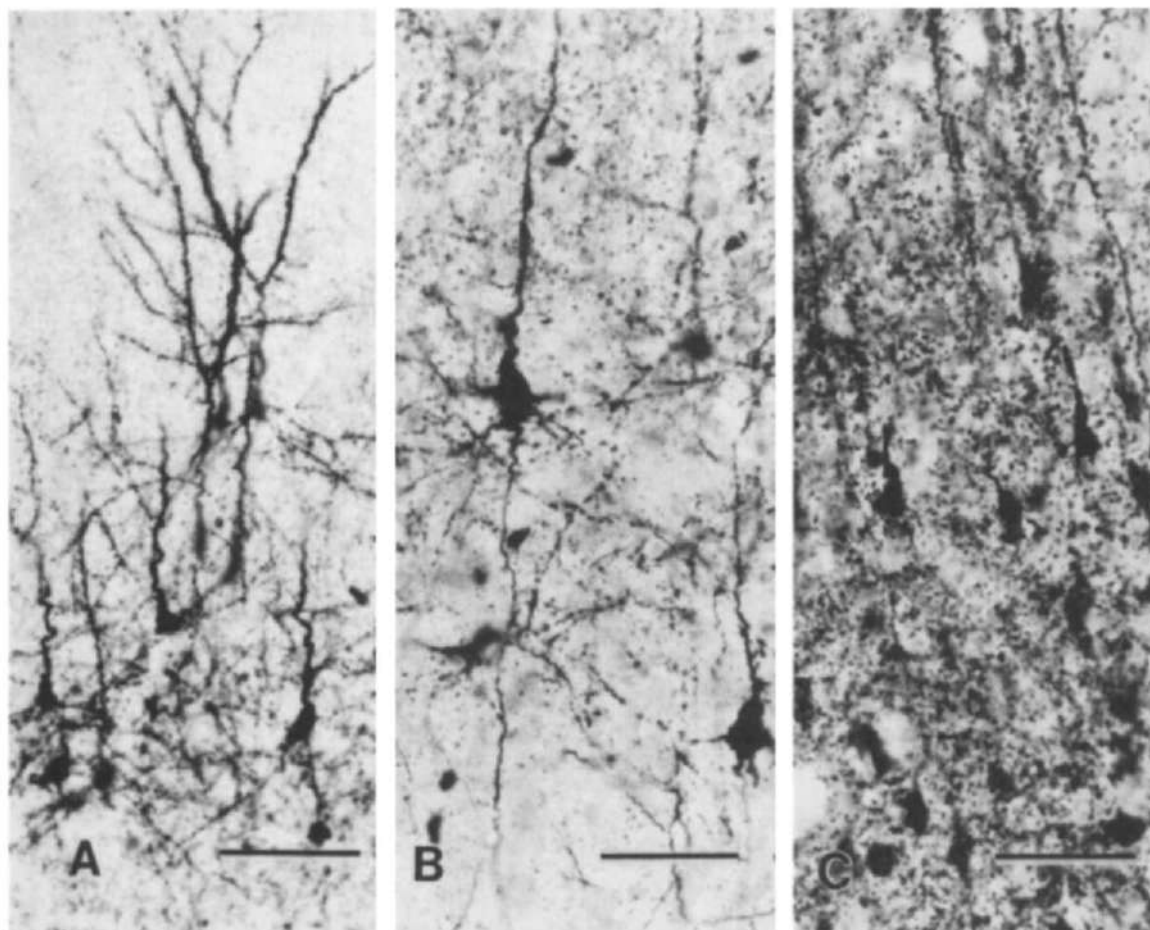
FIG. 5. (A) Golgi-like neuronal profiles in layer I to III of the somatosensory cortex of a Gerbil ischemic brain 6 h after recirculation. Dendritic aborizations are still preserved. Scale bar 200 $\mu$m. (B) and (C) show the progression of the degenerative changes occuring in cell bodies, dendrites, and axons at 24 and 72 h after recirculation, respectively. Scale bar = 100 $\mu$m.

microscopy) does occur between 24 and 48 h post-injection (6,7,47), the first signs of changes and stainability in the involved neurons take place 7-9 days later, acquiring a more conspicuous staining pattern on Days 11 to 13 postinjection (Fig 2B), depending on the neuronal system under study.

In most of the doxorubicin experimental series, the neurons showed conspicuous swelling of their cell bodies and ballooning of their main dendrites, which are filled with coarse silver granules (Fig. 3D). Their axons and terminals which undergo indirect Wallerian degeneration, are also stained with the present procedure and the changes in the axons and collaterals can be detected shortly after the first signs of degeneration appear in the perikarya.

### Trimethyltin Intoxication

Amino cupric silver stained brain sections were obtained from rats that survived between 2-7 days after being intoxicated with a single IP injection of trimethyltin hydroxide (7 mg/kg). The pattern of perikaryal degeneration is very characteristic, in the sense that the affected neurons display swollen cell bodies and main dendrites that appear covered with coarse silver granules (Figs. 1C, 7A,B,C). Their axons and terminals, which undergo indirect Wallerian degeneration, show less distinctive morphological features (Fig. 6A), in comparison with

other patterns of anterograde axonal degeneration (Fig. 6B). In animals surviving only 24 h after a similar treatment, the trimethyltin-intoxicated neurons display much lighter silver deposits that do not involve their axons (Fig. 7 A,B,C). On the other hand, the present protocol allows for the demonstration of a more extensive distribution of damaged brain tissue (Fig. 7 A,B,C) than previously reported by Balaban et al. (4), who used the Carlsen and de Olmos version of the cupric silver (16) technique.

Note that although the protocol just discussed has provided consistent results in our laboratory, all silver procedures may need some calibration depending on mammalian species, age, and the type of pathology produced by the neurotoxicants employed.

### DISCUSSION

The present silver impregnation method for the staining of acute and subacute neuronal degeneration is characterized by outstanding sensitivity and reproducibility, which make it a useful tool for screening location and time course of the pathologic action of a number of agents harmful to the nervous system. Its ability to detect their traumatic action has been tested and confirmed in a series of experiments designed to produce a wide range of neuropathological alterations in
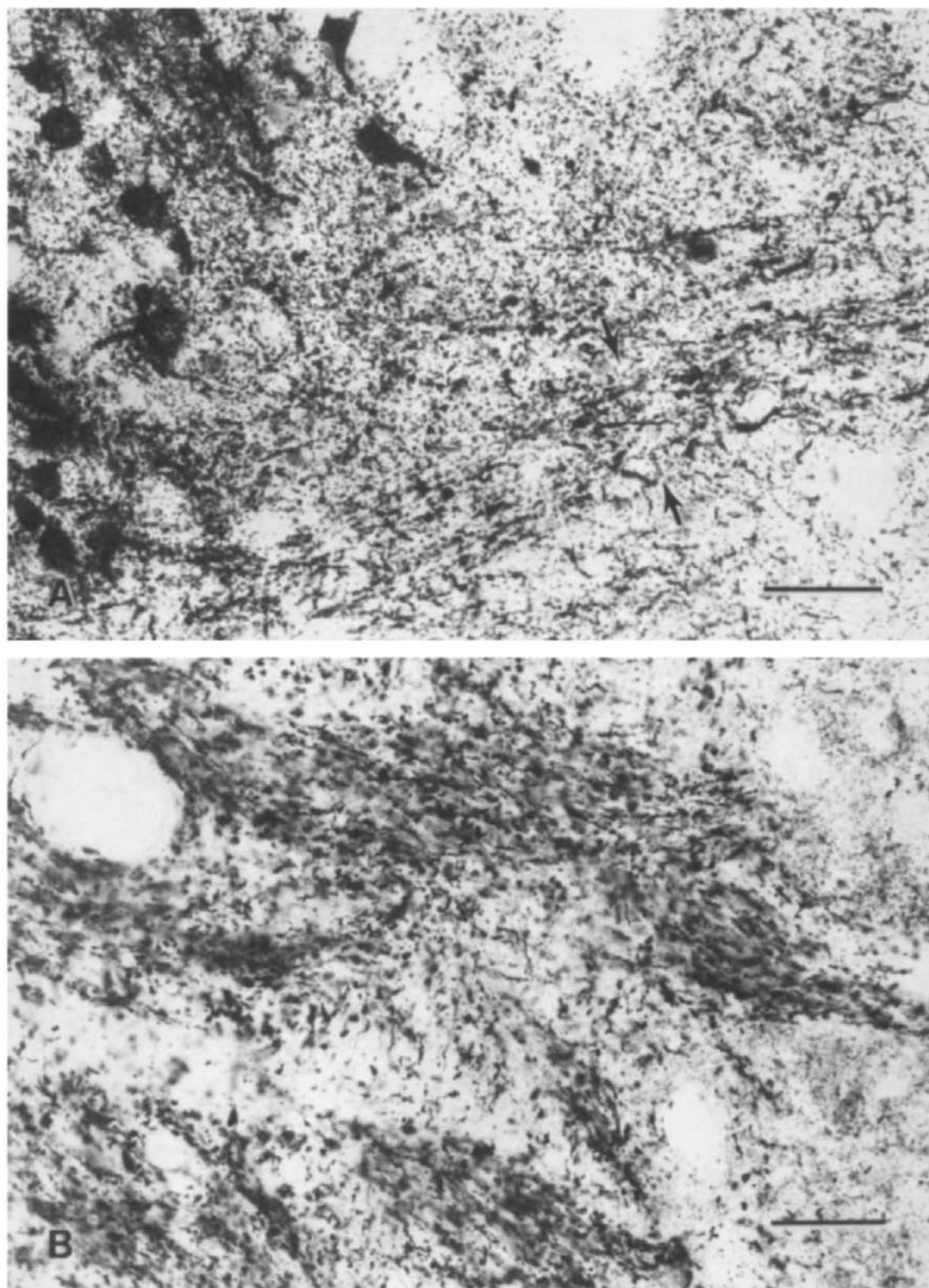
FIG. 6. (A) Axons undergoing indirect Wallerian degeneration (arrows) originated in damaged cells of the ventrolateral entorhinal cortex joining the angular bundle (horizontal section). Trimethyltin intoxication, 7 days survival. Scale bar = 100 μm. (B) Typical pattern of anterograde axonal degeneration in the globus pallidus 48 h after an intrastriatal injection of quinolinate. Scale bar = 100 μm.
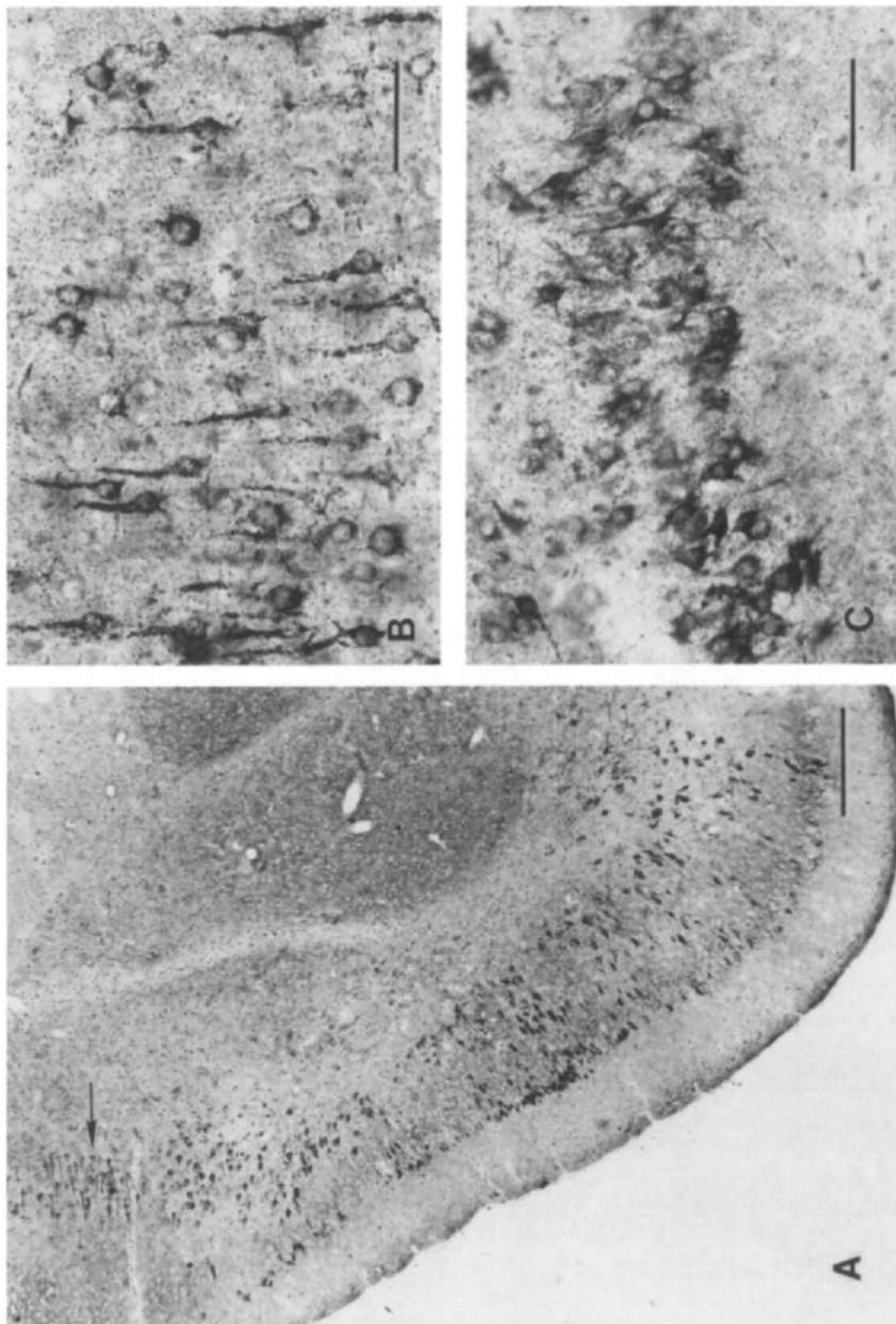
FIG. 7. (A) Sector of coronal section of a rat brain 24 h after an IP injection of trimethyltin. Even at this early survival time cells in the pyriform, perirhinal, and periamygdaloid cortices and in the ventral basolateral amygdaloid nucleus are strongly affected by the toxin. Scale bar = 600 μm. (B) Higher magnification of the sector indicated by the arrow in (A). Typical pattern of coarse granules on the swollen cell bodies and main dendrites. Scale bar = 100 μm. (C) Sector of the hilus of the dentate gyrus of the hippocampus showing typical granular silver reaction. Scale bar = 100 μm.

telencephalic neuronal structures. The various types of traumatic situations included physical trauma (insect pin), anterograde transneuronal degeneration (e.g., olfactory cortex consecutive to complete bulbectomy), local and distant excitotoxic degeneration (e.g., after intrastriatal, intra-amygdaloid, or sublenticular infusions of glutamate analogues), cytotoxic degeneration after "suicidal" retrograde transport of doxorubicine, metabolic injury subsequent to systemic infusion of trimetylthin, and regressive changes resulting from a transient ischemic insult. The technique appears suitable for the demonstration of acute and subacute pathologic processes within a period of time ranging from a few minutes to several weeks or even a few months following the trauma.

Although the amino-cupric silver procedure consistently yields a very clean background that sharply outlines the degenerating structures, brain regions subjected to contusion, electrocoagulation, or other forms of direct local trauma tend to show a patchy, paler staining. Gallyas et al. (36,37,38), based on similar findings obtained with their own silver procedure, speculated that "the rounded outlines and homogeneous inside of the nonargyrophilic patches suggest the possibility that some substance released from damaged blood vessels or damaged parenchymal cells inundates the extracellular territories in question and abolishes the neuronal argentophilia." Furthermore, brain areas that have not been adequately perfused during the fixation can easily be identified by the silver impregnation of red blood cells and/or blood vessels profiles (26) (Fig. 9C).

As is common with a variety of sensitive histochemical procedures, otherwise normal neurons subjected to postfixation trauma are sometimes densely stained showing a Golgi-like appearance. This type of image may lead to misinterpretation by the unwary observer. To avoid postfixation artifacts, a careful regime of perfusion, fixation, and postfixation is mandatory. Similar conclusions were reached by Gallyas et al. (35) using a completely different silver protocol.

In our experiments, the staining of in vivo traumatized neurons may begin shortly, i.e., 5 to 15 min after injury. The time seems to depend on the nature of the inflicted damage and/or the particular sensitivity of the structures involved. In contrast to the unchanging morphological features of postfixation traumatized neurons, the in vivo damaged neurons display progressive modifications of their staining characteristics and of their morphological configuration with increasing survival time. Thus, dendritic and axonic processes are shown to undergo changes that start with a slight argyrophilia and slight beading, followed by fragmentation of processes into rows of pearl-like profiles. This is followed by the appearance of punctate structures having sharply diminished argyrophilia, and finally, by the almost total disappearance of the processes. Meanwhile, the cell bodies and nuclei of the damaged neurons are undergoing various modifications in their shape, size and stainability, leading eventually to their reduction to irregular globular argyrophilic masses. Such a chain of events has been reported as characteristic of neuronal death (16,26, 30,35,63).

Apart from distinguishing artifactually stained normal neurons from pathologically altered ones, the present protocol is capable of differentiating between neurons damaged by various means including in vivo physical trauma, ischemia, or local injections of excitotoxic compounds, on the one hand, and those affected by neurotoxic organic substances on the other. Thus, two different chemicals, doxorubicin and trimethyltin, whose toxic action seems to be exerted through mechanisms other than those involving glutamate receptors (2,8,15,18,32,40,52,55,59) produce silver staining patterns that clearly differ from those caused by the glutamate analogs as shown in the results section (See Figs. 1C, 3D, 7A,B,C). In this context, it is important to consider the time that elapses between the arrival at the cell bodies of the retrogradely transported doxorubicin and the first expression of silver stainability. As emphasized previously, the latter event takes place between 7 and 9 days post injection, or even longer, depending on the neuronal system involved. The onset of this delayed silver staining seems to coincide closely with the time at which the first irreversible degenerative changes have been reported to occur in the cytoplasm of the doxorubicin-intoxicated neurons, as seen with the electron microscope (7,8,18,48,49,58). The fact that the amino-cupric silver technique provides for such a faithful rendition of the chain of events leading to
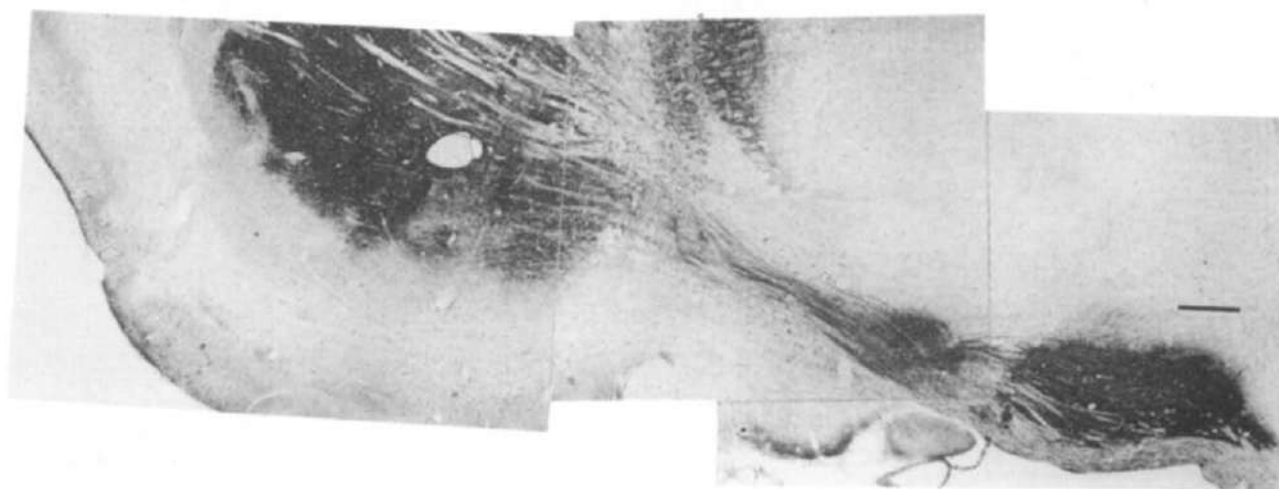


FIG. 8. Composite of a sagittal section of a rat brain with an intrastriatal injection of quinolinate (50 nmol, 24 h survival) illustrating the massive neuronal and terminal degeneration in the striatum from which densely stained degenerating strionigral axons can be followed to their termination in the substantia nigra. Scale bar = 700 $\mu$m.
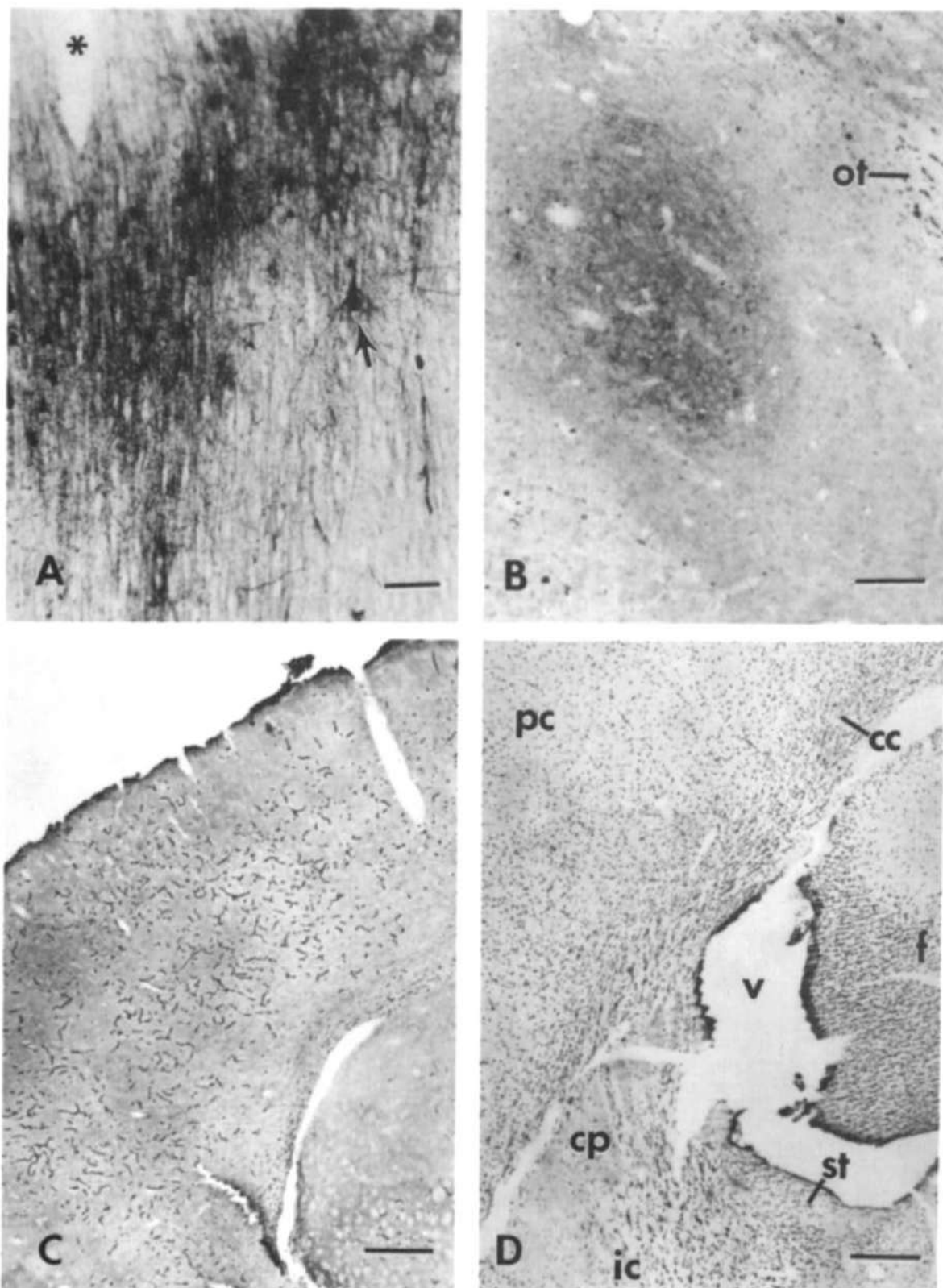
FIG. 9. (A) Low magnification of Figure 1A. The wound left by the insect pin can be observed in the upper left corner (*). The arrow indicates the neuron shown in Figure 1A. Scale bar = 120 μm. (B) Sector of a coronal section showing the pattern of normal argyrophilic granular neuropile in the central amygdaloid nucleus as described in text. Compare this pattern with (A), to appreciate the difference with a pathologic staining. ot: optic tract. Scale bar = 200 μm. (C) Low power magnification of a sector of a gerbil brain cortex to show the staining pattern in the vascular system after an unsuccessful perfussion-fixation. Scale bar = 200 μm. (D) Low magnification of a rat brain section showing glial staining through the section caused by an excess of pyridine in the staining of a young animal material (see text). A dark dotted pattern can be observed both in the gray matter (parietal cortex pc. and caudate-putamen, cp.), and in the white matter (i.e., callosum c. fimbria f. internal capsule, ic. and the tract of the stria terminalis, st.) (v: lateral ventricle). Scale bar = 200 μm.

**1. Preimpregnation**

$H_2O$    $H_2O$    Cu-Ag Aminoacids La, Cd    $H_2O$    Acetone I    Acetone II

1 Hour, 50 C° (or MW to 50 C°)    45 Sec    45 Sec

**2. Impregnation**

AgNH$_4$ EtOH    Reducer 32 C    $H_2O$    Acetic Acid    $H_2O$

45 Min RT    25 Min    2 Min    1 Min    1 Hour *

**3. Bleaching**

I $K_3Fe(CN)_6$ + $KClO_2$    $H_2O$    II $KMnO_4$ + $H_2SO_4$    $H_2O$    $NaS_2O_3$ 2%

30 Sec/1 Min    30 Sec    Until Transparent

**4. Stabilization**

$H_2O$    Rapid Fixer    $H_2O$    $H_2O$    Mount & Counterstain

5 Min    15 Min    1 Hour    As Necessary *

**5. Intensification (optional)**

HAuClH$_4$-HCl    $H_2O$    $NaS_2O_3$ + $NaCO_3$    $H_2O$    $H_2O$    Mount & Counterstain
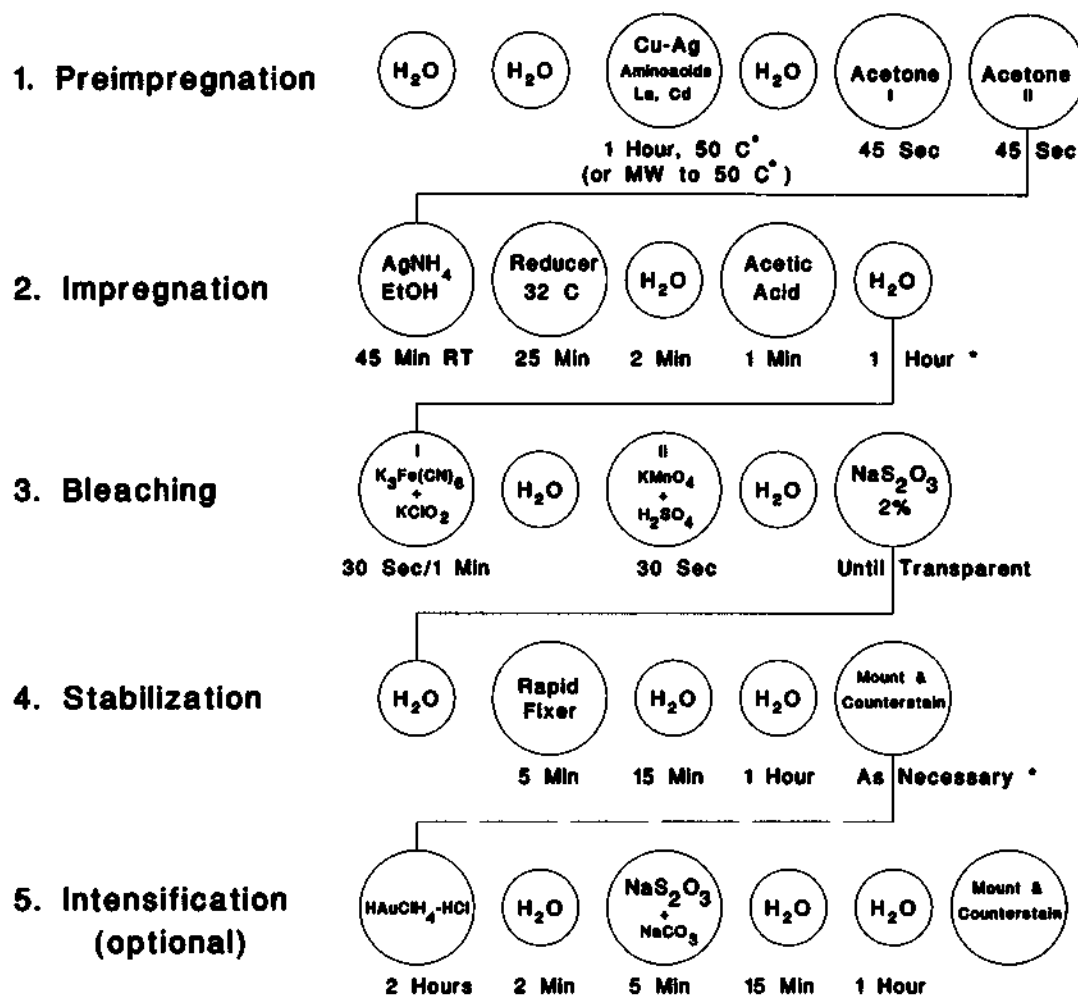
2 Hours    2 Min    5 Min    15 Min    1 Hour

FIG. 10. Flow chart of the steps in the development of the Amino-cupric-silver technique. For the circles labeled $H_2O$ without time indication, a period of 30-s is suggested.

neuronal death after retrograde transport of doxorubicin strongly supports its validity as a tool for the screening of degenerative changes caused by different neurotoxicants.

Finally, note that the impregnation of degenerating axons and their terminals by the amino-cupric silver method is generally comparable to that described for its forerunners (16,23, 25,64) and for the Fink-Heimer method (33). Also it is worth mentioning that axon terminal degeneration independent of the parent cell body death can be observed at very early stages after recirculation in the gerbil (data not shown), and after methamphetamine intoxication (45,46). On the other hand, it seems obvious that to obtain a complete picture of the axonal degeneration elicited in some of the experimental models used in this report (i.e., quinolinate, quisqualate, kainate, ischemia, doxorubicin, and trimethyltin), a wide range of survival times is mandatory. That neurons may show different sensitivities to the damaging action of traumatic agents, thereby causing their axons to undergo anterograde degeneration in untimely fashion, becomes apparent from a critical appraisal of the previous literature (4,21,29,35-38,42,51,63,65).

Further analysis of the data just discussed indicates that the amino-cupric silver procedure may still be profitably used as a tract-tracing tool. Because the method reveals morphological changes in every structural compartment of traumatized neurons, including their axons, collaterals and terminal arborizations (Fig. 8), it can be used to trace the connections of a group of neurons which are undergoing degenerative changes in response to trauma or chemical injury. For example, a group of neurons may be in the process of degeneration because they contain a specific receptor which is the preferred target of a given neurotoxic substance. This approach may therefore provide an alternate means to investigate projections of a chemically homogeneous group of neurons, and, in fact, this may be the only means for such groups when specific antibodies or histochemical stain are unavailable for a particular substance.

In view of the rapid expansion taking place in the fields of neurotoxicology and neuropathology, it becomes important that data concerning degenerative processes in the central and peripheral nervous system be collected as effectively and quickly as possible. Although several histochemical and immunohistochemical procedures have been used for that purpose, they can frequently be applied only under highly specific experimental conditions and furthermore are usually not able

to distinguish between the transient or fatal character of the pathologic changes detected. The sensitivity, reproducibility and rapidity of our procedure makes this amino-cupric silver impregnation method a very promising tool for experimental neuropathology. In the past, silver staining procedures for degeneration often suffered some drawbacks, the most troublesome of these being a lack of consistency. The amino-cupric silver procedure, however, has minimized this defect and additionally offers several advantages that complement other cytochemical markers. A particular advantage is sensitivity; not only does it stain dendritic trees and cell bodies of damaged neurons in very early stages of degeneration (increased argyrophilia), but it also stains their axons in the early stages of neuronal degeneration as well as in later stages which

leads to beading and fragmentation during the subsequent stages of Wallerian degeneration. This feature provides a reliable index for distinguishing irreversible degenerative phenomena from transient cytochemical changes.

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# APPENDIX

## (Additional technical notes)

### Source of Chemicals

*Fisher scientific:* Chloral hydrate, ether, phosphates, nitric acid, cupric nitrate, cadmium nitrate, lanthanum nitrate, isopropanol, ammonium hydroxide, hydrochloric acid, formaline, citric acid monohydrate, glacial acetic acid potassium ferricianide, potassium chlorate, potassium permanganate, sulfuric acid, gelatin, sodium thiosulfate.

*Electron microscopy sciences:* Paraformaldehyde, sodium cacodylate trihydrate, sodium sulfite, borates, neutral red, and ammonium hydroxide.

*Sigma chemicals:* Glutamate analogs (quinolinic acid, quisqualic acid, kainic acid), *dl*-aminobutiric acid, *dl*-alanine, glucose, sucrose, sodium chloride, pyridine, trietanolamine, lithium hydroxide, lactic acid, gold chloride, and sodium carbonate.

*Erba labs:* Doxorubicine

*Abbott labs:* Nembutal

*Fluka:* Silver nitrate, ethanol, and acetone.

*RBI biochemicals:* Trimethyltin

Useable life span of solutions: many of the solutions used in the technique can be stored as long as 6 weeks, that is the case for borate and phosphate buffers, nitric salts of Cu, La, and Cd., LiOH, 10% formaline, citric acid monohydrate, sulfuric acid, Reducer mix, acetic acid sol., Na thiosulfate, Rapid Fixer, 0.2% gold chloride and 0.1N ClH. Those solutions can be preserved on a shelf at room temperature. Neutral red solution: can be stored 6 months at room temperature. Mounting solution: use at room temperature, preserving at 4°C between uses. Life span is 4 weeks.

### Fixation

As pointed out by authors using other staining techniques (13,14,19,35), the use of a delayed autopsy (16–24 h postfixation) after perfusion-fixation avoids almost completely the artifactual silver staining of Cammermeyer's "dark" neurons (11–12). This procedure is therefore highly recommended.

Like its previous versions, the present amino-cupric silver protocol allows for a prolonged storage of the tissue in the refrigerator if needed. However, one of the main advantages of the new procedure resides in its capacity of staining recently prepared sections. This characteristic opens the possibility of using adjacent sections for immunohistochemical studies (57,67). Furthermore, vibratome sections, provided they are between 30 and 35 μm, can also be successfully stained by the present procedure circumventing the need for cryostat sections which are sometimes less suitable for immunohistochemical investigations. As just indicated, phosphate buffering of the fixative does not usually yield optimal results. However, if the sections are postfixed with cacodylate-buffered formalin, the results approximate quite closely those obtained in cacodylate-fixed material. On the other hand, it is important to realize that unless the pH of the fixative is adjusted to the values suggested in materials and methods, the amino-cupric-silver procedure will produce results that are less than optimal. The commonly used formol-saline, fixatives containing osmic acid, or glutaraldehyde appeared to inhibit the staining, and immersion-fixed nervous tissue has not stained well with the present procedure.

### Pretreatment

The use of amino acids in the preparation of silver impregnating baths (or Colosyn) was introduced by Brown and Vogelaar (10) as substitutes for Protargol. In search for improving homogeneity and consistency, we developed a pre-impregnating formula somewhat similar to the Brown and Vogelaar's Colosyn solutions. Important improvements include the use of nitric salts of copper and cadmium and the preservation of the basic pH (8.5) by pyridine. The addition of lanthanum nitrate, neutral red, isopropyl alcohol, and triethanolamine also improved the results.

The addition of neutral red to the preimpregnating solution facilitates the pre-impregnation (53). The triethanolamine included in the pretreatment bath stabilizes the pH and helps to obtain a homogeneous impregnation of the sections by preventing their shrinkage. Its concentration, like that of the pyridine, should be adapted to the type of material being stained. Finally, it was found that microwave irradiation of the amino-cupric-silver bath shortened the preimpregnation step from 45–60 min to a few seconds, which is then followed by a relatively short (1–2 h) *stabilization* step. These modifications appear to help considerably in obtaining the remarkably thorough impregnation of degenerating neurons illustrated in this communication.

### Impregnation

The amino acid-metallo-pyridinic (pre-impregnating) silver solution contains metal ions, which have a suppressive effect on tissue argyrophilia (34). This step, however, can be jeopardized by small variations in the amount of ammonium hydroxide used in the preparation of the silver-diamine (impregnating) solution. Thus, a careful manipulation of the ammonium hydroxide is strongly recommended to avoid unspecific staining.

### Bleaching

The idea of carrying the reducing step to its maximum limit and then using a modified photographic bleaching procedure to unmask the information, that is obscured by the excess silver precipitate, was introduced three decades ago by David et al. (22). These authors applied this strategy, based on the hypothesis that silver binds with different avidity to diverse components of the nervous tissue. Inspired by this idea, we have developed a comparable maneuver which includes two oxidative steps, consisting first of a treatment in an acidic K ferricyanide solution which eliminates most of the staining of normal nervous tissue components, followed by oxidation in an acidic K permanganate solution for the removal of remaining extraneous silver precipitates.

### Gold Toning

This treatment is highly recommended to avoid fading. On the other hand, it may be more difficult to discriminate degenerating axon terminals from the granular argyrophilic neuropil in gold-toned sections. The acidification with hydrochloric acid of the gold chloride solution proved to be the most effective recipe for preventing the reduction or even the partial loss of the impregnation of degenerating structures during the process of substituting gold for silver.

### Counterstaining

Because the addition of any type of buffer to mounting or counterstaining solutions appears to induce some fading of the stain, we recommend aqueous solution of neutral red or other dye if counterstaining is desired.