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Neuronal Death Following Soman Intoxication: Necrosis or Apoptosis?

Robert K. Kan
Christina P. Tompkins
Denise M. Fath
Tracey A. Hamilton
Margaret G. Filbert
Gerald P. H. Ballough

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U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

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14. ABSTRACT
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Abstract

We investigated the temporal progression of apoptotic cell death in the rat piriform cortex after soman intoxication. At various time intervals after seizure onset, animals were euthanized. Brain sections were stained with microtubule-associated protein 2 (MAP-2), Fluoro-Jade B (FJ-B) and in situ oligo ligation (ISOL). MAP-2 immunoreactivity was markedly decreased at 12 hr and 24 hr. FJ-B staining was evident as early as 1 hour after onset of seizures. The proportion of FJ-B positive cells was maximal at 12 hr and declined at 24 hr. The first presentation of ISOL staining was found at 24 hr. Ultrastructurally, no apoptotic cells were readily observed; necrotic neurons were dark and shrunken, with swollen mitochondria and endoplasmic reticulum in the neuronal cytoplasm and neuropil. The decline of MAP-2 staining and the increase in FJ-B labeling over the 24-hr period indicates that brain injury progresses with longer survival times. In addition, initial neuronal death following soman-induced seizures appears to be primarily mediated by necrosis since ISOL staining cells were not evident until 24 hr and FJ-B staining does not discriminate between necrotic and apoptotic cells. Therefore, a subpopulation of neurons surviving the initial necrotic phase dies by apoptosis.

Introduction

Soman (pinacolymethylphosphonofluoridate; O-1,2,2-trimethylpropylmethyl-phosphonofluoridate) is an organophosphonate nerve agent that causes convulsive seizures and death. The toxicity of soman is related to its ability to irreversibly inhibit acetylcholinesterase (AChE), leading to an excessive accumulation of the neurotransmitter acetylcholine (ACh) in neural and myoneural junctions. Increased levels of ACh were observed in the hippocampus and amygdala after soman exposure (Lallement et al., 1992a, 1992b). In addition, the excitatory amino acid glutamate was shown to be elevated in soman-exposed rats (Lallement et al., 1992b; Wade et al., 1987). It has been shown that ACh can potentiate glutamate-induced neurodegeneration by lowering neuronal threshold to the toxicity of glutamate (Mattson et al., 1989). Taken together, the persistence of seizures (status epilepticus) in soman poisoning is thought to be mediated by a release of the excitatory amino acid glutamate following an initial phase of cholinergic hyper-reactivity. This notion of glutamate in maintaining soman-induced seizures has been compellingly demonstrated. McDonough and Shih (1993) reported that when given 40 min after onset of seizures, the anticholinergic drugs scopolamine and atropine failed to block soman-induced seizures, but diazepam and MK-801 (dizocilpine), a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, were effective in terminating seizures. In addition, MK-801 and thienylcylohexylpiperidine (TCP), another noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, were found to arrest seizures and prevent brain damage induced by soman (Carpentier et al., 1994; Sparenborg et al., 1992; Braitman et al., 1989).

Brain areas that sustained considerable damage and neuronal loss after acute soman exposure included the piriform cortex, hippocampus, septum, entorhinal cortex, dentate gyrus, amygdala, and thalamus (Britt et al., 2000; Ballough et al., 1995; Carpentier et al., 1991; McDonough et al., 1989). However, the mechanism by which soman causes cell death remains to be determined. There are two principal cell death pathways, apoptosis and necrosis. Apoptosis, also called programmed cell death, is characterized by chromatin condensation and cell shrinkage with intact plasma membrane. In contrast, necrosis is characterized by cell swelling and a ruptured plasma membrane. The present study was undertaken to examine whether neuronal apoptosis contributes to brain damage in soman-induced seizures.

Materials and Methods

A total of 36 adult male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 250-350 g, were used in the study. Animals were randomly divided into five treatment groups that were euthanized at 1 hr, 3 hr, 6 hr, 12 hr and 24 hr after soman-induced seizure onset. Six animals were used as vehicle controls. All animals were housed in individual cages on a standard 12/12 h light/dark cycle with free access to food and water. All experiments were conducted in compliance with the regulations and standards of the Animal Welfare Act and adherence to the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Soman-Induced Status Epilepticus

One week prior to the study, a few animals were randomly selected from the treatment groups for cortical electrode implantation to confirm seizure activity. On the day of the experiment, animals that were implanted with an electrode were connected to ECG recording apparatus, and baseline ECG activity and behavior were monitored for 15 min; animals that did not have electrode implants remained in their cages. All animals were then pretreated with the oxime HI-6 (125 mg/kg, IP) 30 min prior to soman challenge (180 μg/kg, SC). One minute after soman injection, animals were post-treated with atropine methyl nitrate (AMN) (2.0 mg/kg, IM). HI-6 and AMN were used to decrease the mortality of soman-exposed animals (Shih et al., 1991). The concentration of soman was selected because it has been shown to produce seizures in 100% of the animals (Shih et al., 1991). Vehicle control animals received an equivalent volume of vehicle, HI-6 and AMN.

Brain Tissue Procurement

At the conclusion of the experiment, animals were anesthetized with an overdose of sodium pentobarbital and then transcardially perfused with 0.9% saline, followed by either 10% phosphate buffered formalin (10% PBF) or half Karnovski (1.6% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer). Brains perfusion-fixed with 10% PBF were extracted immediately and postfixed in the same fixative overnight for at least 18 hr at 4°C. The next day, brains were coronally cut into 3-mm slices using a rat brain matrix (ASI Instrument, Warren, MI) and processed in paraffin. Brain sections serially cut at 5 μm were processed for MAP-2 immunohistochemistry, Fluoro-Jade B (FJ-B), in situ oligo ligation (ISOL).

Brains perfusion-fixed with half Karnovsky's were extracted, cut coronally into 3-mm slabs and immersion fixed in half Karnovsky's overnight at 4°C. The following day, brain areas mentioned above were plugged out with a 2-mm biopsy puncher and processed for transmission electron microscopy.

Microwave-Assisted MAP-2 immunohistochemistry

Sections dewaxed in xylene were hydrated in graded ethanol and distilled water. To suppress endogenous peroxidase activity, sections were incubated in 5% hydrogen peroxide at room temperature for 20 min. Following thorough washing in running tap water (5 min), sections were rinsed in dH_2O, boiled 2 times (5 min each time) in 10 mM citric acid (Sigma-Aldrich, St Louis, MO; Lot 30H-0627) in a microwave (Pelco 3440 Max, 800 watts; Ted Pella, Inc, Redding, CA), and cooled at room temperature for 20 min prior to immunohistochemical staining (Kan et al., 2005).

Indirect immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu and co-workers (Hsu et al., 1981). Following microwave antigen retrieval (MAR), brain sections were incubated in 5% horse serum for 30 min at 4°C to block tissue immunoglobulins that could react with secondary antibody. Sections were then incubated in MAP-2 monoclonal antibody (1:100; Clone AP-18; NeoMarkers, Fremont, CA) for 18 hr at 4°C. After thoroughly rinsing in PBS, sections were sequentially incubated in biotinylated secondary

antibody for 1 hr at room temperature (Vector, Burlingame, CA) and ABC solution for 30 min at room temperature (Vector, Burlingame, CA). MAP-2 immunoreactivity was then visualized by incubating sections in diaminobenzidine (DAB) for 5 min at room temperature (Sigma-Aldrich, St. Louis, MO). Negative control sections were simultaneously processed without either MAR or primary antibody to ensure specificity of MAP-2 immunostaining.

Fluoro-Jade B Histochemistry

Fluoro Jade (FJ) (Histochem, Jefferson, AR) is an anionic fluorochrome that selectively stains degenerating neurons resulting from a variety of neurotoxic treatments (Schmued and Hopkins, 2000a; Schmued et al., 1997), seizures (Poirier et al., 2000) and ischemia (Butler et al., 2002). In the present study, FJ-B was chosen as it has a greater specific affinity than FJ for degenerating neurons (Schmued and Hopkins, 2000b). Sections were deparaffinized with EZ-DeWax^{TM} solution (BioGenex, San Ramon CA) and rinsed thoroughly with distilled water. Sections were transferred to 0.06% potassium permanganate for 15 min. Following two rinses in distilled water (2 min each) sections were stained in 0.0004% FJ-B for 30 min at room temperature. After sections were thoroughly rinsed in distilled water, they were dried overnight in the dark, immersed in xylene and mounted with Permount. Sections were examined under a BX61 fluorescence microscope using a fluorescein isothiocyanate (FITC) filter (Olympus).

In Situ Oligo Ligation Staining

In situ oligo ligation assay (Chemicon, Temecula, CA) is a DNA fragment labeling system that specifically recognizes double-stranded DNA breaks, which is a characteristic of apoptosis. Sections were hydrated (Sigma-Aldrich, St Louis, MO) and then incubated in 3% H_2O_2 for 30 min at room temperature (Sigma-Aldrich, St Louis, MO); proteinase K for 15 min at room temperature, equilibrium buffer for 15 sec at room temperature; Oligo A-biotin and T4 DNA ligase solution for 14 hr at 18ºC; and streptavidin-peroxidase for 30 min at room temperature. Ligation product was visualized by incubating sections with freshly prepared diaminobenzidine (Sigma-Aldrich, St. Louis, MO). The extent of brain injury and apoptosis was evaluated under a BX61 microscope (Olympus).

Transmission Electron Microscopy

Brain areas harvested with a 2-mm biopsy puncher were rinsed thoroughly with three changes of 0.1M sodium cacodylate. They were postfixed in 1% osmium tetroxide, dehydrated in graded ethyl alcohol, rinsed in propylene oxide and embedded in epoxy resin (Polybed 812). Semithin sections (1 μm) were made and stained with toluidine blue to identify areas of interest. Ultrathin sections were then collected on copper mesh grids, counterstained with lead citrate and uranyl acetate and analyzed by transmission electron microscopy (JEOL 1200).

Results

Temporal Progression of MAP-2 immunoreactivity

The extent of soman-induced brain damage was evaluated using MAP-2 immunohistochemistry. The development of brain injury was progressive as reflected by the pattern of MAP-2 immunoreactivity at different seizure onset time points (Figure 1). As early as 1 hr after onset of seizures, increased neuronal cell body and dendritic MAP-2 immunoreactivity was found in the piriform cortex as compared to control counterparts. However, this increase in MAP-2 immunoreactivity was accompanied with mild neuronal shrinkage in the piriform cortex. At 3 and 6 hr after seizure onset, immunoreactivity of MAP-2 continued to increase and no obvious loss of MAP-2 immunoreactivity was detected. At 12 hr and 24 hr after seizure onset, loss of MAP-2 immunoreactivity was pronounced in layers II and III. Vehicle control sections showed a typical pattern of MAP-2, predominantly in neuronal dendritic processes.

Temporal Progression of Fluoro-Jade B labeling

The degree of neurodegeneration was examined at different time intervals using FJ-B staining (Figure 2). FJ B labeling was detectable as early as 1 hr after seizure onset. The number of FJ-B stained neurons was maximal at 12 hr, but was reduced at 24 hr. No detectable FJ-B staining was observed in control animals.

Temporal Progression of Apoptosis

The extent of apoptosis was evaluated at different surviving times using ISOL staining procedure. No apoptosis was found in brain areas at 1, 3, 6, and 12 hr after onset of seizures (Figure 3). The first presentation of apoptotic cell death in the piriform cortex was found at 24 hr after seizure onset.

Transmission Electron Microscopy

To investigate the occurrence of apoptosis, ultrastructural characteristics of apoptotic cell death were examined. While apoptotic cells were not readily observed, necrotic cells were frequently found (Figure 4).

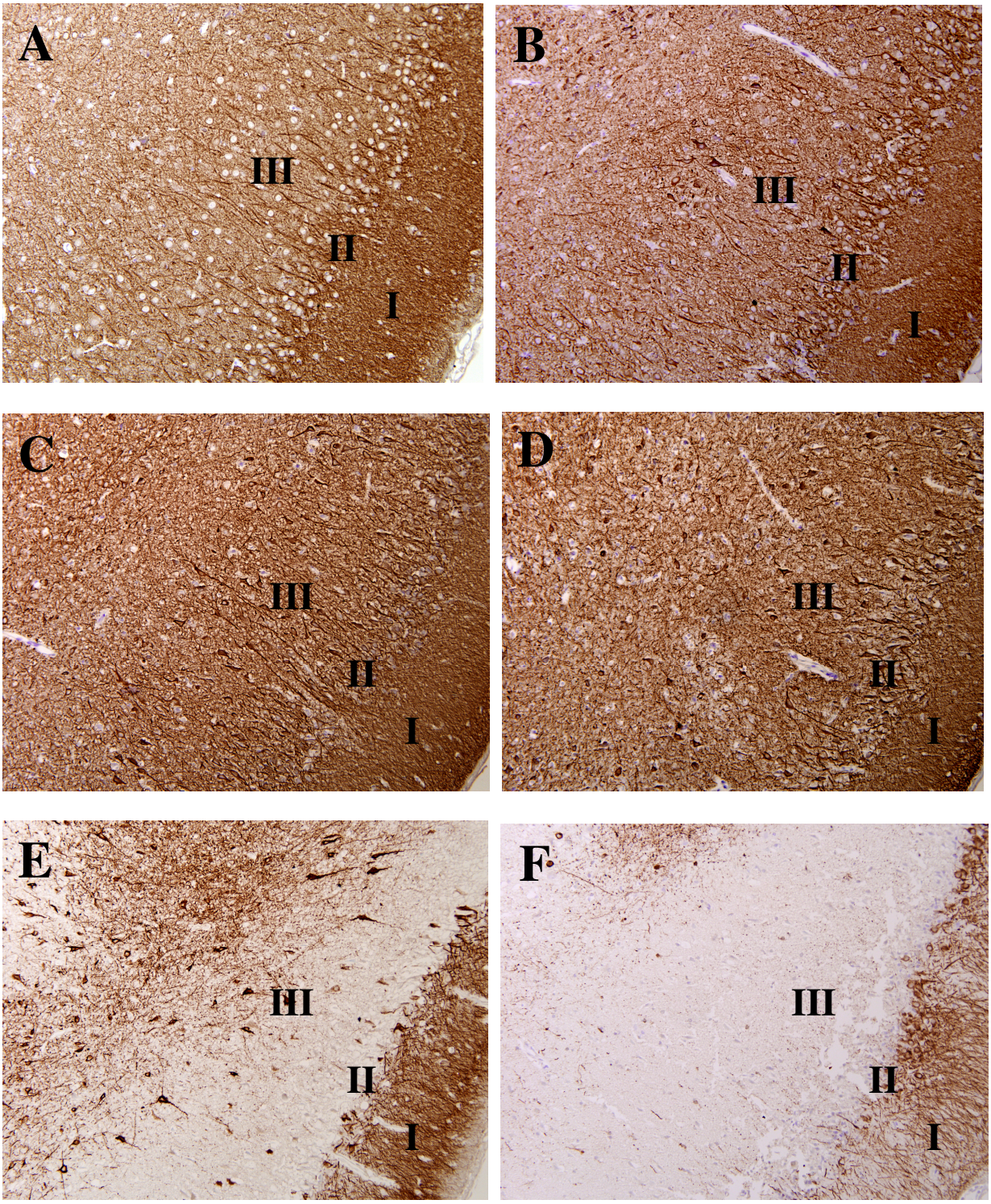

Figure 1. MAP-2 staining pattern in the piriform cortex at different surviving time points after soman-induced seizures. Vehicle control (A) shows MAP-2 staining mostly in dendritic processes and slightly in neuronal cytoplasm. At 1 hr (B) and 3 hr (C) after onset of seizures, increased MAP-2 staining is found in all layers of the piriform cortex, particularly neurons exhibiting altered morphology. At 6 hr (D) after seizure onset, increase in MAP-2 staining is accompanied by focal loss of MAP-2 staining in layers II and III. At 12 hr (E) after onset of seizures, loss of MAP-2 staining expands to all layers. At 24 hr (F) after seizure onset, layer III is completely devoid of MAP-2. Magnification 20X.

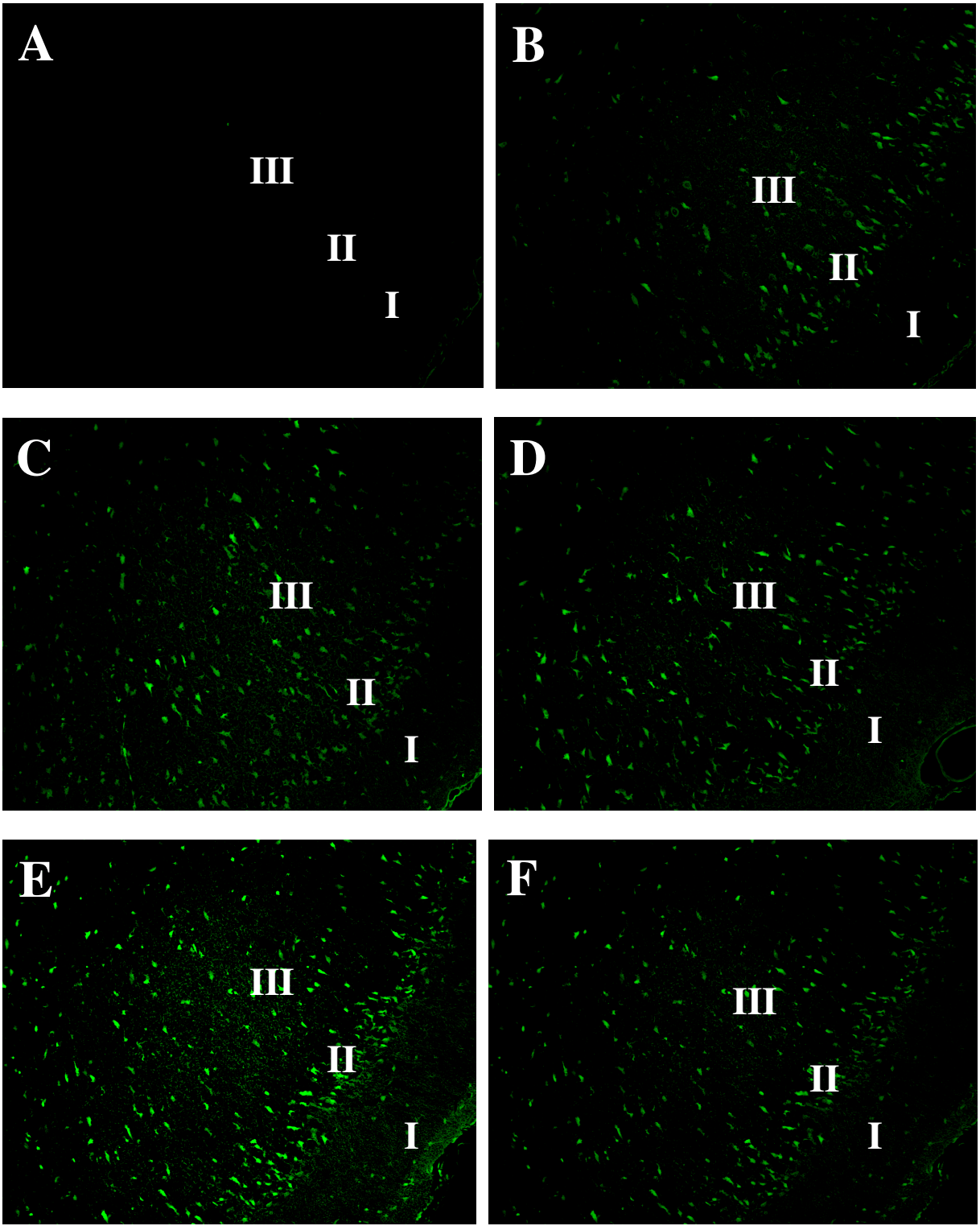

Figure 2. FJ B staining pattern in piriform cortex at different surviving time points after soman-induced seizures: Vehicle control (A), 1 hr (B), 3 hr (C), 6 hr (D), 12 hr (E) and 24 hr (F). FJ-B positive cells are apparent in layers II and III of the piriform cortex as early as 1 hr (B) after seizure onset. The number of FJ-B positive cells increases with longer surviving times, peaks at 12 hr (E) and decreases at 24 hr (F). Magnification 20X.

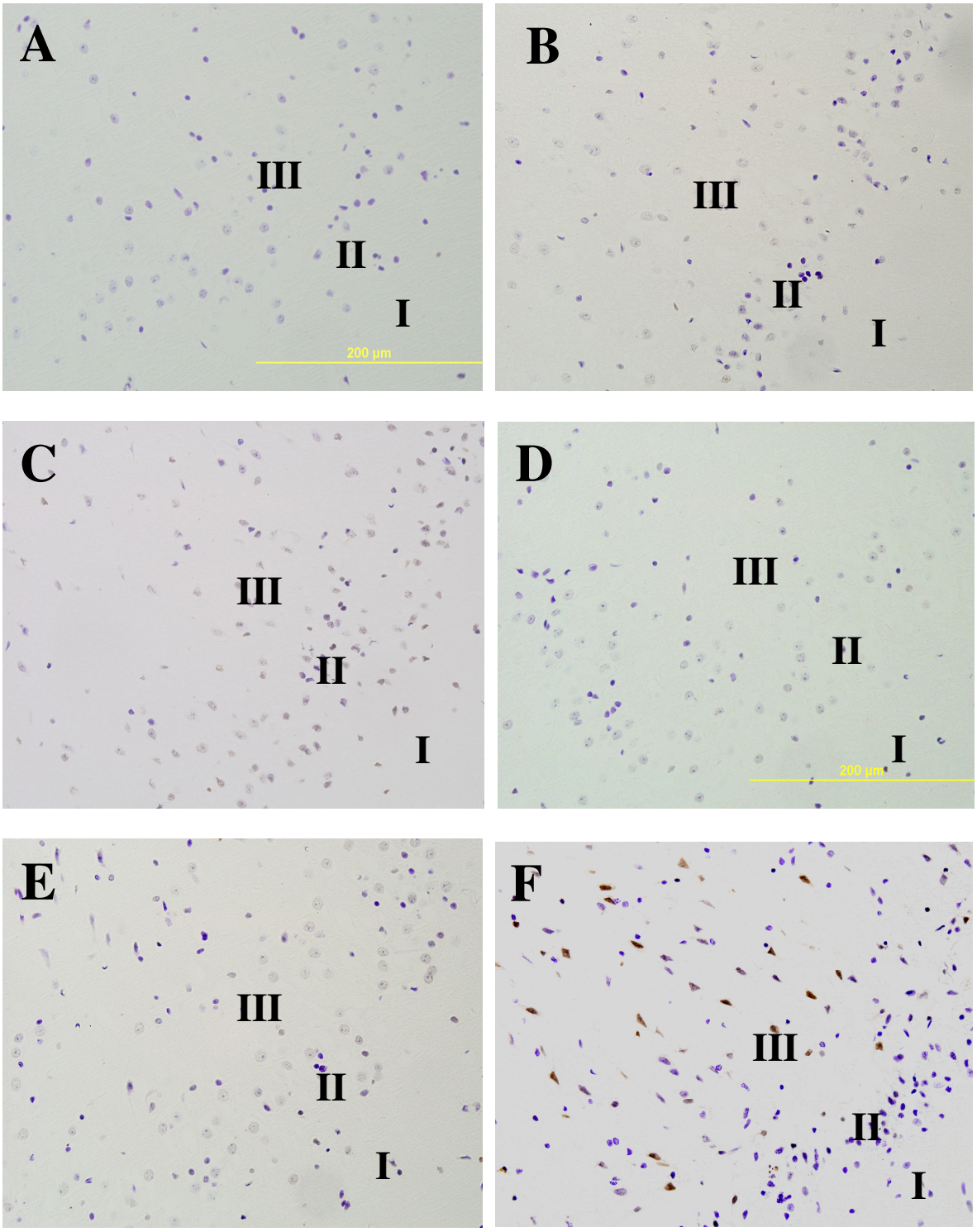

Figure 3. ISOL staining pattern in the piriform cortex at different surviving times after soman-induced seizures: Vehicle control (A), 1 hr (B), 3 hr (C), 6 hr (D), 12 hr (E) and 24 hr (F). No ISOL positive cells are detected until 24 hr (F) after seizure onset. All ISOL positive cells (dark brown) are found in layer III of the piriform cortex. Sections were counterstained with cresyl violet acetate (blue) for topography. Magnification 20X.

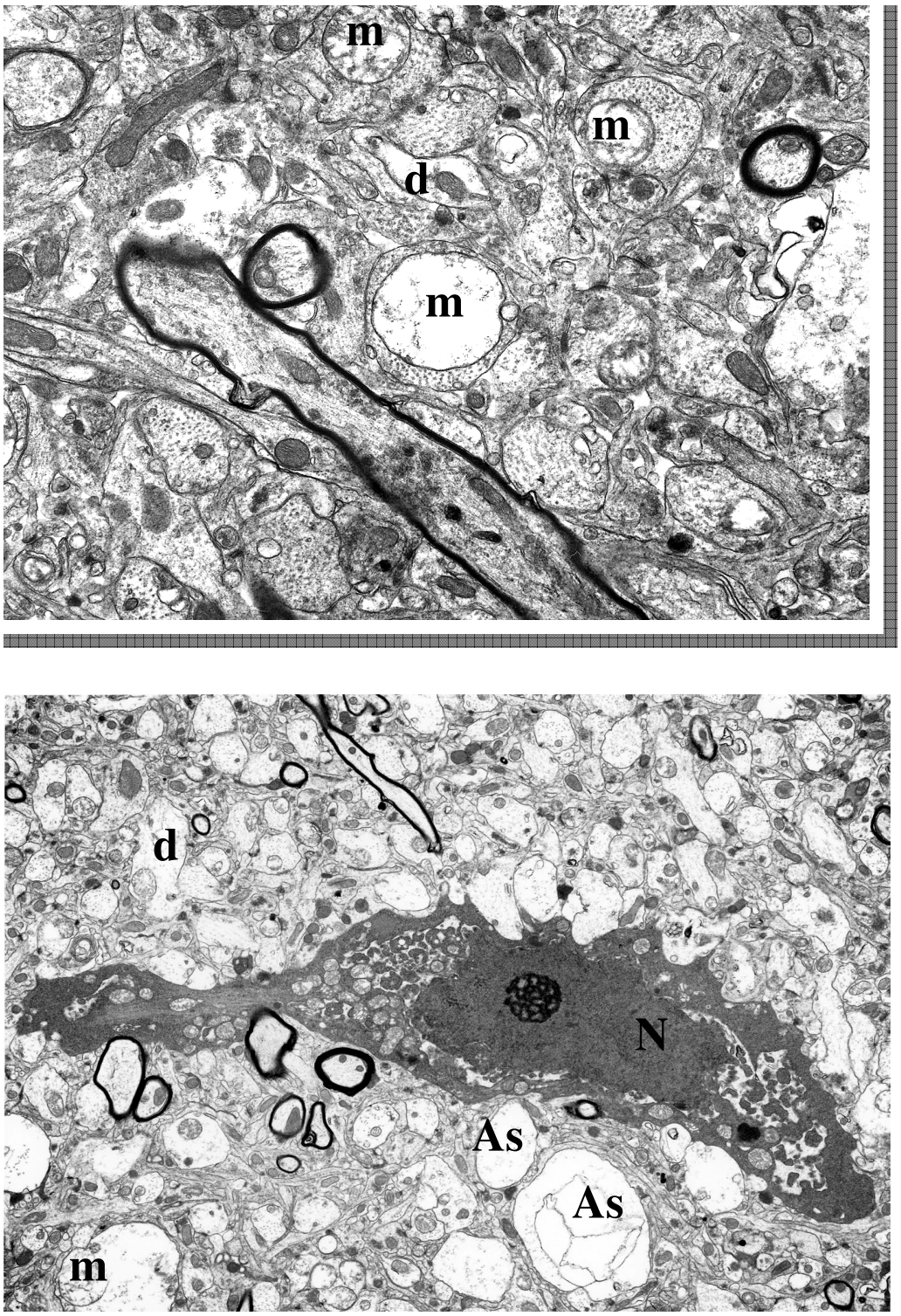

Figure 4. Electronmicrographs showing swollen mitochondria (m), swollen dendrites (d), necrotic neuron (N) and swollen astroglial processes (As) at 6 hr after seizure onset.

Discussion

Loss of MAP-2 staining is widely used as a sensitive indicator of brain damage in various experimental conditions (Folkerts et al., 1998; Posmantur et al., 1996; Ballough et al., 1995; Matesic et al., 1994; Kitagawa et al., 1989). Our results show a time-course pattern of MAP-2 immunostaining. Increased neuronal cell body and dendritic MAP-2 immunoreactivity was found in the piriform cortex at 1 and 3 hr after seizure onset. Since there is no neuronal degeneration, increased MAP-2 immunoreactivity at 1 and 3 hr may represent an increase in neuronal MAP-2 expression as a compensatory response to facilitate recovery of neuronal injury induced by soman-induced seizures. However, loss of MAP-2 immunoreactivity found at 6 hr and longer surviving time points may reflect a failure of neurons to elicit a successful compensation, leading to brain injury and neuronal death.

Fluoro Jade B (FJ-B) has been shown to label degenerating neurons in various seizure models including kainic acid (Riba-Bosch et al., 2004; Hopkins et al., 2000) and lithium-pilocarpine (Voutsinos-Porche et al., 2004; Kubova et al., 2002). Our results showed a strong association between the duration of surviving time and the number of FJ-B positive neurons. Neuronal degeneration as revealed by FJ-B was detected as early as 1 hr and peaks 12 hr following soman-induced seizure onset. Surprisingly, at 24 hr after seizure onset, the number of FJ-B labeled neurons was reduced. The reduction in the number of FJ-B labeled neurons at 24 hr could be due to the severe loss of neurons. The early detection of irreversible neuronal injury by FJ-B staining reinforces the need to terminate seizures soon after induction by soman.

The relationship of the staining pattern of FJ-B and ISOL was examined at different surviving times to investigate the time-course occurrence of apoptosis. It was found that the staining pattern between FJ-B and ISOL was different at different surviving times. FJ-B staining was detected as early as one hour after seizure onset. The number of FJ-B positive cells increased with time and peaked at 12 hr after seizure onset. At 24 hr after seizure onset, the number of FJ-B positive cells decreased, probably due to severe neuronal loss, and the first presentation of apoptotic cells as shown by ISOL staining was evident. FJ-B staining does not discriminate between apoptotic and necrotic cells and only a few scattered apoptotic cells were seen at 24 hr after seizure onset, indicating that necrosis is the primary cell death pathway during the first 24 hr of soman exposure and delayed apoptosis is the cell death process after 24 hr.

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