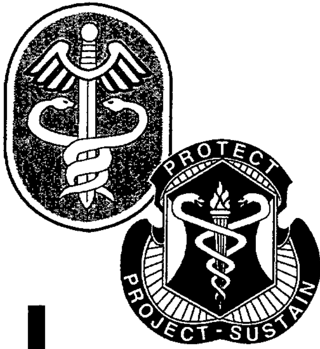

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Determining Optimal Microwave Antigen Retrieval Conditions for Microtubule-associated Protein 2 Immunohistochemistry in the Guinea Pig Brain

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14. ABSTRACT
The present study examined the efficacy of microwave pretreatment on microtubule-associated protein 2 (MAP-2) immunoreactivity in paraffin-embedded sections of formalin-fixed guinea pig brains using different MAP-2 monoclonal antibodies. Brain sections were boiled in sodium citrate, citric acid, Tris hydrochloride, and EDTA solutions with pH values of 2, 4, 6, and 8 in a microwave prior to MAP-2 immunohistochemical staining. Specific MAP-2 immunoreactivity was observed in brain regions when NeoMarkers MAP-2 antibody (clone AP-18) was used in conjunction with citric acid buffer of pH 6.0 as an antigen retrieval solution. No immunoreactivity of MAP-2 was observed in negative control sections. The results suggest that a 10-min boiling in citric acid solution at pH 6.0 is the optimal microwave-assisted AR method for immunolabeling MAP-2 in formalin-fixed, paraffin-processed guinea pig brain samples using NeoMarkers MAP-2 monoclonal antibody (AP-18). This undoubtedly will have important applications in our efforts to conduct retrospective studies on archival guinea pig brain paraffin blocks, ultimately relaxing the use of additional animals to evaluate changes in MAP-2 expression between chemical warfare nerve agent-treated and control samples.

15. SUBJECT TERMS
Microwave antigen retrieval, immunohistochemistry, microtubule associated protein 2 (MAP-2), formalin fixation, paraffin-embedding

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1. INTRODUCTION

Immunohistochemistry (IHC) is increasingly being used as a routine research tool in neurobiology and neuropathology for investigating the expression of cellular proteins in tissue sections. Although immunodetection of brain proteins is historically performed on fresh-frozen cryostat sections, the practice has inherent practical problems associated with storage and may yield poor morphological preservation. Because formalin-fixed, paraffin-embedded sections produce tissue morphology superior to that of frozen sections, they are better suited to correlate immunohistochemical data with morphopathological findings.

However, the usefulness of IHC on formalin-fixed, paraffin-embedded tissues is limited by the difficulty in retrieving the formalin-altered epitopes within the section (Brown, 1998). Formalin-induced intra- and inter-molecular cross-linking between macromolecules alters protein secondary structure, limits antibody penetration of sections, and reduces accessibility of antigenic determinants (Shiurba et al., 1997). Consequently, immunoreactivity is diminished for many antigens. Successful antibody binding and subsequent detection by IHC therefore requires some form of treatment to undo or address these formalin-induced changes.

Different techniques such as enzyme predigestion, formic acid pretreatment, microwave heating, autoclave heating, and ultra-sound pretreatment were developed to overcome this problem (Battifora and Kopinski, 1986; Kitamoto et al., 1987; Shi et al., 1991; Shin et al., 1991; Podkletnova and Alho, 1993). Comparisons between these different antigen retrieval (AR) methods led to the conclusion that microwave-assisted AR is a powerful tool for obtaining optimal immunohistochemical results in formalin-fixed, paraffin-embedded tissue sections (McQuaid et al., 1995). High temperature heating of tissues is believed to be the most critical factor of AR; however, the composition of the AR solution itself has undergone scrutiny, and different AR solutions have been described as optimal for enhancement of different antigens with different antibodies (Shi et al., 1991; Gown et al., 1993). In addition, the pH of the AR solution influences the degree of unmasking of epitopes (Shi et al., 1995). Most antigens are retrievable using aqueous solutions over a range of pH 6.0–8.0, but there are some antigens that can be retrieved at very basic (pH 10.0) or very acidic (pH 2.0) pH values. Therefore, a "test battery" should be used to establish an optimal AR protocol for certain antigens under investigation (Shi et al., 1997).

Our laboratory is interested in evaluating alterations in neuronal cytoskeletal proteins as indicators of neuronal damage using the guinea pig as a model system. Microtubule-associated protein 2 (MAP-2) is the most abundant neuron-specific cytoskeletal protein in the brain, localized mostly in the dendritic processes (Caceres et al., 1984; De Camilli et al., 1984). Loss of MAP-2 immunoreactivity has been shown to be a sensitive marker for nerve agent soman-induced brain damage and for cerebral ischemic damage (Ballough et al., 1995; Kitagawa et al., 1989). To our knowledge, no reports exist on immunohistochemical staining of MAP-2 in routinely formalin-fixed, paraffin brain sections of the guinea pig. Thus, it is important to establish an optimal protocol of AR to obtain maximal enhancement of MAP-2 staining.

The present study was undertaken to evaluate the efficacy of microwave antigen retrieval (MAR) on immunohistochemical staining of MAP-2 in formalin-fixed, paraffin-embedded guinea pig brain samples. A series of AR solutions were tested for their effect on the immunohistochemical staining reactions of different clones of MAP-2 monoclonal antibodies.

2. MATERIALS AND METHODS

2.1 AR Solutions

Four different AR solutions were used. Ten mM citric acid monohydrate (Sigma-Aldrich; St Louis, MO; Lot 30H-0627), 0.1mM EDTA (FisherBiotech, Fair Lawn, NJ; Lot 014990A), 10mM sodium citrate (Spectrum Chemical MFG. Corp., Gardena, CA; Lot AG198), and 0.1mM Tris hydrochloride (Sigma-Aldrich, St. Louis, MO; Lot 64H5768) were prepared according to formula. Solutions were adjusted to pH values of 2.0, 4.0, 6.0, and 8.0 by adding 1 N HCl or 2 N NaOH, while monitoring with a pH meter (Beckman Instruments, Fullerton, CA).

2.2 Tissue Preparation

Routinely formalin-fixed (10% neutral phosphate buffered formalin for 24 hr) guinea pig brains were sectioned coronally in a guinea pig brain matrix (ASI Instruments, Warren, MI). Brain samples were processed in paraffin, sectioned serially at 5µm, and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). Brain sections were allowed to dry at room temperature for 24 hr and then processed for MAR-IHC.

2.3 Microwave Pretreatment

Following dewaxing in xylene and hydration to distilled water, sections were incubated in 3% hydrogen peroxide for 15 min at room temperature to suppress endogenous peroxidase activity. Sections were then rinsed in running tap water and washed thoroughly with distilled water. Areas in the microwave oven (Pelco 3440 Max, Ted Pella, Inc., Redding, CA) with the highest intensity of microwave radiation, or "hot spots," were predetermined by using a Microwave Finder Mat (Ted Pella, Inc., Redding, CA). Two plastic Coplin jars containing one of the four AR solutions were placed on these "hot spots" and brought to a boil. Sections were then transferred to one of the Coplin jars and boiled for 2 5-min cycles, with the power of the microwave set at 100%. Each cycle was broken into two equal time periods of 2.5 min, so that more AR solution could be added to compensate for loss due to boiling over and to avoid drying out the tissue sections (the second Coplin jar served as filling solution for this purpose). The next cycle was repeated in the same manner with fresh AR solution. Caution was made to ensure that Coplin jars were placed on "hot spots" since the location of slides in the microwave produces different staining results. Following two cycles of boiling in the microwave for a total time of 10 min, sections were allowed to cool at room temperature for a minimum of 20 min prior to processing for immunohistochemistry.

2.4 MAP-2 Immunohistochemistry

Indirect MAP-2 immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. (1981). Brain sections, rinsed twice in PBS, pH 7.4 (Sigma-Aldrich, St. Louis, MO; Lot 12K8203), were incubated in 5% normal horse serum for 30 min at 4°C to block non-specific binding sites of tissue immunoglobulins to secondary antibody. Sections were then incubated with mouse monoclonal anti-MAP-2 antibody for 18 hr at 4°C. Following two washes with PBS, sections were incubated with biotinylated secondary

antibody (1:200 dilution) (Vector, Burlingame, CA) for 1 hr at room temperature, washed twice with PBS, and allowed to react with ABC reagent (Vector, Burlingame, CA) for 30 min at room temperature. The presence of MAP-2 immunoreactivity was visualized as a brown precipitate after incubating sections in DAB-H_2O_2 solution (Sigma-Fast DAB tablet sets, Sigma-Aldrich, St. Louis, MO) for 5 min. The tablets were dissolved in 6ml of distilled water instead of 5ml as recommended by the manufacturer, a modification made to attenuate the rate of peroxidase-DAB-H_2O_2 reaction. Finally, sections were counterstained with 0.8% cresyl violet acetate for morphology and topography and mounted with Permount® (Fisher Scientific, Fair Lawn, NJ) for light microscopic examination. Negative control sections were treated in an identical manner except that incubation in primary antibodies or microwave pretreatment was omitted.

Mouse MAP-2 monoclonal antibodies used in this study included clones AP-18 and AP-20 (NeoMarkers, Fremont, CA), AP-20 (PharMingen, San Diego, CA), and HM-2 (Sigma-Aldrich, St. Louis, MO). All primary antibodies were diluted 1:100 with PBS in vertical, 5-slide mailers (Fisher Scientific, Fair Lawn, NJ). The advantage of diluting the antibodies in slide mailers is that primary antibodies can be reused, reducing the cost of purchasing expensive antibodies

2.5 Evaluation of Immunohistochemical Staining Results

Slides were examined using an Olympus BH-2 microscope. Each slide was graded based on four criteria: stain intensity, stain specificity, stain uniformity, and tissue integrity. If all four criteria were met, a grade of ++++ was assigned. Likewise, a grade of +++ was assigned if three of the four criteria were met, ++ if two were met, and + if only one of the criteria was met. Photomicrographs were taken with an Olympus AH-3 microscope with built-in camera and exposure control units. Kodak Ektachrome 64T film was used for color (Eastman Kodak, Rochester, NY).

3. RESULTS

Table I summarizes the results of MAP-2 immunostaining obtained using different MAP-2 monoclonal antibodies, following MAR. The immunoreactivity of MAP-2 was highly influenced by the pH of the AR solution, the type of AR solution, and the clone of the antibody. No MAP-2 immunoreactivity was observed following pretreatment with citric acid solution at pH 2.0 (Figure 1A) and 4.0 (Figure 1B) using NeoMarkers clone AP-18. However, when citric acid at pH 6.0 was used as the AR solution, NeoMarkers clone AP-18 displayed specific dendritic MAP-2 immunoreactivity with excellent tissue morphology (Figure 1C). With citric acid solution at pH 8.0, the edges of the tissue section lifted off the slide, producing uneven MAP-2 immunoreactivity and increased background staining (Figure 1D). Reduction in cresyl violet acetate counterstaining was also observed after AR heating in all solutions tested. At low pH (pH 2.0), loss of cresyl violet stain intensity was exacerbated.

Comparing each of the AR solutions at pH 6.0, using NeoMarkers clone AP-18, citric acid produced superior MAP-2 immunoreactivity (Figure 2A), followed by EDTA (Figure 2B, Tris hydrochloride (Figure 2C), and sodium citrate (Figure 2D). After establishing optimal MAP-2 immunoreactivity using citric acid at pH 6.0, each of the four antibodies was compared.

NeoMarkers clone AP-18 produced the best MAP-2 immunoreactivity (Figure 3A), followed by Sigma clone HM-2 (Figure 3B), and PharMingen clone AP-20 (Figure 3C). Very weak MAP-2 immunoreactivity was produced by NeoMarkers clone AP-20 (Figure 3D). No specific immunoreactivity of MAP-2 was observed in method control (Figure 4A) and negative control (Figure 4B) sections.

Table I. MAP-2 immunoreactivity of four commercially available antibodies following MAR in different buffer solutions of various pH values.

Antibody	AR Solution	pH 2.0	pH 4.0	pH 6.0	pH 8.0
PharMingen AP-20	EDTA	+	++	++	++
PharMingen AP-20	Na Citrate	+	+++	++	+
PharMingen AP-20	Citric acid	+	+	++	++
PharMingen AP-20	Tris HCl	+	++	++	++
NeoMarkers AP-18	EDTA	+	+	+++	+++
NeoMarkers AP-18	Na Citrate	+	++	+++	+++
NeoMarkers AP-18	Citric acid	+	+	++++	+++
NeoMarkers AP-18	Tris HCl	+	+	+++	+++
NeoMarkers AP-20	EDTA	+	+	+	++
NeoMarkers AP-20	Na Citrate	+	+	+	+
NeoMarkers AP-20	Citric acid	+	+	+	++
NeoMarkers AP-20	Tris HCl	+	+	+	+
Sigma HM-2	EDTA	+	+	+++	+
Sigma HM-2	Na Citrate	+	++	++	++
Sigma HM-2	Citric acid	+	+	++	++
Sigma HM-2	Tris HCl	+	+	++	++

4. DISCUSSION

IHC on tissue sections is an important diagnostic and research tool in all branches of pathology. In neuropathology, the localization of structural proteins by IHC is of major importance in determining the etiology or mechanisms of disease processes. However, tissue preparation methods, which are optimal for the best preservation of morphology (formalin and paraffin embedding), cause cross-linking of proteins and are therefore sub-optimal for the preservation of many antigenic epitopes within tissues. MAR has proved to be an important tool in the retrieval of many antigens. The MAR technique has been used increasingly worldwide as a simple, extremely effective, and reliable pretreatment routine for IHC and has become a standard technique for analytical morphology (Boon and Kok, 1995; Cattoretti and Suurmeijer, 1995; Taylor et al, 1996; Shi et al., 1997).

In this study, we utilized different MAR methods for the immunodetection of MAP-2 in the guinea pig brain using four commercially available monoclonal MAP-2 antibodies. The effectiveness of MAR on MAP-2 immunostaining labeled by these antibodies was judged by the intensity and anatomical localization of MAP-2 immunoreactivity, background staining, and tissue integrity. Regardless of which antibody was used, no specific MAP-2 staining was observed following pretreatment with AR solutions at low pH (pH 2.0 and 4.0). Using citric acid solution at pH 6 as the AR solution, only NeoMarkers clone AP-18 generated the strongest intensity following MAR-IHC. The results demonstrate that the optimal MAR is dependent on the type of AR solution and pH value of the AR solution, which are in agreement with the findings of other investigators (Gown et al., 1993; Shi et al., 1994).

The effectiveness of AR is also strongly influenced by the specificity and avidity of the primary antibody. Our results demonstrate that NeoMarkers clone AP-18 MAP-2 antibody is clearly superior to clone AP-20 and to MAP-2 monoclonal antibodies from Sigma and PharMingen. NeoMarkers clone AP-18 antibody, which showed little or no immunoreactivity without microwave pretreatment, showed apparently high sensitive and specific labeling after microwave pretreatment. As seen with Sigma and PharMingen antibodies, background staining may be increased with MAR. Therefore, it is important when evaluating this AR method to carefully compare a number of different clones to select the best antibody for future immunohistochemical research projects.

5. CONCLUSIONS

In summary, the use of a test battery is recommended as a rapid and convenient means of evaluating AR solution and pH. The use of four AR solutions (EDTA, sodium citrate, citric acid, and Tris hydrochloride) and four pH values (2.0, 4.0, 6.0, and 8.0) was adequate in determining optimal immunoreactivity of MAP-2 in the formalin-fixed, paraffin-embedded guinea pig brain. We conclude that boiling sections in 10mM citric acid solution of pH 6.0 for 10 min is optimal for use with NeoMarkers MAP-2 monoclonal antibody (clone AP-18). This application undoubtedly will have important applications in our efforts to conduct retrospective studies on archival guinea pig brain paraffin blocks, ultimately relaxing the use of additional animals to evaluate changes in expression of MAP-2 between chemical warfare nerve agent-treated and control samples.

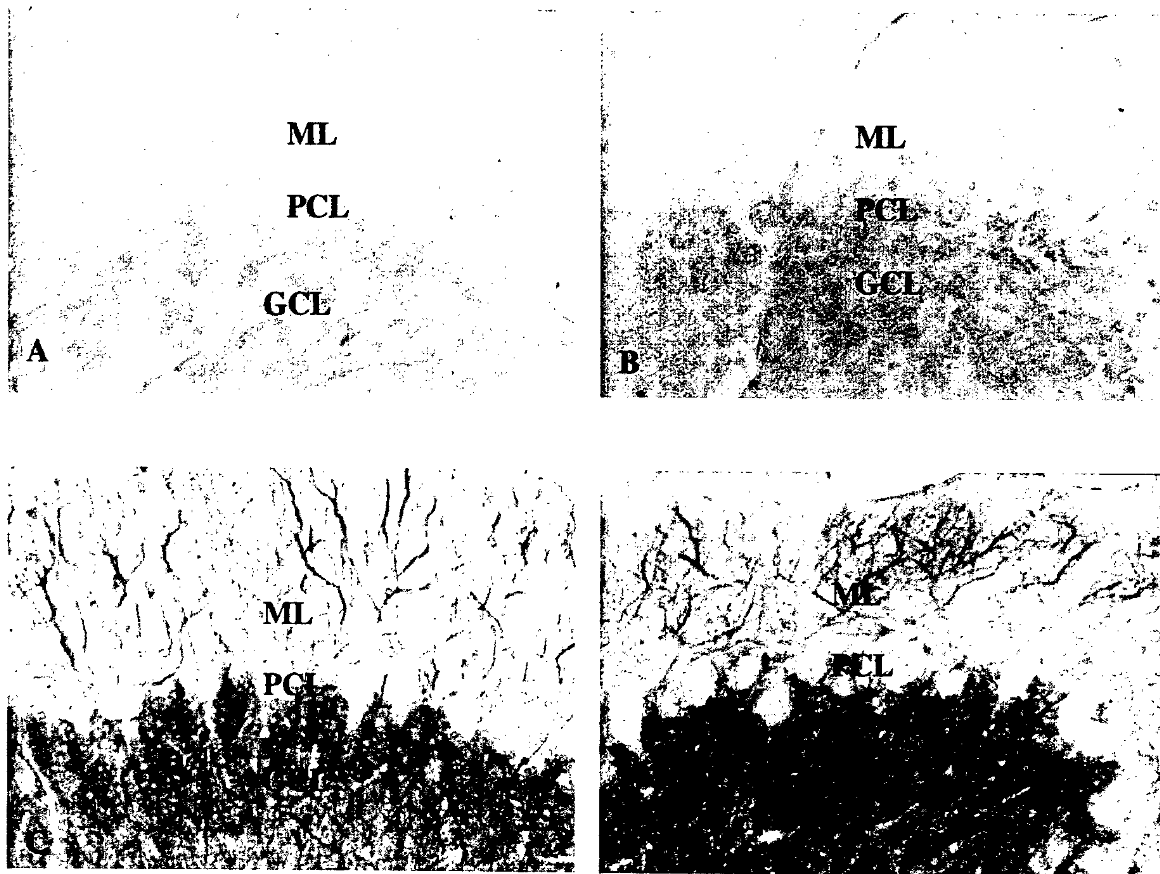

Figure 1. MAP-2 (NeoMarkers, clone AP-18) immunoreactivity in cerebellum processed with microwave pretreatment using citric acid at (A) pH 2.0, (B) pH 4.0, (C) pH 6.0, and (D) pH 8.0. ML, Molecular cell layer; PCL, Purkinje cell layer; GCL, Granule cell layer. Magnification 80X.

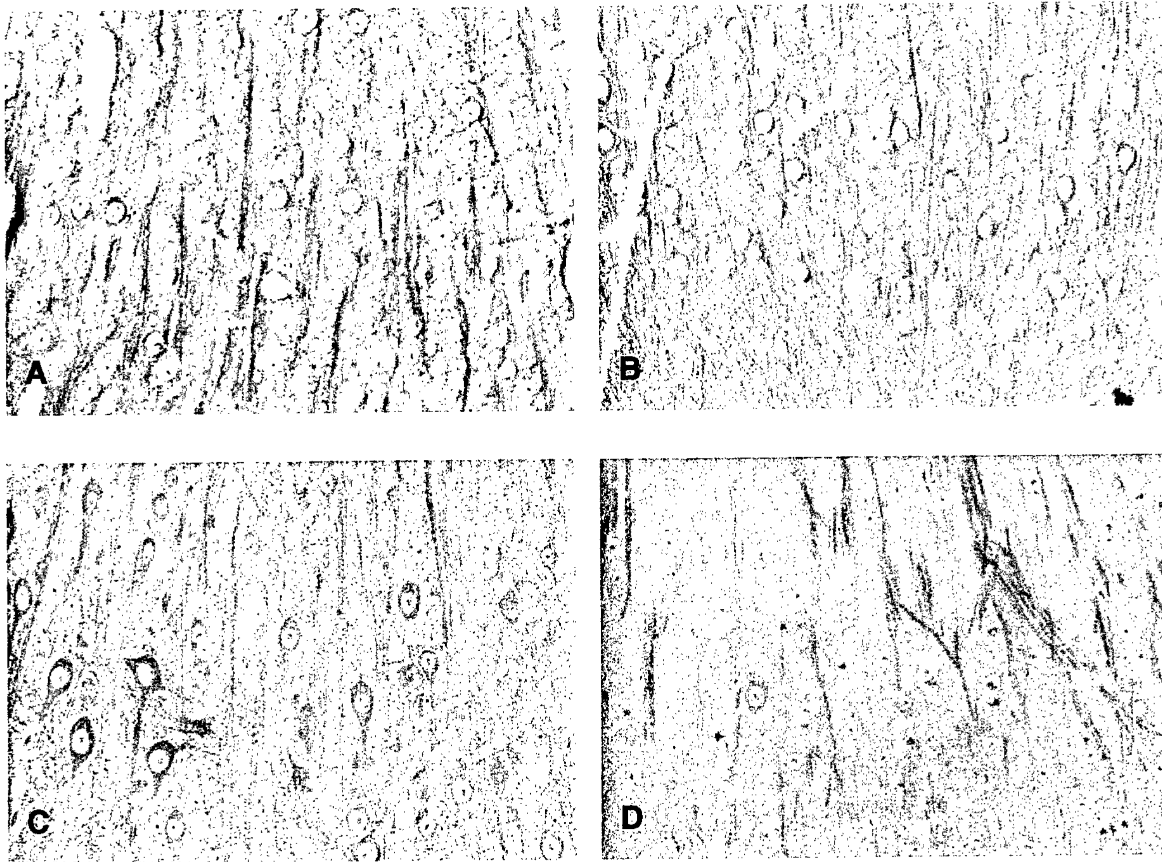

Figure 2. MAP-2 (NeoMarkers, clone AP-18) immunoreactivity in occipital region processed with microwave pretreatment using (A) citric acid, (B) EDTA, (C) Tris hydrochloride, and (D) sodium citrate at pH 6.0 as antigen retrieval solutions. Magnification 80X.

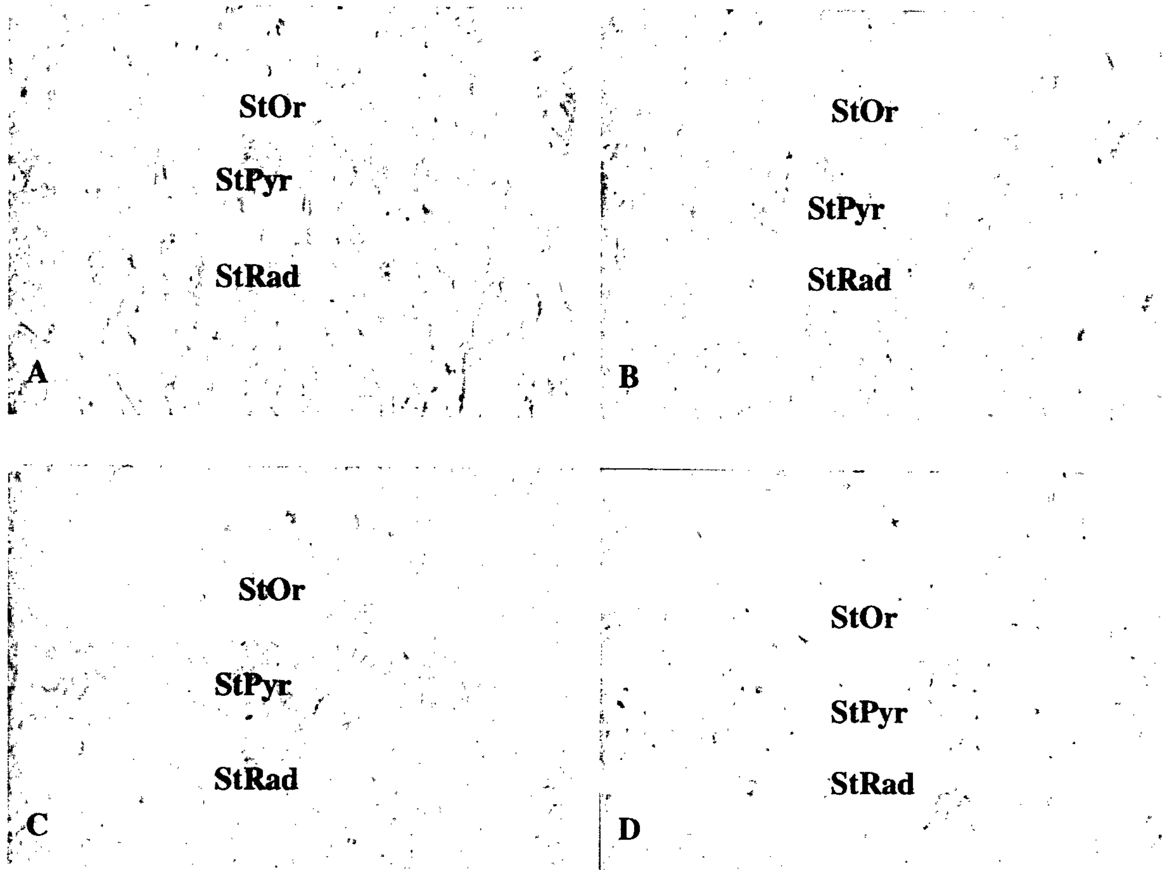

Figure 3. MAP-2 immunoreactivity in CA3 region with (A) NeoMarkers clone AP-18, (B) Sigma clone HM-2, (C) PharMingen clone AP-20, and (D) NeoMarkers clone AP-20 antibodies, following microwave pretreatment with citric acid at pH 6.0. StOr, Stratum orien; StPyr, Stratum pyramidal; StRad, Stratum radiatum. Magnification 80X.

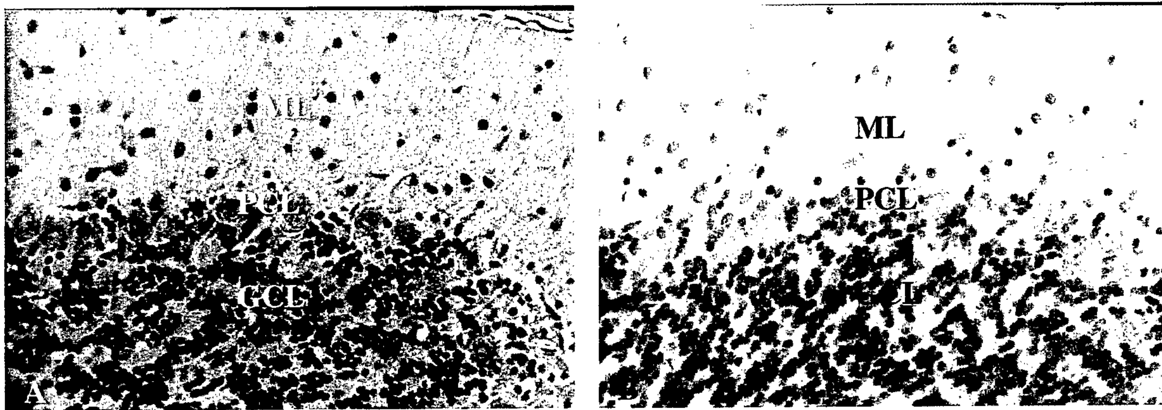

Figure 4. Representative micrographs of the cerebellum processed (A) without microwave antigen retrieval (method control) and (B) without primary antibody (negative control). No MAP-2 immunoreactivity was observed in these control sections. ML, Molecular layer; PCL, Purkinje cell layer; GCL, Granule cell layer. Magnification 80X.

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APPENDIX A

Microwave Antigen Retrieval Technique

1. Deparaffin sections and hydrate to dH_2O.
2.
   A. Xylene (2 changes, 10 min each)
   B. 100% ETOH (2 changes, 3 min each)
   C. 95% ETOH (2 changes, 3 min each)
   D. 70% ETOH (2 changes, 3 min each)
   E. dH_2O (2 changes, 3 min each)

3. Quench sections with 3% H_2O_2 for 15 min to eliminate endogenous peroxidase activity.

   50 ml of 3% H_2O_2 solution: 45ml PBS
   5ml of 30% H_2O_2

4. Rinse in running tap water, 5 min.

5. Rinse in dH_2O, 5 min.

6. Place two Coplin jars containing desired AR solution on microwave "hot spot" and bring to a boil (approximately 2.5 min).

   10mM citric acid: 2.1g citric acid in 1 liter dH_2O
   0.1mM EDTA: .29g EDTA in 1 liter dH_2O
   10mM sodium citrate: 29.4g sodium citrate in 1 liter dH_2O
   0.1mM Tris hydrochloride: 15.7g Tris hydrochloride in 1 liter dH_2O

7. Place slides in one of the Coplin jars and boil sections for 2.5 min.

8. Top off AR solution level with AR solution from the second Coplin jar.

9. Boil sections for another 2.5 min.

10. Repeat steps 5-8 with fresh AR solution.

11. Cool for 20 min after last treatment.

APPENDIX B

ABC Immunohistochemistry Technique

1. Rinse microwave pretreated slides with PBS, 5 min.

2. Incubate sections in blocking serum (5% normal serum from animals that made the secondary antibody) for 30 min at 4°C (Vector, Burlingame, CA).

   10ml solution: 9500μl PBS
   500μl normal serum

3. Incubate sections in primary antibody (1:100 dilution) for 18 hours at 4°C.

   10ml solution: 9900μl PBS
   100μl normal serum [MAP-2 AP-18 and AP-20 (NeoMarkers, Fremont, CA), AP-20 (PharMingen, San Diego, CA), and HM-2 (Sigma-Aldrich, St. Louis, MO)]

4. 2 rinses with PBS, 5 min each.

5. Incubate sections in biotinylated secondary antibody (1:200) dilution at room temperature for 1 hour (Vector, Burlingame, CA).

   5ml solution: 4975μl PBS
   25μl biotinylated antibody solution

6. 2 rinses with PBS, 5 min each.

7. Incubate slides with avidin-biotin-peroxidase complex solution at room temperature for 30 min (Vector, Burlingame, CA). This solution must be made 30 min before use.

   5ml solution: 4800μl PBS
   100μl of reagent "A" (Avidin)
   100μl of reagent "B" (Biotinylated horseradish peroxidase)

8. 2 rinses with PBS, 5 min each.

9. Incubate sections in DAB (3,3′-Diaminobenzidine tetrahydrochloride) solution for 5.5 min (Sigma-Aldrich, St. Louis, MO).

   Dissolve 1 DAB tablet (0.7 mg/ml) and 1 urea hydrogen peroxide tablet (0.2 mg/ml) in 6ml dH_2O.

10. Rinse with dH_2O, 5 min.

11. Counterstain sections with 0.8% Cresyl violet working solution, 15 min.

A. Cresyl violet solution (Sigma-Aldrich, St. Louis, MO)
   Cresyl violet acetate.......4.0g
   dH_2O.....................100.0ml
B. 0.1M acetic acid (Fisher Scientific, Fair Lawn, NJ)
   glacial acetic acid..........3.0ml
   dH_2O.....................500.0ml
C. 0.1M sodium acetate (Sigma-Aldrich, St. Louis, MO)
   sodium acetate.............6.8ml
   dH_2O.....................500.0ml
D. Cresyl violet solution (0.8%)
   0.1M acetic acid.........376.0ml
   0.1M sodium acetate.....24.0ml
   Cresyl violet acetate....100.0ml

12. Rinse with dH_2O, 2 min.

13. Dehydrate and clear.

A. 70% ETOH (1 change, 2 min)
B. 95% ETOH (1 change, 2 min)
C. 100% ETOH (2 changes, 2 min each)
D. Xylene (2 changes, 2 min each)

14. Mount slides with Permount® (Fisher Scientific, Fair Lawn, NJ).