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Research report

Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration

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Abstract

Fluoro-Jade B, like its predecessor Fluoro-Jade, is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration. However, Fluoro-Jade B has an even greater specific affinity for degenerating neurons. This notion is supported by the conspicuous staining of degenerating neuronal elements with minimal background staining. This improved signal-to-noise ratio means that fine neuronal processes including distal dendrites, axons and axon terminals can be more readily detected and documented. Although the staining time and dye concentration are reduced, the method is as rapid, simple and reliable as the original Fluoro-Jade technique. Like Fluoro-Jade, Fluoro-Jade B is compatible with a number of other labeling procedures including immunofluorescent and fluorescent Nissl techniques.

Theme: Cellular and molecular biology

Topic: Staining, tracing, and imaging techniques

Keywords: Fluoro-Jade B; Neuronal degeneration; Kainic acid; Neuropathology

1. Introduction

Recently it was reported that Fluoro-Jade, a novel fluorochrome, could be used for detecting neuronal degeneration [5]. This dye exhibited the advantage over older routine histochemical stains like Nissl or hematoxylin and eosin in that it was specific for degenerating neurons. In this respect, Fluoro-Jade was more like a suppressed silver stain [1,3] which has its own associated advantages and limitations. Advantages include good contrast and permanence, while drawbacks include the labor intensive and capricious nature associated with suppressed silver stains. The general objective in developing Fluoro-Jade B was to develop a tracer that would retain the advantages associated with Fluoro-Jade (e.g. speed, sensitivity and reliability) while further increasing the signal to background ratio, thus improving both contrast and resolution. Such a high affinity tracer would make it easier to detect labeled fine neuronal processes including distal dendrites, axons and terminals. To validate the usefulness of Fluoro-Jade B in detecting neuronal degeneration, lesions were produced in the rat forebrain by exposure to kainic acid, a well characterized neurotoxicant [4].

2. Materials and methods

2.1. Animals

All experiments used adult (3–6 month old) male Sprague–Dawley rats in accordance with the Institutional Animal Care and Use Committee guidelines. Food and water were given ad libitum.

2.2. Treatment

The rats ($n=9$ individuals/group) were dosed with kainic acid (10 mg/kg, i.p.) and sacrificed 4 days later. A control group received an injection of physiological saline. Kainic acid was obtained from Sigma Chem. Co. (St. Louis, MO.).

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2.3. Processing

Following the 4 day survival interval, animals were anesthetized with Ketamine (75 mg/kg) and Rompun (9 mg/kg) and then perfused with 300 ml of 0.1 M neutral phosphate buffered 10% formalin (4% formaldehyde) via the ascending aorta, while clamping off the descending aorta. The brains were post-fixed at least overnight in the same fixative solution plus 20% sucrose. Control and kainic acid exposed tissue was cut on a freezing sliding microtome at a thickness of 25 μm. The majority of sections were collected in 0.1 M neutral phosphate buffer. The sections were typically mounted on 2% gelatin coated slides and then air dried on a slide warmer at 50°C for at least half an hour. The slides were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (20 ml of 5% NaOH added to 80 ml absolute alcohol) for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 10 min, preferably on a shaker table to insure consistent background suppression between sections. The slides were then rinsed in distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution of Fluoro-Jade B (Histo-Chem Inc., Jefferson AR) that was made by adding 10 mg of the dye powder to 100 ml of distilled water. To make up 100 ml of staining solution, 4 ml of the stock solution was added to 96 ml of 0.1% acetic acid vehicle. This results in a final dye concentration of 0.0004%. The stock solution, when stored in the refrigerator was stable for months, whereas the staining solution was prepared within 10 min of use and was not reused. After 20 min in the staining solution, the slides were rinsed for one min in each of three distilled water washes. Excess water was removed by briefly (about 15 s) draining the slides vertically on a paper towel. The slides were then placed on a slide warmer, set at approximately 50°C, until they were fully dry (e.g. 5–10 min). The dry slides were cleared by immersion in xylene for at least a min before coverslipping with DPX (Fluka, Milwaukee WI; or Sigma Chem. Co., St. Louis MO), a non-aqueous, non-fluorescent plastic mounting media.

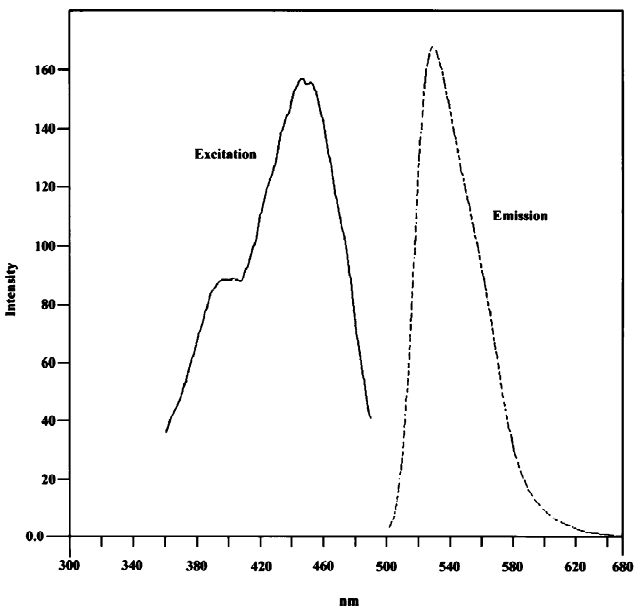


Fig. 1. Emission and excitation profiles for Fluoro-Jade B in distilled water. The emission peak is at 450 nm and the excitation peak is at 530 nm.

2.4. Analysis

The tissue was then examined using an epifluorescent microscope with blue (450–490 nm) or blue-violet (420–490 nm) excitation light. A barrier filter that allows passage of all wavelengths longer than 515 nm will result in a yellow-green emission color, where as a notch filter (e.g. 515–565 nm) will result in a green emission color. Most filters designed for visualizing fluorescein or FITC (e.g. the Nikon B-2A or B-3A filter cubes) will be suitable for visualizing Fluoro-Jade B. The emission and excitation profiles for Fluoro-Jade B are seen in Fig. 1.

2.5. Comparing Fluoro-Jade B with Fluoro-Jade

For comparative purposes, adjacent sections were stained with either Fluoro-Jade or Fluoro-Jade B using the standard Fluoro-Jade staining procedure [5]. The staining procedure was essentially the same as that described in the preceding paragraph. The only difference being that the standard procedure does not pretreat with basic alcohol, and employs greater dye concentrations and incubation times (i.e. 0.001% for 30 min).

2.6. Combining Fluoro-Jade B with fluorescent Nissl stains

It is possible to combine the Fluoro-Jade B technique with a fluorescent Nissl counterstain so that both degenerating and viable neurons are differentially stained. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Chem. Co., St Louis MO) is typically the fluorescent Nissl stain of choice because its staining is not compromised by the basic alcohol or the potassium permanganate pretreatment. A 0.01% stock solution of DAPI (10 mg/100 ml distilled water) is prepared and 2 ml of this stock solution is added to 98 ml of the Fluoro-Jade B staining solution. Blue counterstained normal cell nuclei can be visualized when excited by ultraviolet (330–380 nm) light.

2.7. Combining Fluoro-Jade B with immunofluorescent procedures

It is also possible to combine Fluoro-Jade B staining

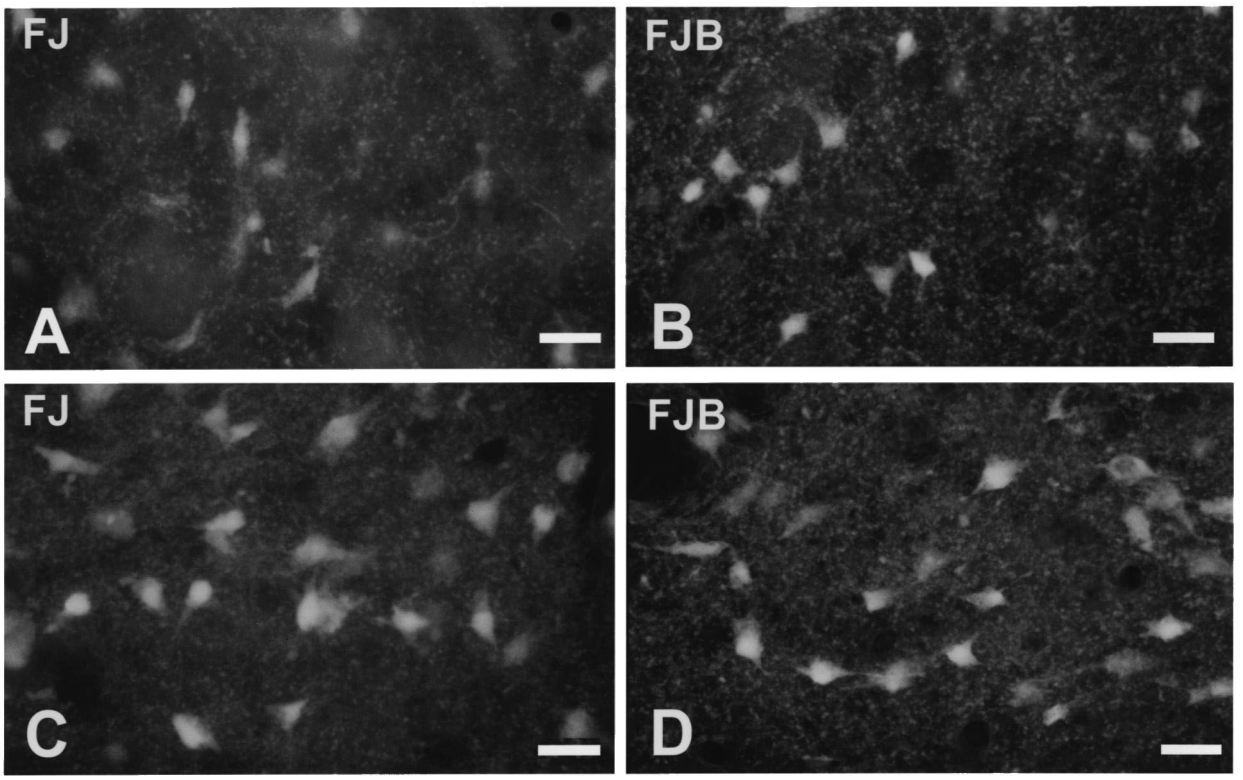


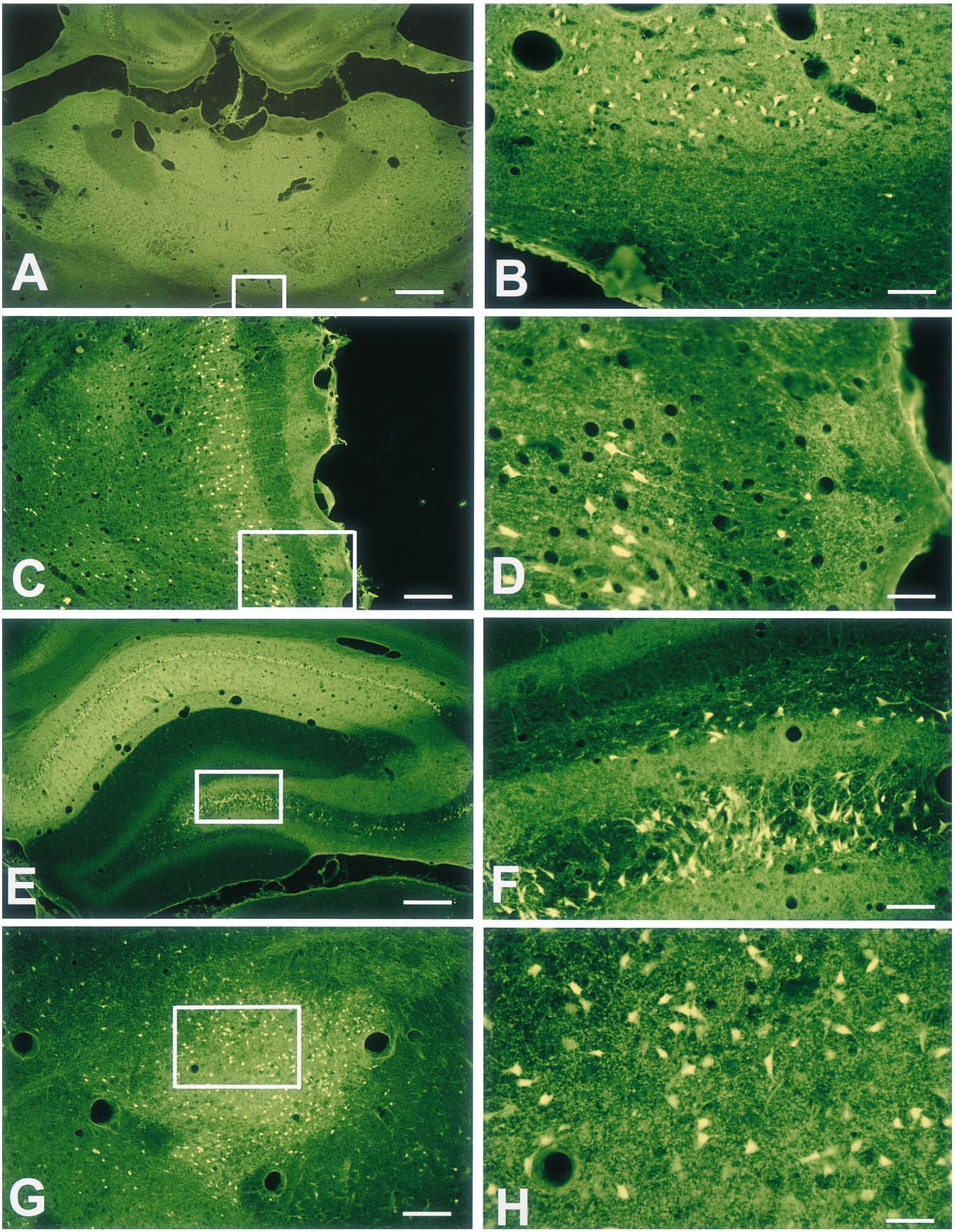
Fig. 2. Comparison of Fluoro-Jade versus Fluoro-Jade B staining of kainic acid induced neuronal degeneration. (A) Fluoro-Jade labeling in the caudal lateral striatum. (B) Fluoro-Jade B labeling of adjacent section in caudal lateral striatum. (C) Fluoro-Jade labeling in the ventral thalamus. (D) Fluoro-Jade B labeling of adjacent section in the ventral thalamus. In general, the Fluoro-Jade B results in high definition staining of cells and terminals, while Fluoro-Jade staining appears more nebulous. Blue light excitation; Magnification bar=42.5 μm.

with immunofluorescent labeling techniques. Rabbit anti-cow glial fibrillary acidic protein (GFAP; DiaSorin, Stillwater MN) primary antibody was used on loose tissue sections first pretreated with basic alcohol and aqueous potassium permanganate. The histological processing was the same as previously described for slide mounted sections except the time in potassium permanganate solution was reduced to 3–5 min. The sections were removed from the distilled water rinse and transferred to the commercially prediluted anti-GFAP antibody. After incubation for 1 day at about 5°C, the sections were rinsed in two 5 min changes of phosphate buffered saline (PBS; 0.02 M, pH 7.4 phosphate buffer in 0.9% saline). The TRITC labeled swine anti-rabbit secondary antibody (Dako Inc, Carpinteria, CA) was used at a 1:30 dilution with PBS. After 30 min in the secondary antibody solution at room temperature, the sections were rinsed for 2 min in each of two distilled water changes. The sections were then mounted on gelatin coated slides and allowed to dry for at least 30 min on a slide warmer. The slides were then placed in the Fluoro-Jade B staining solution and processed as described above. The immunofluorescent labeling was visualized by using green light (500–570 nm) excitation and a barrier filter which allows wavelengths longer than 610 nm (red light) to pass. Barrier filters that allow shorter wavelengths to pass (e.g. 590 nm and over) typically produced noticeable 'bleed through' of Fluoro-Jade B in multiply labeled sections.

3. Results

3.1. Kainic acid induced neuronal degeneration

Brain tissue from kainic acid exposed rats contained numerous Fluoro-Jade B positive neurons. Fig. 3 illustrates the typical pattern and quality of Fluoro-Jade B labeling at both low and high magnifications in the following regions: medial thalamus (Fig. 3A, B), cingulate cortex (Fig. 3C, D), hippocampus (Fig. 3E, F), and the central nucleus of the amydgala (Fig. 3G, H). Staining was characterized by virtually no background labeling of neuropil or normal cells, while degenerating neurons and their processes, including axons (Fig. 4G), axon terminals (Figs. 2B, C, 3D, H, 4D, F) and distal dendritic arborizations (Fig. 4B, C), stained conspicuously. Other regions which contained a lower density of degenerating neurons included the bed nucleus of the stria terminalis, the caudal lateral striatum (Fig. 2B), sensory motor cortex (Fig. 4B, D) and cingulate cortex (Figs. 3C, 4F).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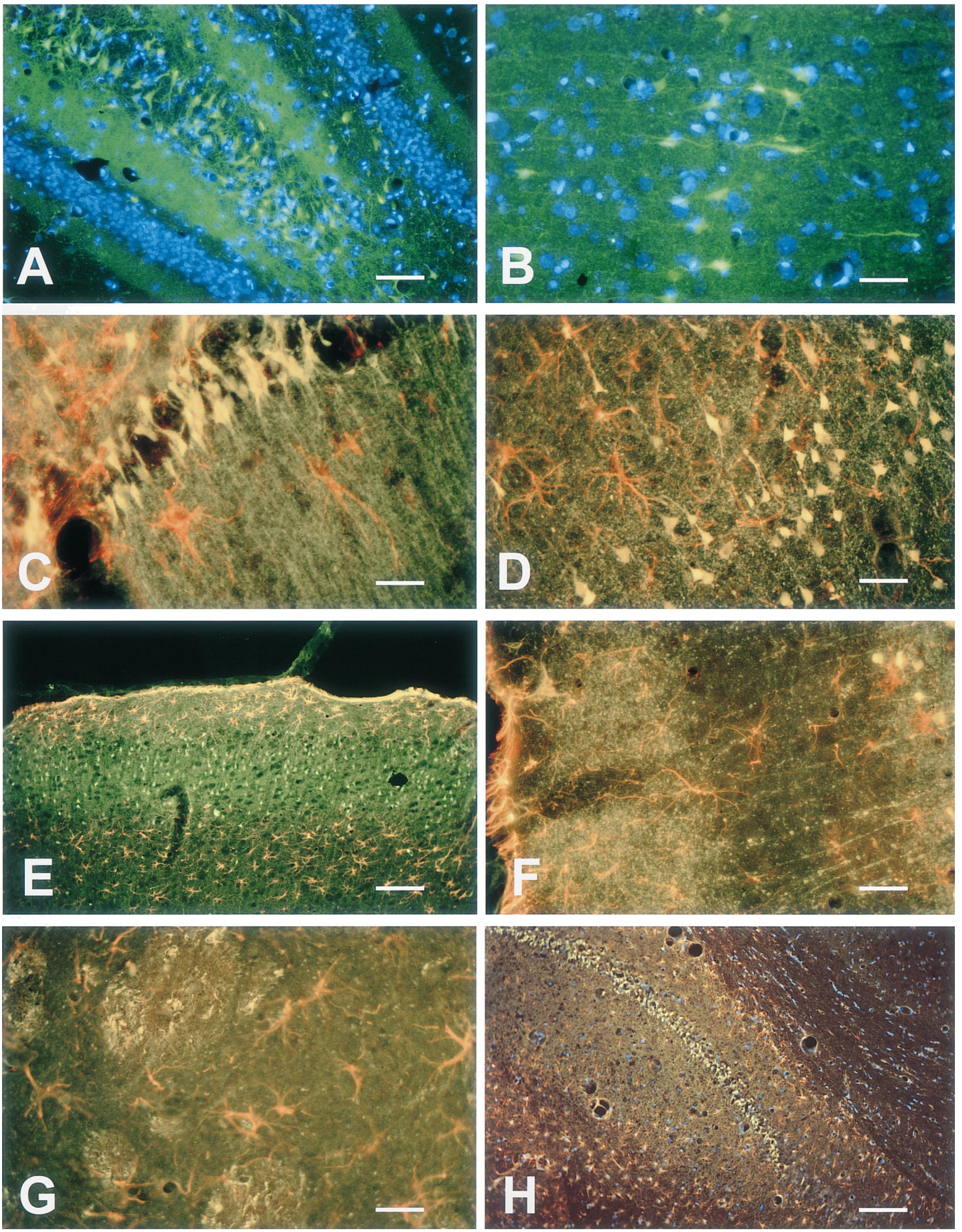
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3.2. Comparison of Fluoro-Jade and Fluoro-Jade B labeling

To compare Fluoro-Jade with Fluoro-Jade B labeling, regions with extensive terminal degeneration were chosen for evaluating contrast and resolution (Fig. 2). When comparing the labeling quality of the two dyes in the lateral dorsal thalamus, the ventral midline thalamus (Fig. 2A, B), the caudal lateral striatum (Fig. 2C, D), and the central nucleus of the amydgala, consistent similarities and differences were seen. The dyes were similar in that both labeled the same number and type of structures (e.g. cells, dendrites, axons and axon terminals). The primary difference was that the background staining was significantly lower with Fluoro-Jade B, making cells and especially terminals stand out conspicuously, as opposed to Fluoro-Jade staining which had a more nebulous appearance. This difference was less noticeable in piriform cortex, which contained extensive necrotic type damage and therefore limited morphological integrity.

3.3. Multiple labeling studies

Fig. 4 illustrates multiple labeling with Fluoro-Jade B and other fluorochromes. When DAPI was incorporated in the Fluoro-Jade B stain solution, the nuclei of normal cells emitted a blue color under ultraviolet illumination, while degenerating neurons appeared green under blue light excitation (Fig. 4A, B). When the Fluoro-Jade B technique was combined with GFAP immunofluorescent techniques, TRITC-labeled astrocytes appeared red under green light excitation, while Fluoro-Jade B positive degenerating neurons appeared green under blue light excitation (Fig. 4C–G). In some brain regions GFAP positive astrocytes can be found adjacent to Fluoro-Jade B positive neurons (Fig. 4C, D, F), while in other regions such as layers II and III of parietal cortex (Fig. 4E) no GFAP positive astrocytes were found near the Fluoro-Jade B positive neurons. Reduced time (e.g. 5 min) in the potassium permanganate pretreatment solution resulted in improved immunofluorescent labeling of astrocytes, although it also resulted in a slightly higher level of Fluoro-Jade B background staining.

Triple labeling was achieved by combining the two aforementioned methodologies. When using a triple band pass filter cube (TRITC, FITC, DAPI), it was possible to simultaneously visualize green Fluoro-Jade B positive degenerating neurons, red GFAP positive astrocytes, and blue cell nuclei of viable cells (Fig. 4H).

Fig. 3. Examples of Fluoro-Jade B staining in the rat brain following exposure to kainic acid. (A) Survey view reveals extensive degeneration throughout the thalamus. Magnification bar=650 μm. (B) High magnification view of area seen in box in previous figure confirms the presence of degenerating cells and terminals in the thalamus (top), but not in the underlying hypothalamus. Magnification bar=42.5 μm. (C) Caudal cingulate and retrosplenial cortex reveals extensive neuronal degeneration. Magnification bar=100 μm. (D) High magnification view of area seen in box in the previous figure reveals cells degenerating in deeper layers (left), a central band which is Fluoro-Jade B negative except for the apical dendrites of aforementioned neurons, and the most superficial layer (right) which contains clusters of terminal like puncta. Magnification bar=42.5 μm. (E) Survey view of the hippocampus reveals extensive neuronal degeneration in the dentate gyrus hilus (CA 4) and the CA 1 region of Ammon's horn. Fewer degenerating neurons are seen in the CA 1 and CA 2 region, while no staining is seen in the granule cell layer of the dentate gyrus. Magnification bar=325 μm. (F) High magnification view of area seen in box in previous figure reveals cellular and terminal degeneration within the dentate hilus fasciae as well as terminal degeneration of the inner 1/3 of the molecular layer. Magnification bar=42.5 μm. (G) Survey view of the central nucleus of the amydgla reveals extensive neuronal degeneration. Magnification bar=100 μm (H) High magnification view of area seen in box in the previous figure reveals cells and conspicuous terminals. Magnification bar=42.5 μm. Blue light excitation.

Fig. 4. Examples of combining Fluoro-Jade B with other fluorescent labels in kainic acid exposed tissue. (A) Fluoro-Jade B and DAPI staining of hippocampal dentate gyrus. The hilus of the dentate gyrus contains numerous Fluoro-Jade B positive cells and terminals as well a population of viable DAPI positive cell nuclei. The granule cell layer contains exclusively DAPI positive nuclei of viable granule cells. The internal molecular layer contains only degenerating perforant path terminals. Combined ultraviolet and blue light excitation. Magnification bar=42.5 μm. (B) Layer III of temporal cortex contains Fluoro-Jade B positive pyramidal cells and axon terminals. The respectively small and large nuclei of viable glial and neuronal cells are DAPI positive. Combined ultraviolet and blue light excitation. Magnification bar=42.5 μm. (C) The CA 1 region of the hippocampus contains yellow appearing Fluoro-Jade B positive degenerating neurons within the stratum pyramidal while the stratum oriens and stratum radiatum on either side contain red immunostained GFAP positive astrocytes. Combined blue and green light excitation. Magnification bar=42.5 μm. (D) Layers III and IV of temporal cortex contain both Fluoro-Jade B positive neurons and axon terminals, and GFAP positive astrocytes. Combined blue and green light excitation. Magnification bar=42.5 μm. (E) Survey view of parietal cortex reveals lamina dependent differential labeling with Fluoro-Jade B and GFAP. Only Fluoro-Jade positive neurons are found within lamina II and III, while only GFAP positive astrocytes are found within lamina I and IV. Illumination with dual pass filter cube for simultaneous visualization of rhodamine (TRITC) and fluorescein (FITC) results in more green appearing Fluoro-Jade B as opposed to the more yellow color obtained with sequential double exposures. Magnification bar=200 μm. (F) Posterior cingulate/retrosplenial cortex contains Fluoro-Jade B positive neurons (upper right), dendrites (center) and axon terminals (left) and well as scattered GFAP positive astrocytes. Combined blue and green light excitation. Magnification bar=42.5 μm. (G) Double exposure of the striatum reveals Fluoro-Jade B positive axons of cortical origin within the penetrating fascicles while the stroma of the striatum contains only GFAP positive astrocytes. Combined blue and green light excitation. Magnification bar=42.5 μm. (H) Triple labeling in the hippocampus reveals green degenerating Fluoro-Jade positive neurons of the CA 1 region, scattered red GFAP positive astrocytes, and blue DAPI stained nuclei of viable cells. Triple pass filter cube (TRITC, FITC, and DAPI) simultaneous excitation. Magnification bar=200 μm.

4. Discussion

4.1. Comparing Fluoro-Jade and Fluoro-Jade B

New methods continue to evolve for the detection of neuronal degeneration. Like Fluoro-Jade [5], Fluoro-Jade B has the advantage of being as reliable and technically simple as a conventional Nissl stain, while being as specific for degenerating neurons as an ideal suppressed silver stain. Also like Fluoro-Jade, Fluoro-Jade B has an affinity for the entire degenerating neuron including cell body, dendrites, axon and axon terminals. Therefore it appears that these two dyes share an affinity for the same biomolecule(s), but differ in relative strength of affinity. The difference observed in their respective staining characteristics would be consistent with the idea that Fluoro-Jade B has a higher affinity for degenerating tissue components than Fluoro-Jade. Also consistent with that notion is the observation that Fluoro-Jade B is used at less than half the concentration required for Fluoro-Jade. Increased specific affinity not only allows the staining to be done faster and with less dye, but more importantly, it confers a sharper and higher definition stain. The presence of comparable staining with either fluorochromes in the piriform cortex may reflect the fact that the morphological integrity of this region is so compromised by necrotic inflammation that there are few fine structures remaining to benefit from the increased resolving power of Fluoro-Jade B.

4.2. Putative chemistry and mode of action

The exact chemical identity of Fluoro-Jade B remains to be confirmed. Thin layer chromatography, using cellulose plates and a mixed solvent system of *n*-propanol, water, and ammonium hydroxide (6:5:2), revealed the presence of one highly fluorescent band and three moderately fluorescent bands. Studies are presently underway to resolve the chemical identity and relative bio-activity of the fluorescent components. As previously postulated [6], the synthesis stoichiometry suggests that the bis-6′-hydroxy-3′-isoxanthenone homologue of Fluoro-Jade as well as the carboxy homologue comprise at least two components of Fluoro-Jade B. Both of these compounds can theoretically exist as two isomers, which may account for the presence of the other two fluorescent chromatographic bands. These homologues are, like Fluoro-Jade, poly-anionic fluorescein derivatives. Based on this fact, one may speculate on the identity of some of the more likely candidate biochemical molecules that Fluoro-Jade B could bind to. This would include molecules with multiple positive charge groups such as the poly-amines. Personal observations revealed that molecules with a strong electrostatic attraction for Fluoro-Jade B in vitro include aminopropyl-butanediamine (spermidine), diaminobutane (putrescine), and diaminopentane (cadaverine). Whether Fluoro-Jade B binds to any of these poly-amines in degenerating neurons remains to be resolved.

Regardless of the mechanism of action, Fluoro-Jade B can be used to resolve a number of questions involving neuronal cell death, including studies on brain pathology, toxicology, development, dementia, and neuroprotection. Although kainic acid exposure was the only insult systematically examined in this study, it is believed that like Fluoro-Jade, Fluoro-Jade B will detect dead neurons regardless of cause of death. This belief is based partially on the structural homology of Fluoro-Jade and Fluoro-Jade B and partially on personal observations of Fluoro-Jade B labeling following other insults including exposure to trimethyltin, substituted amphetamines, domoic acid, and optic tract lesion.

4.3. Further characterization of properties

Although Fig. 1 illustrates the excitation and emission profile for Fluoro-Jade B in distilled water, it should be pointed out that both the staining and emission properties are pH dependent. To achieve selective staining of degenerating neurons, the dye must be used in an acidic (pH 3–4) vehicle. Like fluorescein, the intensity of the emitted light is less at low pH and greater at neutral or basic pH. Glutaraldehyde containing fixatives will not interfere with the staining of degenerating neurons, but typically result in a higher level of background staining. Fluoro-Jade B is quite resistant to fading and therefore the use of anti-fade agents is not required. This resistance to fading is largely attributable to the potassium permanganate pretreatment that not only minimizes background staining, but also confers considerable resistance to fading. For example, no archival fading or deterioration was detected in slides stored in slide boxes at room temperature for over 2 years. Fading resulting from prolonged epi-fluorescent examination was minimal. No fading was observed after 30 min of epi-illumination at magnifications under 40×. Prolonged examination using objective magnifications of 40× or more resulted in subtle, but detectable fading. However, this did not preclude analysis or photography since both labeled cells and background staining faded approximately at the same rate.

Although not fully evaluated, there is no evidence that Fluoro-Jade B is itself toxic. This conclusion is based in part on acute studies in which 200 mg/kg of Fluoro-Jade B injected i.p. in the rat failed to produce any abnormal behavior or pathology (personal observation). It also seems unlikely that Fluoro-Jade B is a potential carcinogen since this is not true of other fluorescein derivatives, and because the molecule does not contains any nitrogen or multivalent metal atoms. The entire procedure is presumably less hazardous than most suppressed silver methods which typically require the use of relatively large quantities of various potential toxicants including arsenate buffered

fixatives, pyridine, uranyl nitrate, potassium ferricyanide, etc. By contrast, Fluoro-Jade is used in milligram quantities and has no demonstrable toxicity. Nevertheless, routine laboratory caution is recommended until its properties are fully characterized.

4.4. Multiple label studies

For some purposes, the usefulness of Fluoro-Jade B can be extended by combining it with a second fluorescent probe. For example, it can be combined with DAPI which will label the nuclei of all viable cells. This serves to both provide anatomical landmarks, and to allow quantitative statements to be made regarding the ratio of viable cells to dead cells. Another potentially useful combination is Fluoro-Jade B and immunofluorescent methodologies. In this study, a GFAP antibody was used to demonstrate the presence or absence of GFAP positive astrocytes adjacent to Fluoro-Jade B positive neurons. When combining Fluoro-Jade B with immunofluorescent methods, several considerations should be kept in mind. First, the secondary antibody should be tagged with a fluorochrome other than FITC or other fluorescent marker with spectral properties that would overlap the Fluoro-Jade B emission profile. In this study good results were obtained with a TRITC conjugated secondary antibody. A second consideration is the fact that the potassium permanganate pretreatment can attenuate the extent of immunofluorescent label, depending on the nature of the antigenic epitope. Therefore, the time in the potassium permanganate solution can be reduced (e.g. to 3–5 min) to improve immunofluorescent labeling. A compromise time can generally be reached so that high quality immunofluorescent label can be obtained, while the background level of Fluoro-Jade staining remains reasonably low. A third consideration is the possibility that neurons no longer express a particular antigen once the cell dies. This was reflected in a previous study [2] in which Fluoro-Jade was combined with tyrosine hydroxylase (TH) immunocytochemistry in brain sections from mice treated with MPTP. Although numerous cells within the substantia nigra and ventral tegmental area were either TH or Fluoro-Jade positive, no double labeled neurons were observed.

In conclusion, Fluoro-Jade B like Fluoro-Jade allows for the rapid, reliable and sensitive detection of neuronal degeneration by itself or in combination with other fluorescent labels. The primary difference between these two related fluorescent dyes is that Fluoro-Jade B generally results in noticeably higher definition and contrast labeling of neuronal processes, while requiring shorter incubation times and lower dye concentrations.

Acknowledgements

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References

[1] J.S. de Olmos, C.A. Beltramino, S. de 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 (1994) 545–561.

[2] T.E. Freyaldenhoven, S.F. Ali, L.C. Schmued, Systemic administration of MPTP induces thalamic neuronal degeneration in mice, Brain Res. 759 (1997) 9–17.

[3] W.J.H. Nauta, P.A. Gygax, Silver impregnation of degenerating axons in the central nervous system: a modified technique, Stain Tech. 29 (1954) 91–93.

[4] J.W. Olney, V. Rhee, O.L. Ho, Kainic acid: a powerful neurotoxic analogue of glutamate, Brain Res. 77 (1974) 507–515.

[5] L.C. Schmued, C. Albertson, W. Slikker Jr., Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration, Brain Res. 751 (1997) 37–46.

[6] L.C. Schmued, W. Slikker Jr., G.J. Wang, Fluoro-Jade B: a bis homolog of Fluoro-Jade with improved degenerate neuron staining properties, Soc. Neurosci. Ab. 24 (1998) 1064.