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Research report

Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration

Larry C. Schmued *, Christopher Albertson, William Slikker Jr.

Division of Neurotoxicology, National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079-9502, USA

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Abstract

Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable method for staining degenerating neurons and their processes. The technique will detect neuronal degeneration resulting from exposure to a variety of neurotoxic insults. Fluoro-Jade can be combined with other fluorescent methodologies including immunofluorescence, fluorescent axonal tract tracing, and fluorescent Nissl counterstaining. Compared to conventional methodologies, Fluoro-Jade is a more sensitive and definitive marker of neuronal degeneration than hematoxylin and eosin (H&E) or Nissl type stains, while being comparably sensitive yet considerably simpler and more reliable than suppressed silver techniques. © 1997 Elsevier Science B.V. All rights reserved.

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1. Introduction

Many neuroscientists including toxicologists, pathologists, pharmacologists, anatomists and histochemists share an interest in detecting neuronal degeneration. The demonstration of this phenomenon, however, is often difficult from both a technical and an interpretational point of view. Conventional techniques such as H&E (hematoxylin and eosin), or Nissl type stains (crystal violet, thionin, etc.) are technically simple and can be used to infer degeneration based on changes such as neuronal shrinkage, vacuolation, and hyperchromatism [5,12,28]. Unfortunately, such changes are not necessarily indicative of neuronal degeneration and may be due to processing artifacts or non-lethal alterations in cellular morphology. Processing artifacts can result in both shrunken and hyperchromatic cells [4,30]. Not only are such techniques prone to false positives, but it is also possible to miss degenerating neurons since all cells stain with these dyes while only relatively subtle morphological differences exist between normal and degenerating neurons. These interpretational difficulties make the analysis of conventionally stained material time consuming. In contrast, suppressed silver techniques [6,9,11,18] are much better from this point of view in that normal neurons remain unstained while degenerating neurons stain black. The main drawbacks associated with suppressed silver techniques lie in their labor intensive and capricious nature.

Because of the limitations associated with the aforementioned methods, the advantages of a histochemical tracer, which is both simple and reliable as well as highly sensitive and easy to interpret, became apparent and provided the impetus for this research. Toward this goal, we screened a variety of fluorescent anionic dyes under a variety of conditions to characterize the optimal tracer and associated technique for detecting degenerating neurons. The rationale for the choice of dyes and staining conditions is further considered in Section 4. Preliminary work [27] indicated Fluoro-Jade to be the compound most suitable for the detection of neuronal degeneration. The characterization and validation of the Fluoro-Jade method is demonstrated with the use of a number of neurotoxic insults.

* Corresponding author. Division of Neurotoxicology, HFT-132, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079-9502, USA. Fax: +1 (501) 543-7745.

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2. Materials and methods

2.1. Animals

All experiments used adult (3–6 months old) male Sprague–Dawley rats except for studies on MPTP which used adult C-57 mice. Animals were given ad libitum food and water. All animals were used in accordance with the Institutional Animal Care and Use Guidelines.

2.2. Drugs

This study employed the following neurotoxicants which were obtained from Sigma Chemical Company, St. Louis, MO, unless otherwise indicated: (1) Kainic acid (9 mg/kg) was administered i.p. and rats were killed 1–7 days later. (2) Domoic acid (1–3 mg/kg), prepared by Dr. Sherwood Hall of the Food and Drug Administration, was administered i.p. and animals were killed 4 h or 7 days post drug administration. (3) Ibogaine (100 mg/kg) was given i.p. and the animals were killed 1 day or 1 week post drug administration. (4) PCP (phencyclidine) (50 mg/kg) and MK-801 (5 mg/kg), from Research Biochemicals International, Natick, MA, were both given i.p. and the animals were killed 1 day or 1 week after drug exposure. (5) 3-NPA (3-nitropropionic acid) was given as a single subcutaneous dose of 30 mg/kg and the animals were perfused 1–5 days later. (6) MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) from Research Biochemicals International, Natick, MA, was administered as a single 50 mg/kg i.p. dose and the animals were perfused 1, 2, or 5 days later.

2.3. Stereotaxic injection

Six animals were injected with 0.05 μl of 0.05 M manganese acetate or manganese chloride into the striatum, while a similar volume and concentration of iron chloride or iron sulfate was injected into the contralateral striatum. These multivalent metal salts were injected stereotaxically with a Hamilton microsyringe over a 10 min time interval while animals were under deep ketamine (75 mg/kg) and Rompun (9 mg/kg) anesthesia. The animals were perfused 2 days later.

2.4. Enucleation

Two animals were anesthetized with the aforementioned anesthetic and the left eye was enucleated. One week later the animals were perfused with buffered formalin and their brains were removed and postfixed in fixative solution plus 20% sucrose.

2.5. Controls

Six animals received a single i.p. injection of physiological saline and were perfused 2 days later.

2.6. Histological processing

Animals were anesthetized with ketamine and Rompun, as described above, and the majority were perfused with 500 ml of 0.1 M neutral phosphate buffered formaldehyde (4%). A few of the kainic acid dosed animals were perfused with 4% formaldehyde in carbonate buffer (pH 11, 0.01 M), and a few animals were anesthetized and their brains removed without fixation. Brains to be cut on a freezing sliding microtome were postfixed in the fixative solution plus 20% sucrose and cut into 20–40 μm thick sections which were collected in buffer solution. Cryostat sections were cut at 15 μm thickness and mounted directly on gelatin coated slides. Unfrozen vibratome cut sections were cut at 50 μm thickness and collected in buffer solution. Tissue for paraffin embedding was dehydrated with gradated alcohols, cleared with xylene, and infiltrated with paraffin overnight. This paraffin embedded tissue was then cut into 8 μm thick sections which were mounted on slides, deparaffinized with xylene and rehydrated in gradated alcohols prior to staining.

2.7. Fluoro-Jade dye

Fluoro-Jade can be obtained from Histo-Chem Inc., P.O. Box 183, Jefferson, AR 72079, USA. Fluoro-Jade is an anionic tribasic fluorescein derivative with a molecular weight of 445 daltons. It has an emission peak at 550 nm and excitation peaks at 362 and 390 nm respectively. The dry powder is stable when stored in the dark in an air tight container. The 0.01% stock solution is stable for at least 2 months when stored in the refrigerator, in contrast to the working solution which should be used the same day as prepared.

2.8. Fluoro-Jade staining procedure

Brain sections were mounted with distilled water onto gelatin coated slides and dried on a slide warmer at 45°C. The tissue was fully dry within 20 min at which time it was immersed in 100% ethyl alcohol for 3 min followed by a 1 min change in 70% alcohol and a 1 min change in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 15 min and were gently shaken on a rotating platform. This solution when kept in a sealed glass container remains usable for a period of about 1 week. The slides were rinsed for 1 min in distilled water and were then transferred to the Fluoro-Jade staining solution where they are gently agitated for 30 min. A 0.01% stock solution of the dye was prepared by dissolving 10 mg Fluoro-Jade in 100 ml of distilled water. The 0.001% working solution of Fluoro-Jade was prepared by adding 10 ml of the stock Fluoro-Jade solution to 90 ml of 0.1% acetic acid in distilled water. After staining, the sections were rinsed with three 1 min changes of distilled water. Excess water was drained off, and the slides were

rapidly air dried on a slide warmer or with a hot air gun. When dry, the slides were immersed in xylene and then coversliped with D.P.X. (Aldrich Chem. Co., Milwaukee, WI) mounting media. Sections were examined with an epifluorescence microscope using a filter system suitable for visualizing fluorescein or FITC. The resulting slides are quite stable and require no special storage conditions or anti-quench agents. The potassium permanganate pretreatment further enhances the permanence of the preparation resulting in extremely slow fading, even under high magnification epifluorescent illumination.

2.9. Staining variations for multiple label studies

Although the aforementioned staining protocol has been deemed to be of optimal sensitivity, resolution, and contrast, it may be that some aspects of the pretreatment procedure are incompatible with other desired histological endpoints. It should therefore be emphasized that the procedure is basically quite flexible. The pretreatment procedure with ethanol and potassium permanganate serves to reduce background staining, but may also inhibit costaining with immunofluorescent, fluorescent Nissl, and fluorescent axonally transported tracers. The pretreatment steps may be omitted if alternative measures are taken to reduce background staining. Alternative ways to reduce background staining include the use of high pH formalin fixation (e.g. 0.1 M, pH 11 sodium carbonate vehicle), low dye concentration (e.g. 0.0001%), low temperature (e.g. 5°C) of staining solution, and long post staining rinses with distilled water (e.g. 3 changes of 3 min each). Multiple labeling with fluorescent retrograde axonally transported tracers such as Fluoro-Gold (Fluorochrome Inc., Englewood, CO) was achieved by combining the standard procedure used to demonstrate retrograde axonal transport of Fluoro-Gold [25] with the aforementioned Fluoro-Jade procedure. Briefly, animals were anesthetized with ketamine (75 mg/kg) and Rompun (20 mg/ml) and then received 0.1 μl of 2.5% Fluoro-Gold injected stereotaxically into the hippocampus or the lateral septum. Two days later the animals were given kainic acid and were subsequently perfused with neutral buffered formalin fixative the following day. Sections containing retrogradely transported Fluoro-Gold were then stained with Fluoro-Jade without ethanol and potassium permanganate pretreatment. Fluorescent Nissl counterstaining of non-degenerating cells was accomplished with the use of ethidium bromide or DAPI as previously described [24]. Briefly, a 0.01% stock solution of ethidium bromide or DAPI (4,6-diamidino-2-phinylindole) from Aldrich Chemical Company, Milwaukee, WI, was prepared in distilled water, and the appropriate amount was added directly to the Fluoro-Jade staining solution. The final DAPI concentration should be 0.0002% whereas the final ethidium bromide concentration should be 0.00005%. Nuclear counterstaining with DAPI can be accomplished following pretreatment with potassium permanganate while ethidium bromide Nissl counterstaining requires omission of the pretreatment step. Another multiple labeling technique involved combining standard immunofluorescent methods with Fluoro-Jade histochemistry. For this study, loose or slide mounted tissue was first rinsed with two 5 min changes of vehicle (phosphate buffered saline with 2% normal swine serum) and then incubated in a primary antibody for 1–3 days at 5°C. For this study, an antibody to GFAP (Dako A/S, Denmark) was used at a dilution of 1:200 in vehicle. The sections were then rinsed with two 5 min changes of vehicle, and subsequently immersed for 45 min in a room temperature secondary antibody solution consisting of a rhodamine conjugated swine anti-rabbit antibody (Dako A/S, Denmark) diluted 1:30 in vehicle. The tissue was then rinsed with two 5 min changes of 0.9% saline before being processed for Fluoro-Jade histochemistry. Pretreatment with ethanol and potassium permanganate tended to reduce the immunoreactivity of the GFAP immunocytochemistry and therefore was generally omitted. The background staining was minimized by using a staining solution of low dye concentration (0.0001%) and a low temperature (5°C).

2.10. Conventional brightfield techniques

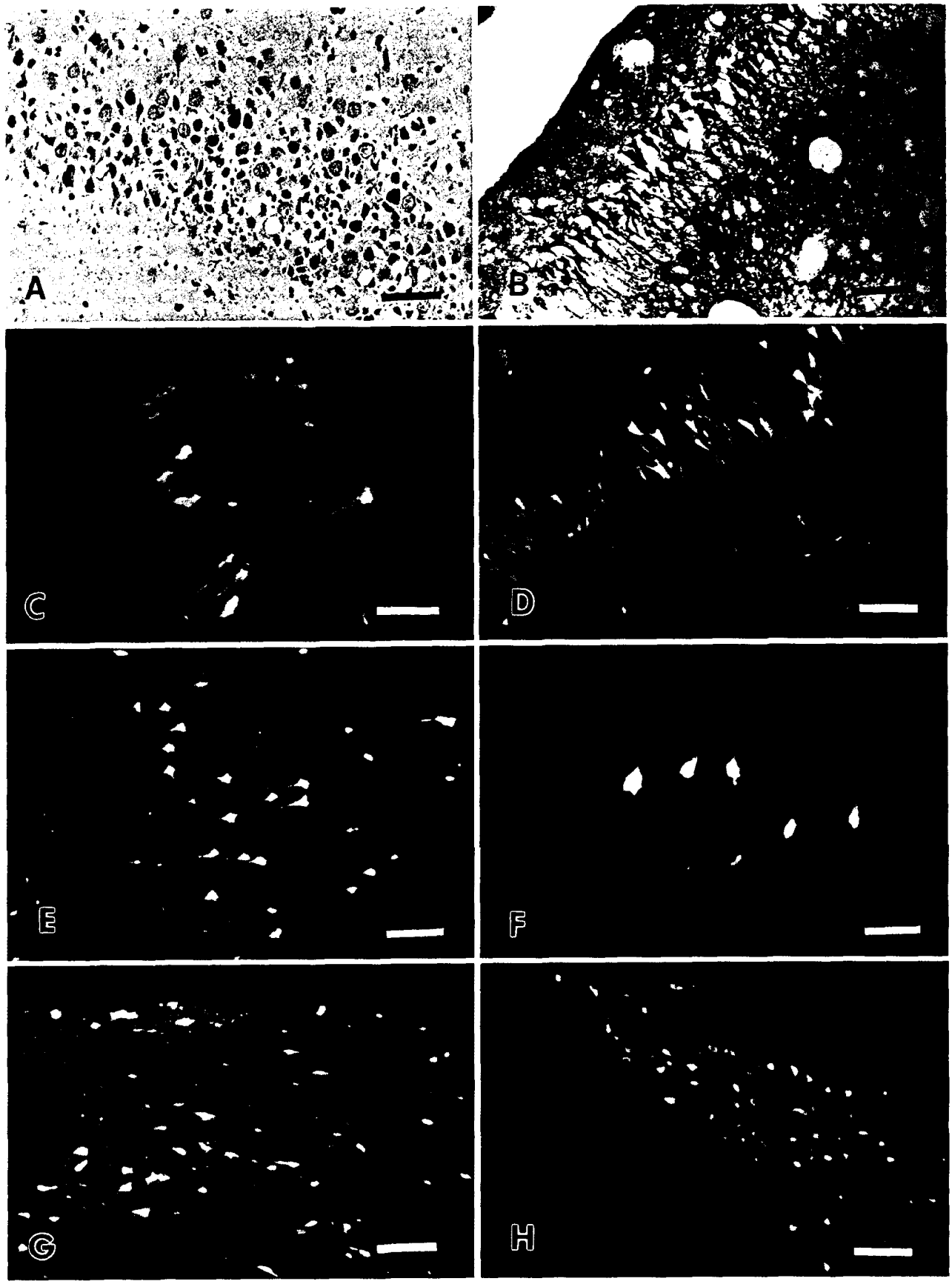
For purposes of comparison, two conventional brightfield histological procedures were applied to the hippocampus of the same kainic acid dosed animals that were examined with the Fluoro-Jade staining procedure. H&E staining of paraffin processed 4–8 μm thick tissue sections employed the standard unacidified variant [17]. Suppressed silver staining of neuronal degeneration was achieved by application of the de Olmos' cupric silver technique [6] to 35 μm thick frozen sections.

3. Results

3.1. Conventional histological methods

(1) H&E staining of paraffin processed tissue resulted in the staining of all brain components. Nuclei are typically stained blue with hematoxylin, while the cell cytoplasm and neuropil stain red with eosin. Kainic acid exposure resulted in the altered morphology of some hippocampal pyramidal neurons of the CA1 region (Fig. 1A). Some neurons exhibit large round nuclei with conspicuous nucleoli and a difficult to define cytoplasm. Other neurons exhibit a more shrunken and hyperchromatic nucleus which may or may not be accompanied by a hypereosinophilic cytoplasm.

(2) The de Olmos' cupric-silver method resulted in the selective staining of a subset of hippocampal neurons (Fig. 1B) in animals dosed systemically with kainic acid. These neurons, their proximal dendrites and terminal-like puncta appeared opaque against a light background. Non-specific staining often resulted in some artifactual staining of mito-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chondria, nucleoli, and myelin. This was seen most frequently in tissue that had been postfixed for less than 1 month.

3.2. Fluoro-Jade staining

In general the staining pattern seen with Fluoro-Jade corresponds to the pattern of argyrophilia seen with an ideal suppressed silver method. Fluoro-Jade stains the cell bodies, dendrites, axons and axon terminals of degenerating neurons but does not stain healthy neurons, myelin, vascular elements or neuropil. Cells of the meninges and the choroid plexus exhibit an affinity for Fluoro-Jade. Unlike the silver methods in which degenerating neurons appear dark against a light background, degenerating neurons stained with Fluoro-Jade appear bright green against a dark background (Fig. 1C–H). Thicker sections (40–100 μm) allow for the visualization of dendritic arborizations (Fig. 1D and Fig. 2A), while thinner sections (15–40 μm) provide the best resolution of terminal puncta (Fig. 1F, 1G, and Fig. 2D). The exact extent and pattern of Fluoro-Jade labeling depend on the nature of the neurotoxic insult and the ensuing survival interval. Some degeneration was seen at all time intervals examined. The following is a more detailed description of the staining pattern observed following exposure to the following neurotoxic insults:

3.2.1. Kainic acid

This potent excitotoxin resulted in extensive Fluoro-Jade labeling within the brain. The densest labeling was found throughout the pyramidal cells of the hippocampus (Fig. 1C, 1D, and Fig. 2H, 2G) and piriform cortex. Survival intervals of 1–4 days were optimal for revealing cellular degeneration, while terminal degeneration was optimally seen 4–7 days. Other structures with conspicuous labeling included the dorsal thalamus (Fig. 1G), the septum, the central nucleus of the amygdala, and the substantia nigra pars reticulata. Also, portions of the cortex exhibited a patchy distribution of Fluoro-Jade labeling with relatively extensive labeling of cingulate and temporal cortex regions and a more patchy distribution of layer II, III, and V pyramidal cells (Fig. 1F) throughout parietal and sensorymotor cortex (Fig. 1E).

3.2.2. Domoic acid

This seafood contaminant was relatively similar to kainic acid in terms of the resulting distribution of Fluoro-Jade stained cells. Labeled cells were detected at times as short as 4 h, or as long as 7 days after drug exposure. Lower doses of domoic acid (e.g. 1.5 mg/kg) occasionally produced a very localized patch of terminal degeneration within the stratum oriens near the CA1–CA3 boundary. This may or may not be accompanied by a few Fluoro-Jade positive cell bodies.

3.2.3. Ibogaine

This antidepressant alkaloid produced a very restricted pattern of Fluoro-Jade labeling. Specifically, only small clusters of labeled Purkinje cells and their dendrites could be found in the paravermal region of the cerebellum (Fig. 2A, 2B).

3.2.4. MK-801 / PCP

These NMDA receptor blocking anesthetics also resulted in a restricted Fluoro-Jade labeling pattern. Specifically, small stellate cells of deep retrosplenial and cingulate cortex were positively stained.

3.2.5. 3-NPA

Administration of this inhibitor of metabolic respiration resulted in extensive degeneration throughout the basal ganglia (Fig. 2E), the thalamus, the hippocampus (mostly CA1), the deep nuclei of the cerebellum, and the cochlear nuclei. 3-NPA induced degeneration was typically characterized by a staining of virtually all neurons and neuropil within a specific structure or substructure. Degenerating cell body profiles were most apparent following a 1 day survival interval, while degenerating axonal profiles were most apparent following a 5 day survival interval.

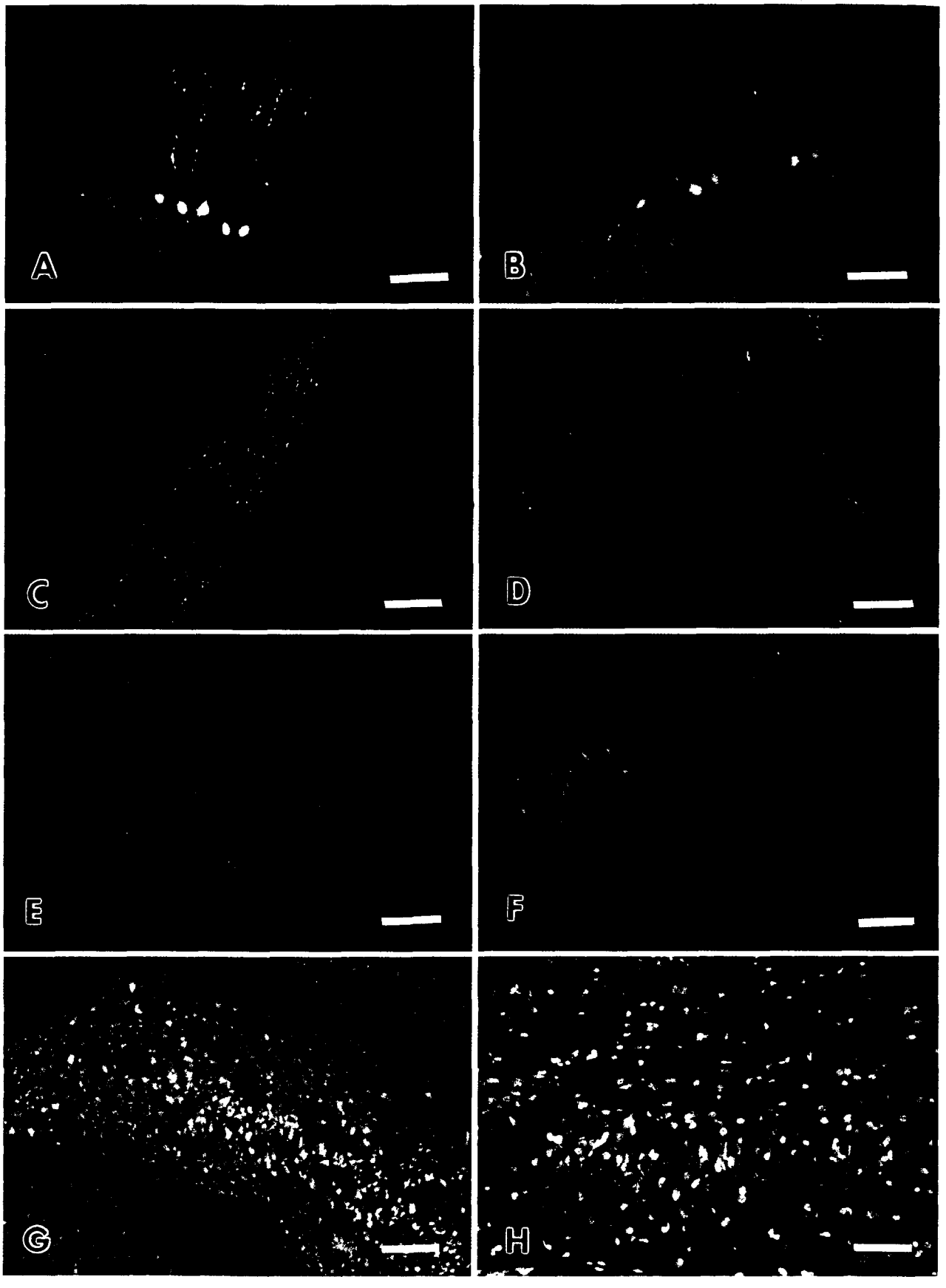
3.2.6. MPTP

A single dose resulted in labeled fusiform cells throughout the substantia nigra pars compacta and the ventral tegmental area in the mouse (Fig. 1H).

3.2.7. Stereotaxic injection of multivalent metals

The injection of iron or manganese salts into the striatum resulted in extensive Fluoro-Jade labeling of the cen-

Fig. 1. A: hematoxylin and eosin were used to stain this paraffin embedded section of the hippocampus of a rat 24 h after exposure to kainic acid. Note the variety of cellular morphologies and dye affinities making interpretation difficult. Brightfield; magnification bar = 200 μm. B: the de Olmos suppressed cupric-silver technique was used to label degenerating pyramidal cells in frozen sections taken from the hippocampus contralateral to that seen in (A). Degenerating neurons appear black, while normal neurons are unstained. Brightfield; magnification bar = 200 μm. C: Fluoro-Jade was also used to stain the paraffin embedded kainic acid treated tissue seen in (A). Degenerating hippocampal pyramidal cells appear green, while normal neurons appear even darker than background with a lightly staining nucleoli. FITC filter; magnification bar = 100 μm. D: Fluoro-Jade was used to stain degenerating hippocampal neurons from the same kianic acid treated frozen sections seen in (B). Degenerating neurons and their dendrites exhibit a green fluorescence while healthy neurons are unstained. FITC filter; magnification bar = 200 μm. E: kainic acid exposure also produced Fluoro-Jade positive pyramidal cells seen here in parietal cortex. FITC filter; magnification bar = 200 μm. F: higher magnification of cortical degeneration seen in (E) reveals proximal dendrites and terminal-like puncta. FITC filter; orientation: 90 degree rotation; magnification bar = 100 μm. G: The dorsal lateral thalamus of a rat treated with kainic acid 4 days prior to sacrifice reveals numerous Fluoro-Jade positive cells and terminals. FITC filter; magnification bar = 200 μm. H: the substantia nigra of a mouse treated with MPTP reveals numerous Fluoro-Jade positive neurons within the pars compacta and ventral tegmental region. FITC filter; magnification bar = 200 μm.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tral one half to two thirds of the entire striatum. Degenerating cellular profiles could be detected against labeled neuropil. Labeled cellular profiles were most apparent around the circumference of the injection site. Iron salts produced the largest lesion, as well as a Parkinson disease-like post-operative rigidity.

3.2.8. Enucleation

Unilateral enucleation resulted in axonal label within the entire contralateral optic tract and primary terminal fields. Specifically, labeled axons within the contralateral optic tract (Fig. 2C) could be followed from the optic chiasm dorsally and laterally to the lateral geniculate in which many fibers could be seen radiating amidst numerous terminal-like puncta (Fig. 2D). A portion of the optic tract continued posteriorly and ended in relatively superficial terminal fields within the superior colliculus. Other nuclei in which terminal labeling could be observed were the suprachiasmatic nuclei and the medial terminal nucleus of the accessory optic system. Only the occasional labeled axon could be found in the ipsilateral optic tract. No labeled cell bodies were seen in any brain region.

3.2.9. Controls

Animals receiving only saline exhibited an absence of Fluoro-Jade stained neurons, glia, neuropil, or myelin. Only the choroid plexus and the meninges were Fluoro-Jade positive.

3.3. Multiple label studies

3.3.1. Fluoro-Jade and fluorescent Nissl stains

This combination allowed one to simultaneously localize in the same tissue section both degenerating and viable cells. Fluoro-Jade and ethidium bromide resulted in good color contrast whereby degenerating neurons appeared green, and viable neurons appeared red (Fig. 2B, 2G). The Fluoro-Jade background level was somewhat higher since ethidium bromide staining was not compatible with potassium permanganate pretreatment. Although DAPI was compatible with the pretreatment procedure, it served to alter the staining characteristics so that nuclear rather than Nissl staining resulted. Either counterstain contrasted well with the green Fluoro-Jade labeled degenerating neurons. Unlike Fluoro-Jade which stained the entire degenerating cell, the fluorescent counterstains labeled only the nucleic acids of normal cells [24].

3.3.2. Fluoro-Jade and fluorescent axonal tracers

When Fluoro-Gold was injected into the septal nucleus and kainic acid was subsequently administered systemically, gold colored Fluoro-Gold retrogradely labeled hippocampal pyramidal cells were seen in the same field as Fluoro-Jade labeled neurons. However, no double labeled cells were found which contained both fluorochromes. Potassium permanganate pretreatment was found to quench the Fluoro-Gold fluorescence.

3.3.3. Fluoro-Jade and immunofluorescence

Activated glial astrocytes were localized in the hippocampus with an antibody directed against GFAP. A rhodamine conjugated secondary antibody resulted in red colored astrocytes while Fluoro-Jade labeled degenerating neurons appeared green (Fig. 2F). GFAP staining was most intense in regions surrounding degenerating neurons, although GFAP labeled astrocytes were not observed immediately adjacent to degenerating neurons. Potassium permanganate pretreatment moderately reduced the intensity of the GFAP immunoreactivity.

4. Discussion

The findings of this study support the hypothesis that Fluoro-Jade can be used for the simple and reliable demonstration of degenerating neurons and their processes. This

Fig. 2. A: selected patches of Purkinje cells and their dendrites in the medial cerebellum indicate ibogaine induced degeneration revealed by Fluoro-Jade labeling. Sagittal orientation; FITC filter, magnification bar = 200 μm. B: the same region of cerebellum seen in (A) with ethidium bromide counterstain. This double exposure illustrates the small orange granule cells at bottom, larger pale normal Purkinje cell bodies (open arrow), and the light green and somewhat shrunken degenerating Purkinje cells (solid arrow). Sagittal section; combined FITC/RITC filters; magnification bar = 200 μm. C: the optic tract 1 week following enucleation of the contralateral eye illustrates extensive Fluoro-Jade labeling of axons. FITC filter; magnification bar = 200 μm. D: the same brain as seen in (C) reveals an attenuated optic tract (arrow) at this most lateral aspect of the caudal diencephalon. Medially, Fluoro-Jade positive axons and terminal-like puncta can be seen throughout the lateral geniculate nucleus contralateral to the enucleated eye. FITC filter; magnification bar = 200 μm. E: following treatment with 3-NPA, this brain reveals extensive degeneration throughout the basal ganglia including the ventral half of the striatum illustrated here. Degenerating neurons and neuropil are Fluoro-Jade positive, while surrounding tissue and the penetrating myelinated fiber bundles are unstained. FITC filter; magnification bar = 400 μm. F: this kainic acid treated section illustrates multiple labeling in the CA3 region of the hippocampus. Degenerating neurons are Fluoro-Jade positive, while activated astrocytes appear red following immunofluorescent localization of GFAP with a rhodamine conjugated secondary antibody. Combined FITC and RITC filters; magnification bar = 200 μm. G: multiple labeling within the dentate gyrus of a kainic acid treated animal reveals both degenerating and healthy neurons. An ethidium bromide counterstain results in all granule cells emitting a red color. Centrally, Fluoro-Jade labeled polymorph cells of the hilar region emit a light green color. Combined FITC/RITC filters; magnification bar = 200 μm. H: another example of combining a fluorescent Nissl counterstain with Fluoro-Jade to simultaneously demonstrate healthy and degenerating neurons. In this case, DAPI is used to label healthy neurons of the CA3 region (seen as a band of mostly blue cells below the middle of the figure) and some hilar cells found more dorsally. In both regions, degenerating light green Fluoro-Jade positive cells can be colocalized following kainic acid administration. Combined FITC/wide band ultraviolet filters; magnification bar = 200 μm.

conclusion is based largely on the close correlation observed between the labeling pattern seen with Fluoro-Jade and the pattern of argyrophilia seen with an ideal suppressed silver stain. This correlation holds true for all neurotoxic insults examined including kainic acid [22], domoic acid [26,31], 3-NPA [3,14], ibogaine [19,23], PCP [20], MK-801 [10], MPTP [15], intracranial injection of manganese or iron salts [29], or enucleation [13]. For example, when adjacent kainic acid treated tissue sections are stained respectively with suppressed silver and Fluoro-Jade, the distribution and staining of cells, dendrites and terminals are virtually identical. One relatively subtle difference is that brain regions which are so necrotic that the tissue is only partially intact, such as the pyriform cortex of kainic acid treated animals or the core of the striatal lesion of a 3-NPA treated animal, can be detected with Fluoro-Jade but remain unstained with the cupric-silver method. Fluoro-Jade also stained degenerating myelinated fiber tracts (e.g. optic tract, fimbria) better than cupric-silver methods. Concerning nonspecific staining, silver methods can stain a number of elements artifactually including subcellular elements, myelinated tracts and neuropil. This is in contrast to Fluoro-Jade which in addition to labeling degenerating neurons will only stain certain non-neuronal elements adjacent to the brain surfaces (e.g. meninges and choroid plexus).

A relatively large and diverse selection of neurotoxic insults was chosen to validate Fluoro-Jade's ability to detect neuronal degeneration arising from a variety of different mechanisms. Neuronal cell death was detected by the following insults and their respective putative mechanism of action: (1) kainic acid and domoic acid which are believed to act by excitotoxic mechanisms via interaction with the NMDA receptor [22], (2) iron and manganese compounds which are thought to act by the generation of oxidative free radicals [1,8], (3) MK-801 and PCP which act to block NMDA receptors [21], (4) ibogaine which is thought to produce excitotoxic damage to select Purkinje cells via activation of the inferior olive [19], (5) 3-NPA which blocks oxidative respiration via inhibition of succinate dehydrogenase [14], (6) MPTP which is converted to MPP^+ and then sequestered by the dopamine uptake system where it is thought to inhibit mitochondrial complex 1 [7,16], and (7) enucleation which results in Wallerian degeneration of axons and terminals throughout all primary optic nuclei [13]. Thus, it appears that regardless of the mechanism by which a nerve cell dies, it will be labeled by Fluoro-Jade. It is not, however, within the scope of this study to examine every type of neuronal degeneration at all time points. Some of the more specialized forms of neuronal degeneration such as apoptotic cell death, ischemia, peripheral neuropathies, tissue culture studies, or chronic human degenerative disease states remain to be examined for Fluoro-Jade affinity.

It is also apparent that Fluoro-Jade stains degenerating neurons in their entirety including cell bodies, dendrites, axons and terminals. Although terminal-like puncta were frequently observed near or adjacent to degenerating neurons, it was necessary to prove unequivocally that the puncta observed were actually axon terminals, and not merely fragments of the dendritic arborization of nearby labeled neurons. Enucleation was therefore used to demonstrate axon and terminal label in the absence of cellular or dendritic label.

The exact mechanism by which Fluoro-Jade stains degenerating neurons is not known. However, some inferences can be made concerning the histochemical specificity of Fluoro-Jade based on its chemical properties. For example, a degenerating neuron presumably expresses a strongly basic molecule since it has an affinity for the strongly acidic Fluoro-Jade, and an aversion for the basic dyes used for Nissl counterstaining purposes. Such an electrostatic interaction would be consistent with the observation [2] that acid fuchsin can serve as a brightfield stain of degenerating neuronal cell bodies in paraffin processed tissue, as well as our unpublished observation that a number of acidic dyes may stain degenerating neurons (although low resolution, high background levels, or rapid fading limit practical applications). It also seems likely that this 'degeneration molecule' is actually produced during the degeneration process and is not simply a normally occurring molecule that labels only when the Fluoro-Jade gains access via a disrupted plasma membrane. This is inferred from the observation that treatments which can permeabilize membranes (e.g. freezing, solvent extraction, and detergent exposure) fail to alter the characteristic staining patterns. Another property of this putative 'degeneration molecule' is that it is resistant to solvent extraction and strong oxidation by potassium permanganate. It is tempting to speculate that the potassium permanganate pretreatment acts by similar mechanism as the potassium permanganate pretreatment step of Nauta's suppressed silver technique [18]. This, however, seems unlikely as the Nauta technique involves subsequent reduction of the tissue with oxalic acid, a procedure which will negate the beneficial effect of potassium permanganate on the suppression of background staining with Fluoro-Jade. Potassium permanganate pretreatment not only suppresses background staining, but also significantly reduces fading of the fluorochrome. However, a drawback associated with the pretreatment procedure may occur during multiple label studies since exposure to potassium permanganate may eliminate or degrade fluorescent counterstains, immunofluorescent tags or axonally transported fluorochromes. Concerning multiple labeling studies with the retrograde axonal tracer Fluoro-Gold, it would appear that degenerating neurons do not retain the fluorochrome since populations of neurons containing either Fluoro-Jade or Fluoro-Gold are frequently seen, but double labeled cells are never observed.

Applications for Fluoro-Jade are generally similar to the applications of an optimal suppressed silver stain. Fluoro-

Jade does, however, appear to have a number of advantages over traditional silver methods. For example, Fluoro-Jade is fast and simple. The stain requires no more than an hour, as opposed to the cupric-silver technique which typically involves 2 days of intensive labor. Suppressed silver methods also typically require long (weeks to months) post fixation intervals to minimize background staining. Most silver methods also are limited to a relatively small number of loose sections which are processed individually. This is in contrast to Fluoro-Jade in which large numbers of slide mounted sections can be processed simultaneously. Another significant advantage of Fluoro-Jade is its reliability. This is in contrast to suppressed silver methods which are notoriously capricious in nature. Another advantage associated with Fluoro-Jade over silver methods is its suitability for multiple label studies. Silver methods do not generally lend themselves to multiple label studies since the extensive histochemical processing tends to preclude most other histochemical techniques and because the opaque silver reaction product tends to mask other brightfield labels. In conclusion, Fluoro-Jade provides a simple, sensitive, and reliable new method for demonstrating and localizing neuronal degeneration.

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