Dystrophic Neurites in the APP/PS1 rat model of Alzheimer's Disease

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Abstract

Among the three Fluoro-Jade dyes, Fluoro-Jade C (FJC) is the most sensitive fluorescent marker of neuronal degeneration, allowing for the localization not only of degenerating neurons, but also dendrites, axons and axon-terminals. FJC also has a high binding affinity for the core of plaques observed in Alzheimer's disease (AD), which mainly consists of the amyloid beta (Aβ) peptide. Never characterized before is FJC's ability to bind to dystrophic neurites (DNs; swollen neuritic processes). Here, we propose the presence of DNs may correlate with synaptic impairments in AD. Several studies have indicated the mechanism of Aβ deposition in AD; however, little attention has been paid to the location of DNs in AD brains.

DNs in the AD brain are morphologically recognized by immunohistochemical staining with antibodies specific to amyloid precursor protein (APP) and reticulon 3 (RTN3). DNs also contain ubiquitin (UBI), β-Site APP cleaving enzyme-1 (BACE1; enriched within a distinct subtype of DNs), endosome/lysosome marker lysosomal associated membrane protein 1 (LAMP-1), and/or CD68. In this study, we localized DNs using FJC in the APP/PS1 rat model of AD. Colocalization immunolabeling on FJC-stained tissues with LAMP-1, CD-68, APP, BACE1, RTN3, and UBI were performed. To determine whether FJC binds to microglia, colocalization with light chain ferritin was used. Moreover, to discriminate what location in the plaques the oligomeric form of Aβ and FJC colocalization occurs as well as the location of axonal degeneration, NU-1 and myelin basic protein (MBP) staining were also performed. Data indicate 84% or more of FJC colocalization with all markers occurs in the diffuse periphery (DNs) of the Aβ plaques, with less colocalization in the plaque core (i.e.-intense central staining). Excluding MBP (only observed in the periphery), of the total area occupied by the plaque cores, the majority colocalized with markers of DNs, microglia, and the oligomeric form of Aβ. Disregarding the distinction between core and peripheral plaque morphology, the intensity and area of colocalization was highest in ferritin and NU-1, while being lowest in CD68. In conclusion, FJC could be used to detect DNs and axonal swelling in the APP/PS1 rat model of AD.

Introduction

Various neuropathological conditions have been demonstrated by using Fluoro-Jade since its discovery in 1997[1].The highly sensitive FJC binds with higher affinity to the "neurodegenerative molecule" to which these dyes bind. DNs are known to occur around neuritic plaques early in clinical progression of AD, but the mechanism underlying their development remains unclear[2]. It is possible that Aβ plaques cause a local toxic effect that generates presynaptic DNs by disrupting microtubules and impairing transport, thus resulting in BACE1 and APP accumulation, which further contributes to Aβ generation and plaque growth in a feed-forward mechanism[3]. Although briefly reported previously[4], here we thoroughly characterize DNs stained by FJC.

Disclaimer
This poster has been reviewed and approved for presentation. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

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Materials and Methods

Animals- Tissue sections (25µm) were obtained from 16-month-old APP/PS1 Swedish double mutation transgenic (APP/PS1 Tg) rats, which are a model for AD[5] (NCTR breeding colony). All animal procedures were approved by the Institutional Animal Care and Use Committee at the National Center for Toxicological Research and were conducted in full accordance with the general principles for animal husbandry outlined in the Guide for the Care and Use of Laboratory Animals.

Combined FJC and immunolabeling: Sections were cut on a cryostat, starting at the genu of the corpus callosum and extending caudally to the posterior mid brain, collected in 0.1 M PB (pH 7.4; phosphate buffer) and stored at −20°C in a cryoprotectant solution (mixture of 25% glycerol, 30% ethylene glycol and 25% 0.1 M PB, 20% distilled water) until subsequent processing. Briefly, floating sections were processed in a scintillation vial and were first washed in neutral 0.1 M PB solution 3 times for 5 min each followed by incubation in 0.5% Triton-X +PB (PBT) for 30 min to permeabilize the tissue for antibody penetration. To reduce non-specific binding of the primary antibody, the sections were treated with 5% normal goat serum in PB for 20 min and then incubated in polyclonal rabbit antiserum against primary antibodies (table 1) for 2 days at 4°C on a shaker. Sections were then rinsed three times with PBT for 3 min per rinse and incubated in biotinylated secondary IgG for 3-4 h at room temperature. Following three 5-min washes in PBT, the sections were incubated in Streptavidin- TRITC for 3 hours at room temperature. After a brief wash in PBT, sections were mounted on gelatin coated glass slides and labeled for FJC with slight modification[6,7]. To avoid loss of immunolabeling, pretreatment in basic alcohol and 70% alcohol was omitted and the sections were incubated in KMnO4 for 2 -6 min to suppress background noise. The sections were then briefly rinsed in distilled water, incubated in FJC for 10 min and then rinsed in 3 changes of distilled water. Sections were then air dried on a slide warmer, cleared in xylene and cover slipped in DPX mountant. The tissue was examined with a Nikon epifluorescent microscope using the following set of filters: for TRITC streptavidin, excitation of 540–590 nm, band pass of 596 nm and emission of 600–660 nm; for FJC, excitation of 460–500 nm, band pass of 505 nm and emission of 510– 560 nm. Thus, structures positive for FJC exhibited green fluorescence while APP, BACE, RTN3, LAMP-1, CD68, Ferritin, Ubiquitin, and NU-1 positive structures exhibited red fluorescence.

Analysis:
Images were taken at 20x and subsequently analyzed in NIS Elements Advanced Research. Red channel threshold was set between 89-255 with 3X smoothing and size restrictions of 5.45-595µm. Green channel threshold was set between 83-255 with 3X smoothing and size restrictions of 5.45-595µm. To quantify colocalization, a binary mask was created for areas where green and red thresholds overlap, which was named "colocal". The object data for the binary was exported to excel and statistical analysis was performed in GraphPad Prism 6. For FJC core quantification, the green threshold layer was selected, and channel threshold was set between 160-255 with 3X smoothing, size restrictions of 5.45-595µm, circularity between 0-0.74, and saved as "core FJC." To determine what portions of the core were colocalized, addition of the two binaries was performed. To determine what portions of the core were not colocalized, subtraction of the two binaries was performed. Data were exported to Excel and statistical analysis was performed in GraphPad Prism 6. One or two-way ANOVA with Holm-Sidak post-hoc was performed; in all cases α = 0.05.

Primary antibody (source/dilution)	Secondary antibody (Thermofisher/1:100)	Streptavidin conjugate (Jackson Immunoresearch)
Rabbit APP (Sigma/1:200)	Biotinylated Goat anti-Rabbit IgG	Streptavidin TRITC (1:200)
Rabbit BACE (CST/1:200)	Biotinylated Goat anti-Rabbit IgG	Streptavidin TRITC (1:200)
Rabbit RTN3 (Millipore/1:100)	Biotinylated Goat anti-Rabbit IgG	Streptavidin TRITC (1:200)
Mouse anti- Rat CD68 (BioRad/1:500)	Biotinylated Goat anti-mouse IgG	Streptavidin TRITC (1:200)
Mouse anti- Rat LAMP-1 (Developmental Studies Hybridoma Bank/1:200)	Biotinylated Goat anti-mouse IgM	Streptavidin TRITC (1:200)
Rabbit myelin basic protein (MBP) (Novus/1:200)	Biotinylated Goat anti-rabbit IgG	Streptavidin TRITC (1:200)
Rabbit NU-1 (Gift from Dr. William Klein/1:500)	Biotinylated Goat anti-rabbit IgG	Streptavidin TRITC (1:200)
Anti-Ubiquitin rabbit antibody (DAKO/1:100)	Biotinylated Goat anti-rabbit IgG	Streptavidin TRITC (1:250)
Rabbit Ferritin (L) (Sigma/1:300)	Biotinylated Goat anti-rabbit IgG	Streptavidin TRITC (1:250)

Table 1. Antibody source and dilution factor

Results and Discussion

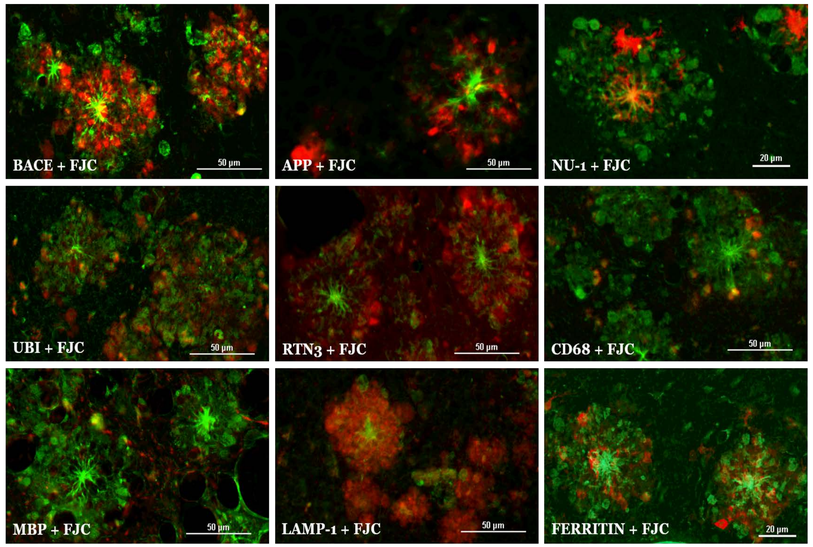

Figure 1. Colocalization of markers with FJC in the cortex. Images (20x) of immunohistochemical markers for DNs, oligomeric Aβ, and microglia are in red. FJC is in green.

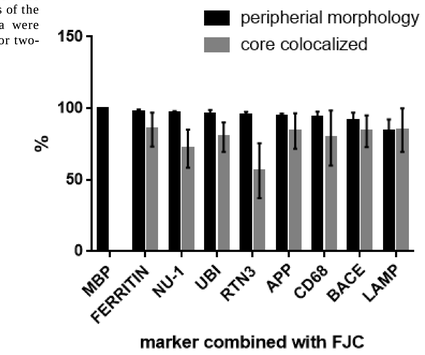

Figure 2. Graphical characterization of peripheral and core components

Marker colocalizing with FJC	% peripherial morphology	% core morphology	% of core colocalized
MBP	100.0	0.0	0.0
FERRITIN	97.5	4.9	85.2
NU-1	96.7	4.7	71.9
UBI	95.9	8.2	80.0
RTN3	95.4	7.4	56.4
APP	94.2	7.8	84.2
CD68	93.4	13.2	79.4
BACE	91.2	11.8	84.0
LAMP	84.0	16.0	84.8

Table 2. Numerical characterization of peripheral and core components

- The plaque peripheries in the cortex displayed: DNs (APP, RTN3) active in multiple pathways (UBI, BACE1, LAMP-1, CD68), axonal degeneration (MBP), the oligomeric form of Aβ (NU-1), as well as microglia (ferritin).
- Where plaques with densely FJC-stained cores were observed, DNs, microglia, and oligomeric Aβ were also evident.
- In the cortex, 84% or more of stain colocalized with FJC in the plaque periphery. Colocalization with MBP was solely in the periphery.
- FJC has a highest affinity for the oligomeric form of Aβ (NU-1) and microglia (light chain ferritin).
- The frequency of plaques present varied by brain region (data not shown).

Conclusion

- ✓ FJC labeled DNs located in the plaque periphery also contain a variety of substances suggesting a myriad of pathways may be involved in the accumulation around plaques and eventual synaptic dysfunction. These processes may be involved in the progression of AD; thus, one of these pathways may serve as a therapeutic target to mitigate disease progression.
- ✓ Colocalization of multiple markers remains the best form of identification for DNs as FJC has a higher affinity for other structures and, in this study, the total area occupied by colocalization of FJC with any other marker in the cortex comprised less than 3% of the 20x FOV.
- ✓ Axonal degeneration (MBP) is unique to the plaque periphery.

References
1. Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra-high resolution and contrast labeling of degenerating neurons. Brain Res (2005) 1035 (1):24-31
2. Yan XX. Formation of Dystrophic Neurites in Alzheimer's Disease. FASEB (2018) S1:91.3-91.3
3. Sadlier KR, Kandalepas PC, Buggia-Prevot V, Nicholson DA, Thankaran G, Vassar R. Presynaptic dystrophic neurites surrounding amyloid plaques are sites of microtubule disruption, BACE1 elevation, and increase Aβ generation in Alzheimer's disease. Acta Neuropathol (2016) 132:235-256.
4. Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, Manalastas A, Hilfiker M, Pfister S, Schwerdel C, Carsten R, Meyer U, Knuesel I. Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. J Neuroinflamm (2012) 9:151
5. Cohen, R.M., Rezai-Zadeh, K., Weitz, T.M., Rentsendorj, A., Gate, D., Spivak, I., Bholat, Y., Vasilevko, V., Glabe, C.G., Breunig, J.J., Rakic, P., Davtyan, H., Agadjanyan, M.G., Kepe, V., Barrio, J.R., Bannykh, S., Szekely, C.A., Pechnick, R.N., Town, T., 2013. A transgenic Alzheimer rat with plaques, tau pathology, behavioral impairment, oligomeric abeta, and frank neuronal loss. J Neurosci. 33, 6245-56.
6. Sarkar S, Schmued L. Kainic acid and 3-Nitropropionic acid induced expression of laminin in vascular elements of the rat brain. Brain Res (2010) 17;1352:239-47
7. Sarkar S, Raymick J, Schmued L. Temporal Progression of Kainic Acid Induced Changes in Vascular Laminin Expression in Rat Brain with Neuronal and Glial Correlates. Current Neurovascular Res (2012) 9;110-119.