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RESEARCH ARTICLE

High Contrast and Resolution Labeling of Amyloid Plaques in Tissue Sections from APP-PS1 Mice and Humans with Alzheimer's Disease with the Zinc Chelator HQ-O: Practical and Theoretical Considerations

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ARTICLE HISTORY

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Abstract: ***Background*:** Various methodologies have been employed for the localization of amyloid plaques in numerous studies on Alzheimer's disease. The majority of these stains are thought to label the plaques by virtue of their affinity for aggregated Aβ. However, plaques are known to contain numerous other components, including multivalent metals such as zinc.

***Objective*:** This investigates whether it is possible to localize the presence of zinc in parenchymal and vascular amyloid plaques in afflicted brains. To accomplish this, a novel fluorescent zinc chelator, HQ-O, was investigated to determine its mechanism of binding and to optimize a stain for the high contrast and resolution histological localization of amyloid plaques.

***Methods*:** A novel zinc chelator, HQ-O, was developed for localizing zinc within amyloid plaques. The histology involves incubating tissue sections in a dilute aqueous solution of HQ-O. Its compatibility with a variety of other fluorescent methodologies is described.

***Results*:** All amyloid plaques are stained in fine detail and appear bright green under blue light excitation. The staining of parenchymal plaques correlates closely with that seen following staining with antibodies to Aβ, however, the HQ-O sometimes also label additional globular structures within blood vessels. In situ mechanistic studies revealed that fluorescent plaque-like structures are only observed with HQ-O when synthetic Aβx-42 is aggregated in the presence of zinc.

***Conclusion*:** Zinc is intimately bound to all amyloid plaques, which was demonstrated by its histological localization using a novel fluorescent zinc chelator, HQ-O. Additionally, the tracer is also capable of labeling intravascular leucocytes due to their high zinc content.

Keywords: Amyloid plaques, Alzheimer's disease, zinc chelator, brain pathology, Aβ, Amylo-Glo.

1. INTRODUCTION

In 1906, Alois Alzheimer first described the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles within the brains of patients with the neurodegenerative disease that bears his name. Subsequently, various histochemical methods were developed to localize these pathologies. One of the first organic dyes used for this purpose was Congo Red [1], which when applied in a basic solution results in the red staining of amyloid plaques, which becomes birefringent when viewed under polarized illumination. Congo Red can also be used at the fluorescent level, although the brightness and resolution are relatively low. The first dye used specifically as a fluorescent probe for amyloid plaques was Thioflavin S [2], which appears green under blue light excitation and is also capable of staining intracellular Neurofibrillary Tangles (NFTs). The contrast and resolution are of an intermediate level and the tracer exhibits a very broad excitation and emission profile, making it unsuitable for most multiple labeling studies. Amylo-Glo [3] is used in a basic aqueous vehicle and results in the bright, high resolution, high contrast staining of amyloid plaques under UV excitation. Another styryl-benzene based dye, FSB [4], exhibits similar properties, aside from fluorescing under both UV and blue light excitation, making its use for multiple labeling studies more limited. These techniques typically show the classic plaque morphologies similar to that seen following Aβx-42 immunohistochemistry. By contrast, Aβ immunolabeling of vascular plaques is much more limited than the pattern seen following Amylo-Glo staining. It is also worth noting that amyloid plaques are far from homogeneous and other components have been co-localized including various enzymes, gangliosides, neuritic, and transition metals, just to list a few. With regards to the latter, zinc [5] iron [6], and copper [7] have all been found in association with amyloid plaques.

HQ-O is the oxalate salt of 8-hydroxyquinoline which, like its protonated precursor, exhibits diverse properties and

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applications [8]. The majority of 8-hydroxyquinoline applications are based on its property as a chelator of numerous multivalent metals. Its chelate with aluminum has been used industrially to make organic light emitting diodes. It has also been used clinically as a topical disinfectant, and its halogenated derivatives, such as clioquinol and aluminum sulfate, have both been used to treat intestinal parasites in humans. It has been reported to bind to at least 26 multivalent metals with those of biological significance being Ca, Cu, Co, Fe, Mg, Mn, and Zn; and of these, only Zn, Ca, and Mg form fluorescent complexes, with zinc exhibiting the highest binding constant [9]. Although Smith *et al.* [9] used the tracer for the localization of granules containing zinc within leucocytes, another group used it to locate zinc in sections of the rat prostate [10]. The present study represents its first use in brain tissue. Although another fluorescent zinc chelator, TSQ (6-methyl-8-quinoyl-toluene-sulfonamide), has been shown to label amyloid plaques, the resulting low resolution staining revealed few morphological details of the amyloid plaques [5] in an APP-PS1 mouse model of Alzheimer's disease (AD).

2. METHODS

2.1. Tissue

Tissue sections were obtained from 12 months old wild type mice, the BGC3-Tg(APPswePSEN1dE9)85Dbo/J strain of APP/PS1 transgenic mouse model of AD (APP-PS1) (Jackson Labs, West Grove PA), and autopsy tissue from humans diagnosed with AD. De-identified fixed AD-confirmed human tissue samples were obtained from the University of Maryland Brain and Tissue Bank (Baltimore, MD) and the University of Miami Brain Endowment Bank (Miami, FL), under protocol E07631.01. Each of the brain banks listed above operated under their institution's IRB approval. All experimental procedures described here were approved by the appropriate committees at the National Center for Toxicological Research/FDA, including the NCTR Office of Research, the Regulatory Compliance and Risk Management Director and the FDA Research Involving Human Subjects Committee (RIHSC). As determined by the FDA RIHSC, this study did not reach the definition of "Human Subject Research" at 45 CFR 46.102(f) and thus, 45 CFR Part 46 does not apply. Both frozen-cut and paraffin-embedded tissues were used and were cut at thicknesses of 25 and 10 microns respectively. All tissue was formalin-fixed prior to processing and staining.

2.2. HQ-O Staining

The frozen sections were mounted onto gelatin-coated slides and allowed to dry on a slide warmer for at least 20 min. Typically, the slides are first immersed in 100% ethanol for 5 min followed by 3 min in 70% ethanol and 3 min in distilled water. Should exposure to solvents be counter-indicated, this step may be omitted and the sections may simply be rehydrated in distilled water for 3 min. The slides are then transferred to the HQ-O staining solution which is prepared by adding 33 mg of HQ-O (Histo-Chem, Jefferson AR) to 100 ml of distilled water vehicle. The optimal staining time is temperature dependent, therefore overnight (16-24 hrs) incubation is required when staining fresh tissue sections at room temperature, while only 3 hrs. incubation time is required at 60°C. The staining solution should be used within 24 hrs. of preparation. Paraffin-embedded sections are first deparaffinized in xylene and then rehydrated with a graded series of alcohols and finally in distilled water. The hydrated slides are then transferred to the same HQ-O staining solution as described above. At room temperature, the paraffin-processed tissue will require around 3 hrs. to fully stain, while tissue sections incubated at 60°C will require only about 45 min to stain. Following staining, all slides are rinsed for 3 min through 2 changes of distilled water and then allowed to air dry on a slide warmer. Slides are cleared by brief (1-2 min) immersion in xylene and then coverslipped with DPX (Sigma-Aldrich, St Louis MO), a non-polar and non-fluorescent mounting media. Slides are visualized under blue light excitation.

2.3. Multiple Labeling Studies

HQ-O staining lends itself to multiple labeling studies and in such studies, the tissue was first stained with HQ-O, as indicated above, and then double labeled with one of the following staining procedures:

2.3.1. Aβ Immunohistochemistry

Following HQ-O staining as described above, a set of sections were stained with one of three antibodies directed against different epitopes of Aβ. All sections were processed on-the-slide and were first washed in neutral Phosphate Buffered Saline (PBS) 3 times for 5 min followed by a 30 min incubation in 0.5% Triton-X to permeabilize the tissue for antibody penetration. Then, sections were incubated in 10% normal horse serum for 15 min to prevent non-specific binding. The sections were then incubated in one of three primary antibodies, specifically those directed against Aβx-42, Aβx-40 and Aβx-40/42. The primary Aβx-42 rabbit antibody (Abcam, Cambridge MA) was diluted 1:200 with phosphate buffered saline plus 0.5% Triton-X, (PBS-T), incubated overnight and then rinsed with 3 changes of PBS-T for 3 min per change. The secondary antibody, biotinylated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove PA) was diluted 1:200 in PBS-T and incubated with the tissue for 3 hr. Following three 5 min washes in PBS-T, the sections were incubated in TRITC conjugated streptavidin (Vector Laboratories, Burlingame, CA) diluted 1:250 in PBS-T for 1 hour and subsequently rinsed through 3 changes of PBS-T, briefly (30 seconds) rinsed in distilled water, dried on a slide warmer, cleared by 1-2 min immersion in xylene and then coverslipped with DPX (Sigma-Aldrich, St Louis MO) mounting media. Essentially the same procedure was used for staining the other epitopes of Aβ, except primary antibodies directed against Aβx-40 (Invitrogen, Carlsbad CA) were diluted 1:200 in PBS-T while monoclonal antibodies against Aβx-40/42 (Biosensis, Temecula CA) were diluted 1:500 with PBS-T. The latter also used a secondary biotinylated goat anti-mouse antibody (Jackson Immunoresearch, West Grove PA) diluted 1:200 in PBS-T.

2.3.2. Amylo-Glo

Following HQ-O staining, Amylo-Glo fluorescent labeling of amyloid plaques was accomplished using the previously described method [3]. In brief, this involved immersing slide mounted tissue sections in 70% ethanol for 5 min

and then immersion for 20 min in a staining solution of Amylo-Glo (Histo-Chem, Jefferson AR) with the working solution being prepared by adding 1 ml of the stock solution to 99 ml of 0.9% saline. Sections are rinsed in distilled water for 3 min and then air dried on a slide warmer. Slides are briefly cleared by immersion in xylene and then coverslipped with DPX mounting media. Amylo-Glo staining of plaques is visualized under UV light excitation.

2.3.3. Ethidium Bromide Fluorescent Nissl-like Stain

Following staining with HQ-O, slides were briefly rinsed in distilled water and then transferred to a solution containing 0.0001% ethidium bromide (Sigma, St Louis MO) dissolved in a 0.1% acetic acid vehicle for 3-5 min. They were then briefly rinsed in distilled water, air dried, xylene cleared and coverslipped with DPX mounting media. This tracer is typically visualized using green light excitation, although its emission peak is broad enough to allow simultaneous visualization with the HQ-O under blue light excitation and broad band excitation and emission filters.

2.3.4. DAPI Fluorescent Nuclear Stain

Following staining with HQ-O, DAPI (MP Biomedicals, Solon OH) fluorescent counterstaining of all cellular nuclei was achieved by immersing the tissue in a solution of 0.00005% DAPI dissolved in a 0.1% acetic acid vehicle for 3-5 min. Tissue sections were then rinsed in distilled water for 3 min, air dried on a slide warmer, briefly cleared by immersion in xylene and coverslipped with DPX mounting media. DAPI staining is visualized under UV light excitation.

2.3.5. Anti-GFAP Immunofluorescent Stain

The following staining with HQ-O, anti-GFAP immunofluorescent staining was achieved by on-the-slide staining procedures in which the various solutions are applied directly to the tissue sections that are kept in a humidity chamber. A blocking solution consisting of 10% horse serum in PBS-T is applied directly onto the tissue sections for 20 min and then rinsed with two 3min changes of saline. The primary polyclonal rabbit anti-GFAP antibody (Dako, Carpentaria CA) was used at a 1:500-1:1000 solution in PBS-T and was allowed to stay on the section overnight at room temperature. The slides were then rinsed with 2 changes of PBS and then incubated in the biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove PA) at a dilution of 1: 200 for 2h. Following two PBS rinses, the sections were incubated in an avidin-TRITC conjugate solution in PBS at a dilution of 1: 250 for 1 hr. Sections were rinsed twice with PBS, briefly rinsed in distilled water, air dried, briefly cleared in xylene and then coverslipped with DPX mounting media.

2.4. *In Situ* Binding Studies

1 mg of Aβ (Tocris) was dissolved in 1 ml of physiological saline plus 60 ul of ammonium hydroxide. 0.5 ml of this solution was then "puddled" onto a glass slide in a humidity chamber heated to 37 ^{o}C. A second slide received the same Aβ solution plus 0.01% zinc acetate. The third slide designed to serve as a control contained just saline and 0.01% zinc acetate. All three slides were incubated for 3 days. After equilibration to room temperature, the slides were rinsed for 3 min with distilled water, flooded with the same HQ-O staining solution as described above for 10 min, rinsed for another 3 min in distilled water and then dried on a slide warmer.

3. RESULTS

3.1. HQ-O Staining

Examination of brain sections from year old wild type mice under blue light excitation exhibited no labeling (Fig. **2C**), while sections from APP-PS1 mice revealed numerous plaque-like structures scattered throughout all layers of the neocortex (Fig. **1A**), piriform cortex and the hippocampal complex in both paraffin-processed (Figs. **1A, B**) and frozen-cut tissue sections (Fig. **1C**). The plaques revealed somewhat diverse morphologies including dense core, condensed and diffuse appearing structures (Figs. **1A-F**). Examination of the autopsy tissue from humans with AD also revealed both condensed and dense core plaque morphologies throughout neocortical and allocortical brain regions (Fig. **1E**). The autopsy material did not reveal NFTs within adjacent neurons, but only a relatively small number of cells were available for examination. Vascular plaques were frequently seen in the brains of both the transgenic mice (Fig. **1D**) as well as humans with Alzheimer's disease (Fig. **1F**). Although not all brain blood vessels contain plaques, they are most frequently seen in the meninges and the penetrating vessels of the cerebral cortex (Figs. **1D**; **2A-B**). The vascular plaques exhibit more variation in distribution and morphology than the parenchymal plaques. When present, labeled vascular structures typically appear as either discontinuous circumferential striations (Fig. **1F**) or as globular structures (Fig. **1D**). The globular appearing structures are often correlated with the DAPI staining of cell nuclei (Fig. **1D**). When comparing the staining of vascular plaques with HQ-O (Fig. **2A**) vs Amylo-Glo (Fig. **2B**), both tracers stain select meningeal vessels comparably, while HQ-O stains the penetrating vessels more extensively than the Amylo-Glo.

3.2. Fluorescent Nissl-like Counterstaining

The use of fluorescent counterstains allows for the localization and quantification of neurons relative to adjacent amyloid plaques. Post-staining with ethidium bromide resulted in the fluorescent red staining of the nucleolus of all cells and both the nucleolus and Nissl substance of all neurons (Figs. **3A** and **B**). It also allows investigators to evaluate the impact of the amyloid plaques on adjacent neurons. Thus, it is possible to appreciate how a prominent plaque in the hilus of the dentate gyrus has infringed on the viability of the adjacent granule cells (Fig. **3B**). Post-staining with DAPI resulted in the fluorescent blue staining of nuclear components (Fig. **3C**). This also allows the investigator to localize plaques relative to cellular landmarks. Multiple labeling with HQ-O and DAPI (Fig. **3C**) suggest that the HQ-O labeling of parenchymal plaques is not likely to be cellular in nature.

3.3. GFAP Counterstaining of Activated Astrocytes

By combining HQ-O staining with immunofluorescent GFAP staining of activated astrocytes it is possible to visualize reactive astrocytes relative to the amyloid plaques. As expected, activated astrocytes can be seen surrounding the majority of amyloid plaques (Fig. **3D**).

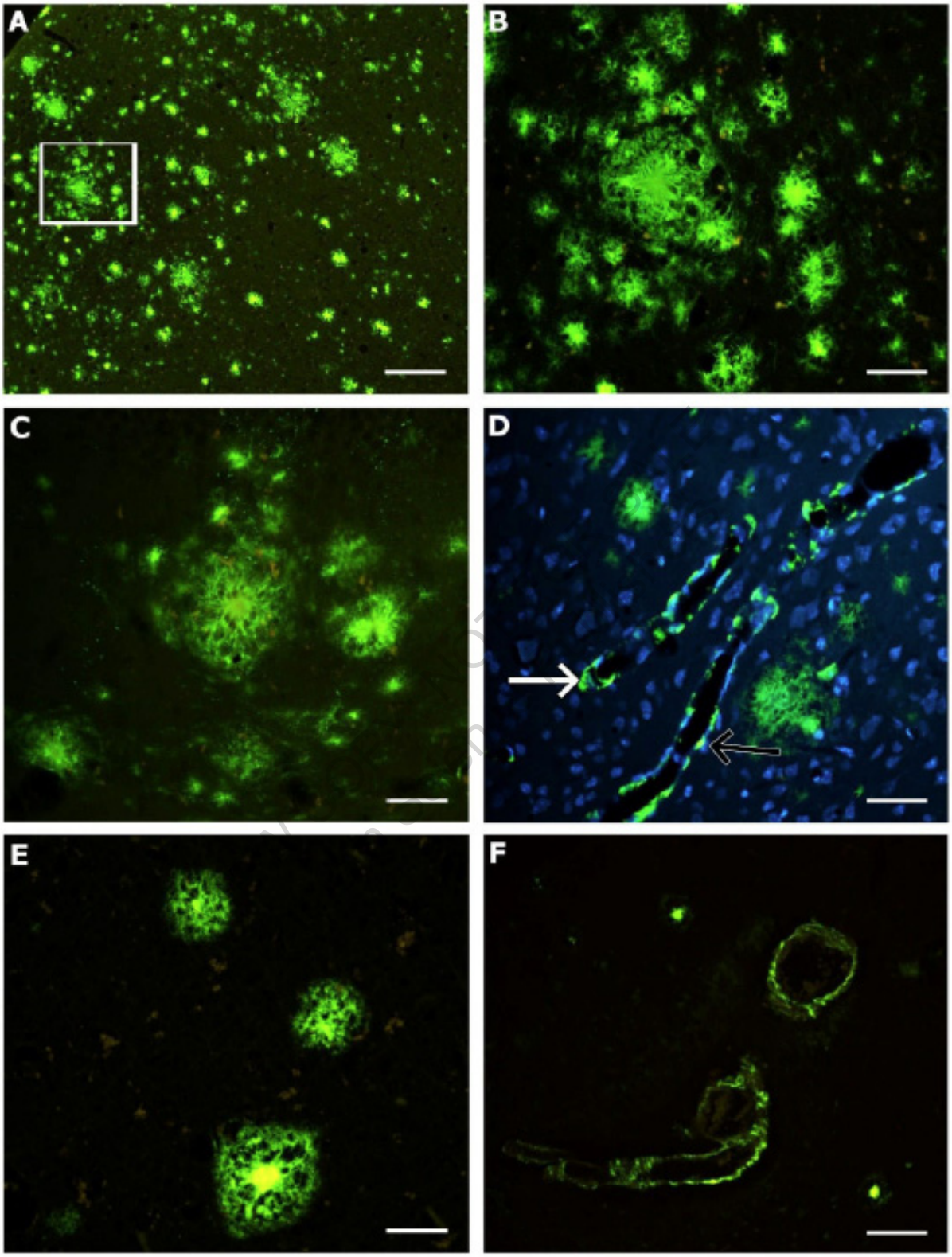

Fig. (1). Examples of HQ-O plaque labeling in APP-PS1 mice and Alzheimer's disease patients: Parenchymal plaques can be seen throughout a survey view of the cortex of a paraffin-processed APP-PS1 mouse brain, with the pial surface at the upper left (**A**). The region circumscribed by a white rectangle is also illustrated at high magnification (**B**). An example of HQ-O localized plaques in frozen mouse tissue sections is illustrated (**C**). An example of vascular plaque labeling in the APP-PS1 mouse cortex is illustrated (**D**) along with DAPI counterstaining of adjacent cellular nuclei. The white arrow identifies a vascular plaque labeled only with HQ-O, while the black core arrow indicates multiple labeling with both HQ-O and DAPI. Cortical parenchymal plaques from an AD patient are localized in a paraffin-processed section (**E**). An example of a vascular plaque in the cortex of a human AD patient is shown (**F**). Mag bar for 1A = 200 um; Mag bars for 1B-F = 40 um.

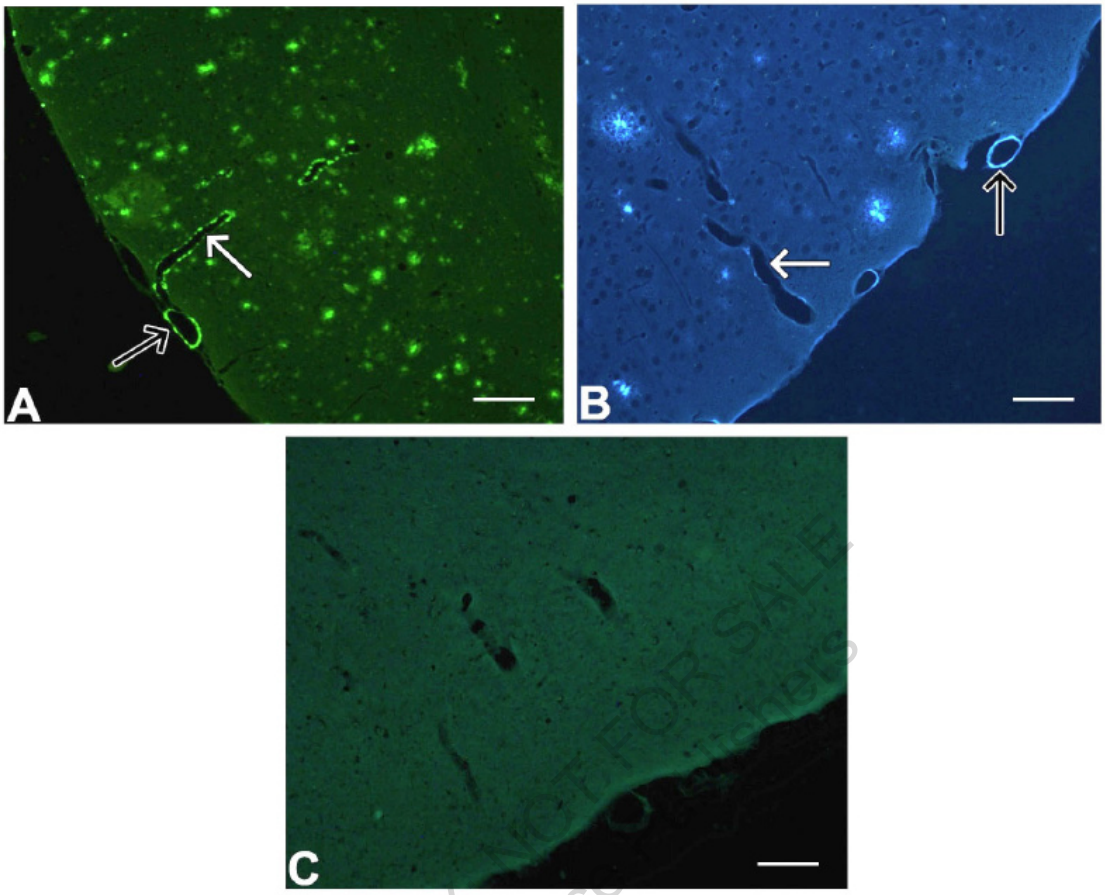

Fig. (2). Comparison of vascular staining with HQ-O (**A**) and Amylo-Glo (**B**) in the cortex of the one-year old APP-PS1 AD mouse. Arrows with black cores indicate transverse cut meningeal vessels which are comparably stained with both tracers, while solid white arrows reveal significantly more labeling of penetrating vessels with HQ-O than with Amylo-Glo. The cortex of a 1-year old wild type mouse (**C**) exhibits no HQ-O staining of either parenchymal or vascular plaques. Mag bars for 2A-C = 200 um.

3.4. Co-localization with Amylo-Glo Histochemistry

Double labeling with Amylo-Glo and HQ-O resulted in the high resolution and contrast staining of the entire amyloid plaque. Comparison of the 2 stains (Figs. **4A-C**) reveals virtually a total correlation between the 2 stains. Both stains label in fine detail a variety of plaques including those with dense core, condensed and diffuse morphologies. They also both stain vascular plaques, although not identically (Figs. **2A** and **B**).

3.5. Co-localization with Anti-Aβ Immunofluorescence

For the labeling of parenchymal plaques, antibodies directed against 3 different epitopes of Aβ showed a generally similar fibular pattern of labeling (Figs. **4E, H** and **K**). However, some subtle differences were observed. Specifically, the Aβx-42 antibody resulted in the most complete labeling of the plaques (Fig. **4K**) and correlated the closest with the HQ-O staining (Figs. **4D, G** and **J**). By comparison, labeling with the monoclonal Aβx-40/42 antibody tended to label the plaque cores more than the fine peripheral components (Fig. **4E**), while labeling with antibodies directed against Aβx-40 resulted in noticeably fewer detectable plaques (Figs. **4H, I**).

Although the pattern of staining of parenchymal plaques with HQ-O correlates relatively closely to the staining seen with antibodies against Aβ, similar correspondence was not observed in the blood vessels. Specifically, HQ-O staining (Figs. **4G, I, J** and **L**) resulted in the conspicuous labeling of some, but not all blood vessels. This staining typically appears as a non-continuous banding of adjacent globular structures surrounding what appears to be the abluminal side of the vessel (Figs. **4G, J, 1D** and **F**). Although labeled blood vessels could be seen throughout the forebrain, they were most commonly seen in the cortex and hippocampal complex. This labeling was typically in an all-or-nothing manner with heavily labeled vessels commonly found adjacent to completely unlabeled vessels (Figs. **4G, H** and **I**).

3.6. *In Situ* Binding Studies

HQ-O staining of the slide incubated with only saline and zinc revealed a fine background of thin rod-shaped structures (Fig. **5A**). The slide incubated only with the Aβ showed minimal labeling with HQ-O. Occasionally, a small faintly labeled undifferentiated structure could be seen whose morphology did not resemble that of typical amyloid plaques (Fig. **5B**). Examination of the slide incubated with the Aβ and zinc acetate combination revealed a variety of brightly labeled structures (Fig. **5C**). The most common morphologies were fibular, rosette and partial and complete thread-like loops.

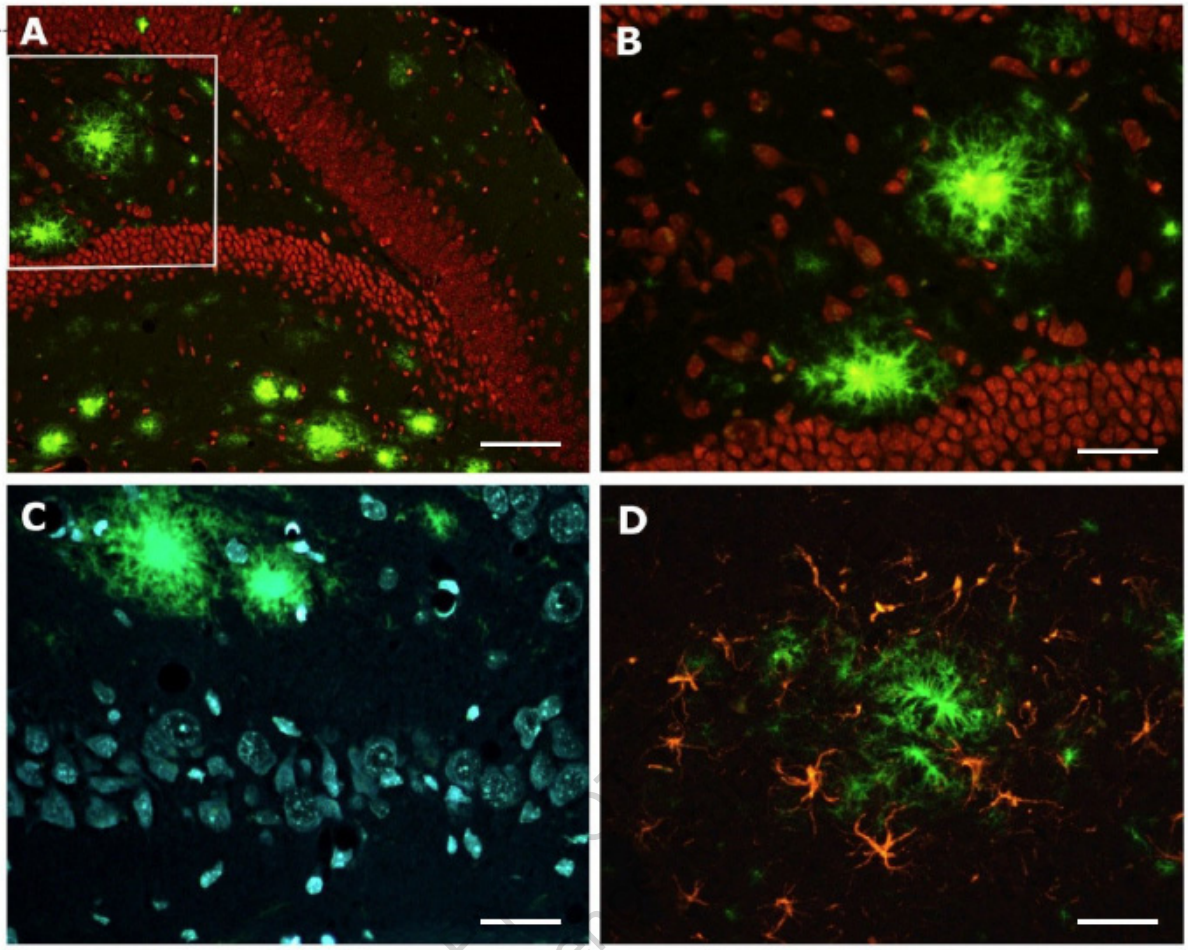

Fig. (3). Examples of HQ-O labeled plaques in relationship to various counterstained anatomical structures are illustrated in the APP-PS1 transgenic mouse. Double labeling with ethidium bromide allows localization of green colored plaques relative to red colored Nissl-like stained cells of the dentate gyrus (**A**). The area within the white rectangle at the upper left is shown at higher magnification (**B**) where it is apparent that one of two amyloid plaques within the hilar region has impinged on the underlying ventral blade of the granule cells. Counterstaining with DAPI resulted in the blue staining of nuclear components of all cells including the granule and polymorphic neurons of the dentate gyrus (**C**). Also, within the CA4 region of the hippocampus, co-labeling using GFAP immunofluorescent methods demonstrates the relationship between hypertrophied astrocytes and the amyloid plaques that they typically surround (**D**). Mag bar for 3A = 200 um; Mag bars for 3B-D = 40 um.

4. DISCUSSION

Resolving the anatomical structures within the brain that exhibit a histochemical affinity for HQ-O is relatively straight forward, since its distribution and morphology correlate with that of amyloid plaques, plus they are only seen in brains from aged APP-PS1 mice and humans diagnosed with Alzheimer's disease. HQ-O labeling of parenchymal plaques correlates very closely with that seen following staining with bis-styryl-benzene derivatives such as Amylo-Glo or FSB. Similarly, the staining of parenchymal plaques with antibodies directed against Aβx-42 also closely resembles that seen following staining with HQ-O although the latter exhibited more staining of the fine neurite-like peripheral structures. It is not known if this actually represents a differential affinity or simply the HQ-O exhibiting more contrast. In agreement with the literature [11], staining with antibodies directed against Aβx-40 resulted in a much more restricted labeling pattern. This antibody resulted in the labeling of fewer plaques and an absence of labeled diffuse plaques. Somewhat surprisingly, although the labeling of vascular plaques was comparable between Aβx-40 *vs* Aβx-42, both were quite different from the pattern seen following HQ-O staining. This would suggest that HQ-O not only labels Zn containing circumferential amyloid plaques but also the Zn containing leucocytes within the vessel's lumen. However, when interpreting the staining with antibodies directed against diverse peptide lengths, it is worth considering that no single antibody can distinguish Aβx-40 or Aβx-42 from the various N-truncated peptides having the same C-terminus.

Although all the aforementioned probes resulted in the labeling of vascular plaques, there was significant variation in the extent and pattern of labeling observed. The most obvious difference was that although all markers labeled the restricted circumferential band-like plaques, HQ-O staining also labeled globular appearing structures. This could be interpreted as evidence for all probes labeling the Aβ aggregates, either directly or indirectly, while the HQ-O also labeled the leucocytes lining the lumen of certain vessels. Both

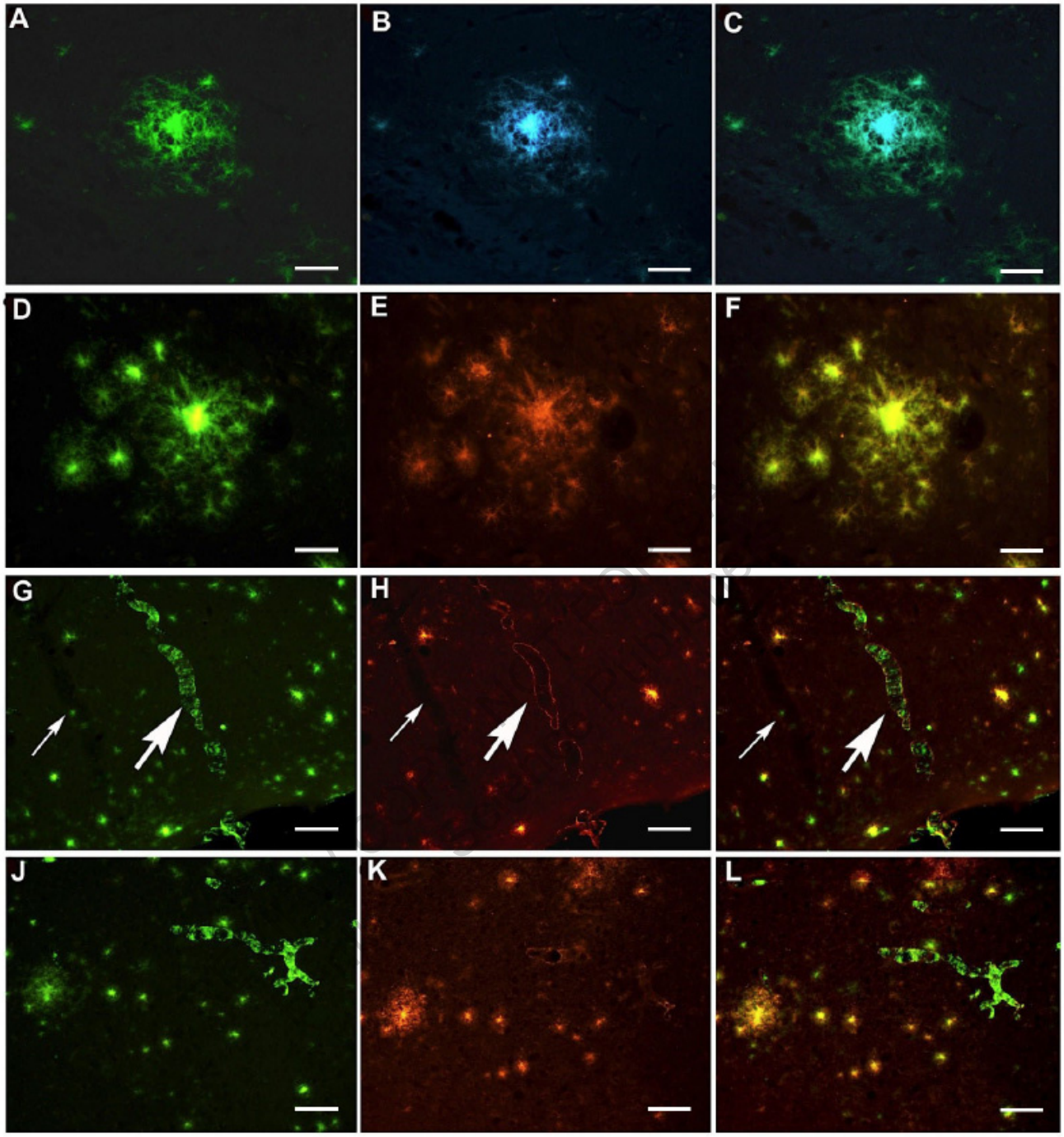

Fig. (4). Examples of co-labeling of parenchymal and vascular plaques with HQ-O and other markers of amyloid plaques in the one-year old APP-PS1 mouse are compared. In all cases, the column at left represents HQ-O staining, the middle column shows the same field of view stained with an alternative amyloid plaque specific tracer and the column at right shows the merged images. Typical HQ-O labeling of a parenchymal plaque is seen in the hilar region of the dentate gyrus under blue light (**A**). The same field imaged with UV light reveals that this plaque is also Amylo-Glo positive (**B**). This is confirmed by merging the 2 images (**C**). HQ-O stained plaques can be seen within the CA-1 region of the hippocampus (**D**). This same field of view stained with immunofluorescent methods using the Aβx-40/42 antibody reveals a similar but somewhat more restricted labeling of the same amyloid plaques (**E**), which is apparent when the images are merged (**F**). Both parenchymal and vascular plaques in the cortex are labeled with HQ-O in the parietal cortex (**G**). The larger arrow indicates a penetrating blood vessel that is conspicuously labeled with HQ-O, while the smaller arrow indicates a similar vessel except it is HQ-O negative. The same field following immunofluorescent staining with antibodies against Aβx-40 reveal a similar staining of the parenchymal plaques but very different labeling of the vascular plaques (**H**). These similarities and differences can be appreciated in the merged image (**I**). HQ-O staining reveals the presence of both parenchymal and vascular plaques in the temporal cortex (**J**). The same field is seen after staining with antibodies directed against Aβx-42 (**K**). Although parenchymal plaques stain comparably with both tracers, vascular staining is much more limited with the Aβx-42 immunofluorescent labeling compared with the HQ-O labeling (**L**). Mag bars for 4A-L = 200 um.

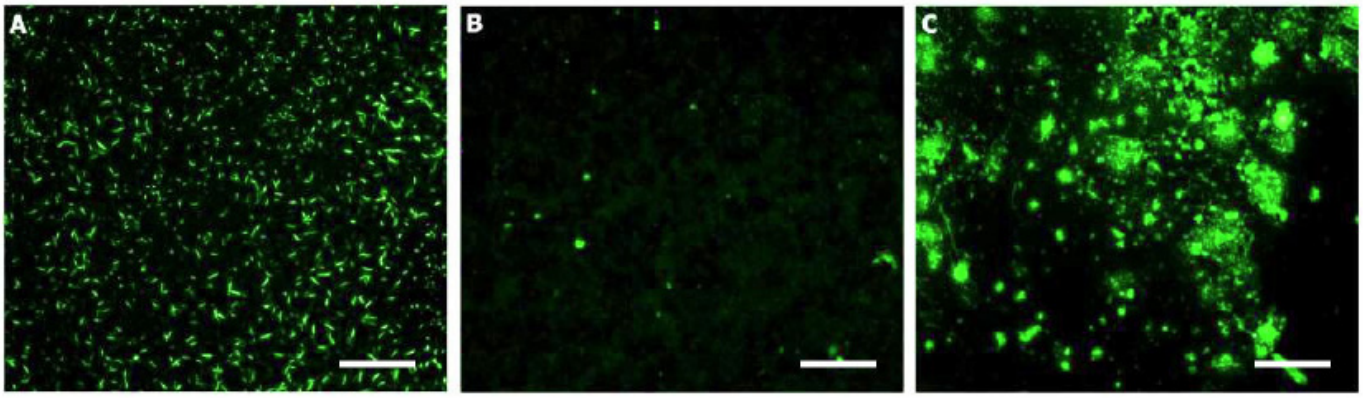

Fig. (5). *In situ* studies on the mechanism underlying the binding of HQ-O to amyloid plaques reveal the following: After incubation in a zinc acetate solution, HQ-O staining reveals numerous fine needle-like fluorescent structures (**A**). Aβ aggregates fail to show significant labeling following staining with HQ-O alone (**B**). Aβ aggregates in the presence of zinc acetate reveal a variety of plaque-like structures following staining with HQ-O (**C**). Mag bars for 5A-C = 40 um.

types of labeling by HQ-O would be consistent with a mechanism whereby it binds to the zinc known to be associated with both amyloid aggregates and blood borne leucocytes.

Although the identity of the anatomical structure which HQ-O labels seems fairly obvious, understanding the underlying molecular mechanism of binding is much more challenging. However, it is possible to propose a mechanism that is consistent with all of the results seen in this study. All data suggest that HQ-O likely binds to zinc molecules known to be found in amyloid plaques, thus becoming fluorescent. This is supported by the *in situ* studies which showed that only when Aβ is aggregated in the presence of zinc, did appreciable labeling result from HQ-O.

Although understanding the underlying molecular mechanism of binding is challenging, it is possible to speculate on the more probable underlying binding mechanisms. One candidate mode of action would be direct binding to the misfolded Aβ aggregates. Support for this idea would include the observation that the HQ-O staining of parenchymal plaques very closely resembles the labeling pattern seen following Aβx-42 immunofluorescence as well as that of Amylo-Glo. Additionally, NMR-studies reported that a different Cu and Zn chelator, bathocuproine, can bind to Aβ dimmers in the absence of any transition metals [12]. However, these observations do not explain the fact that HQ-O itself is virtually non-fluorescent and only its chelation to certain multivalent metals has been shown to result in the generation of a fluorophore. Therefore, a potentially more plausible mode of tracer binding is one that involves the compound's ability to chelate metals. As previously noted, HQ-O can bind to at least 26 different multivalent metals. Of these Ca, Mg, Co, Cu, Fe, Mn and Zn are of biological importance, while only Ca, Mg, and Zn form fluorescent chelates, with Zn showing the highest binding affinity of all [8]. It is also worth noting that Zn, Cu, and Fe are all found in the neuropil, but only Zn is significantly elevated in the plaques themselves, as revealed by X-ray fluorescence microscopy [13]. Therefore, a plausible mechanism of action could be that, since Zn has been localized in amyloid plaques, the HQ-O could simply bind to the Zn already bound to the plaques where it would form an insoluble fluorescent precipitate. Although attempts to eliminate or attenuate the staining by incubating the tissue in various acids such as 5% HCl or EDTA were all unsuccessful, this would be consistent with studies using another fluorescent Zn chelator, TSQ. Staining with this tracer also could not be significantly reduced by treatment with dithizone [5], suggesting the Zn may be too tightly bound to be removed. Also, when comparing the labeling pattern for TSQ with HQ-O, one is struck by the fact that although both unquestionably label amyloid plaques, the respective staining morphologies look fairly different, with the TSQ showing much lower resolution and detail. One interpretation could be that the tracers are not staining the same components of the plaque. However, another interpretation could be that they are both staining the same Zn containing plaque components, but the TSQ staining being at a much lower resolution than seen with HQ-O staining. This would be consistent with the relatively low resolution illustration for Congo Red labeling provided in the same publication, which could be attributed to the fact that the study used unfixed tissue.

Regardless of its underlying mechanism of binding, HQ-O has practical applications for the histological localization of amyloid plaques in the brains of both rodent models of Alzheimer's disease and postmortem human AD patients. Attributes include the simplicity of the stain, since the staining procedure is essentially just a matter of incubating the tissue sections in an aqueous HQ-O solution. Another attribute includes the relative speed of the method, although the specific staining times required depend on several factors including temperature, tissue section thickness and mode of tissue processing. Presumably the tracer binding involves an endothermic reaction since heating the solution from room temperature to 60^{o}C results in reducing the staining time by at least one quarter. The reason why the paraffin-processed tissue requires less than a quarter of the staining time needed for the frozen-cut sections probably is due to the use of thinner tissue sections, the paraffin-embedding process or both. Other positive attributes of the novel probe are related to the quality of the stain itself, including its high degree of brightness, contrast and resolution. Another advantage relates to the fact that the tracer is only visible under blue light excita-

tion and its emission will not bleed through when using UV or green light excitation. This, combined with its inherent stability, makes it ideal for multiple labeling studies. The stability of the labeling also translates to a lack of photo bleaching during examination as well as long term archival permanence.

When comparing the pros and cons of diverse histochemical stains for localizing amyloid plaques, one sees both similarities and differences in their staining properties. The traditional histological dyes such as Congo Red or Thioflavin S typically stain plaques in their entirety, albeit at relatively low brightness, contrast and resolution. A second limitation of Thioflavin S is its broad emission spectrum, making it of limited value for multiple labeling studies. Aβ immunofluorescence reveals the distribution of Aβ aggregates with conspicuous labeling of plaque cores and the proximal neurites, but reduced labeling of distal neurites and diffuse plaques, possibly suggesting that the Aβ immunofluorescent method may be staining a substructure of the entire plaque. This would be consistent with the fact that amyloid plaques also contain many biomolecules in addition to the beta-pleated form of Aβ aggregates. The presence of non-Aβ components in amyloid plaques is far too numerous to fully discuss here, although a partial list of compounds includes multivalent metals, enzymes, neurites and gangliosides. The qualitative differences in vascular staining between HQ-O and antibodies to Aβ are much more pronounced and unexpected. One possibility for this difference is that in addition to labeling a thin layer of Aβ positive material on the edges of select vessels, the HQ-O also conspicuously labels the interrupted bands or striations and the more globular looking structures encircling the same Aβ positive blood vessels. The significance of these HQ-O positive vascular structures to the progression of the disease remains to be elucidated. Although the exact mechanism whereby HQ-O results in the labeling of amyloid plaques has not been resolved definitively, the following hypothesis is consistent with all present observations: Aβ aggregates, presumably due to the relative position of their positive and negative charged amino acids, chelate extracellular Zn which can then itself form insoluble but fluorescent HQ-O chelates. The role Zn plays in the pathology of Alzheimer's disease is both interesting and controversial. For example, inorganic Zn has been shown to significantly increase the rate of Aβ aggregation in situ [14]. Furthermore, it has been reported that the incubation of AD patient autopsy brain tissue sections in solutions containing certain chelators resulted in the removal of plaques, which would be consistent with the authors' theory that certain metals such as Zn are involved in the generation and configuration of Aβ aggregates [12]. Since metal chelation typically involves the coordination of both positive and negative charges around a multivalent metal, it is easy to see how the high composition of both positively and negatively charged amino acids in Aβ (one Aβ molecule contains 6 negatively and 6 positively charged amino acids) could bind Zn. The bound Zn could then serve to link together adjacent Aβ molecules, while chelation could potentially reverse this process by metal removal.

CONCLUSION

In summary, this novel use of HQ-O for the localization of amyloid plaques in brain tissue sections should be applicable to a variety of studies investigating the diagnosis, understanding and potential treatments for Alzheimer's disease. Advantages of the method include speed, simplicity, water solubility, narrow excitation and emission profiles, stability, brightness, specificity, contrast and resolution. The affinity HQ-O exhibits for amyloid plaques raises the possibility that related compounds could be of therapeutic value in the treatment of AD. Although HQ-O itself would presumably not have clinical applications because of its limited blood brain barrier permeability and significant nephrotoxicity, its halogenated derivatives such as cliquinol have been shown to reduce the amyloid plaque burden by about half in APP-PS1 mice [15, 16], although high doses in humans have been reported to result in subacute myleoptic neuropathy [17]. Promising as Zn chelation may be for the treatment of Alzheimer's disease, there are inherent limitations since Zn is a necessary trace metal which is essential for numerous functions throughout the body and the brain. Therefore, a good therapeutic target may be the Zn transporters that control the import (ZIP), and export (ZNT), of this metal into and out of the brain and its cells [18].

There are several possible follow-up studies that could be informative. One such study would be to resolve unequivocally the molecular binding site of HQ-O to amyloid plaques and also confirm that the process is Zn dependent. Another useful follow-up study would be to determine the exact binding constant of the tracer for its target. It should also be interesting to unequivocally determine if HQ-O can label intraneuronal neurofibrillary tangles like the other stains for amyloid plaques, including Thioflavin S and various bis-styryl-benzene tracers, or if it does not share this staining property, possibly due to an absence of Zn in the NFTs. It would also be of significant value to resolve whether the presence of elevated Zn levels in the plaques of the brains of Alzheimer's disease patients represents a cause or an effect of the disease process.

DISCLAIMER

This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the ethic committee at the National Center for Toxicological Research/FDA, including the NCTR Office of Research, the Regulatory Compliance and Risk Management Director and the FDA Research Involving Human Subjects Committee (RIHSC), USA.

HUMAN AND ANIMAL RIGHTS

Not Applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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