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Staining of Amyloid Beta (Abeta) Using (Immuno) Histochemical Techniques and Abeta42 Specific Peptides

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1. Introduction

In the elderly, Alzheimer's disease (AD) is the most common form of dementia (Hebert et al., 2003). The two pathologies that characterize the disease are the presence of large numbers of intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques in the brain (e.g., Braak and Braak, 1991; 1998; Selkoe, 2001). Neurofibrillary tangles consist of hyperphosphorylated, twisted filaments of the cytoskeletal protein tau (e.g., Duff, 2006), whereas plaques are primarily made up of amyloid β(Aβ [Selkoe, 2001; Dickson and Vickers, 2002]), a 39-43 amino acid long peptide derived from the proteolytic processing of the amyloid precursor protein (APP [Selkoe, 2001; Vetrivel and Thinakaran, 2006]). When APP is sequentially cleaved by the β-secretase and γ-secretase, one of the resulting breakdown product is Aβ, in contrast, initial cleavage by α-secretase (in the middle of the Aβ sequence) leads to production of $APP_s\alpha$ and the C83 peptide (Selkoe, 2001).
Most cases of AD are sporadic, however approximately 5 % of AD cases are familial (Price and Sisodia, 1995; Selkoe, 2001), these cases are related to mutations in the genes for APP, and presenilin 1 and 2 (PS1 and PS2 [Price and Sisodia, 1995; Hardy, 1997; Selkoe, 2001]). Transgenic mice expressing mutated human AD genes offer a powerful model to study the role of Aβ in the development of pathology (e.g., Duff and Suleman, 2004; McGowan et al, 2006). The present study employs three lines of transgenic mice expressing both human APPswe and/or PS1 mutations. These lines of mice develop elevated levels of Aβ42 at different ages, and at different locations (Van Groen et al., 2005; Wang et al., 2003).

2. Materials and methods

2.1 Animals

Two lines of APP and PS1 single and double transgenic mice (**AP/PS**) were used in the present study. The first line of mice was generated from matings between APPswe and

HuPS1-A246E transgenic mice, this mouse line was originally produced at the Johns Hopkins University (Borchelt et al., 1996), and was bred locally on a C57BL/6J background. The second line of APP/PS1 mice was the APPswe+PS1Δ9 line, originally produced at the Johns Hopkins University (Jankowsky et al., 2001), we acquired these mice from JAX at the age of six weeks. The animals were housed 4/cage in our facility; in a controlled environment (temperature 22°C, humidity 50-60%, light from 07:00-19:00), with food and water were available *ad libitum*. All procedures were conducted in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

2.2 Peptides

In short, a mirror image phage display approach was used to identify novel and highly specific ligands for Alzheimer's disease amyloid peptide Aβ1-42 (Wiesehan et al., 2003). In short, a randomized 12-mer peptide library presented on M13 phages was screened for peptides with binding affinity for the mirror image of Aβ1-42 (Wiesehan et al., 2003). After four rounds of selection and amplification the peptides were enriched with a dominating consensus sequence. The mirror image of the most representative peptide (i.e., D1) was shown to bind Aβ1-42 with a dissociation constant in the submicromolar range (Wiesehan et al., 2003). The D2 and D3 peptides come from two other phage display selections against Aβ42. The D1 peptide has a higher affinity for Aβ42 monomers, D2 has a low specificity, whereas D3 has a high affinity for Aβ42 oligomers (Funke et al, 2010). To study the binding characteristics of the D-peptides in more detail, an L-peptide version of the D1-peptide was made, and a scrambled (sequence) peptide of similar length was also made. In the binding experiments the peptides that were used had been conjugated with a FITC molecule for visualization purposes, except in a few experiments. In those experiments D1 conjugated with other fluorophores were tested to study the interaction of the fluorescent moiety with binding characteristics of the D1 peptide.

2.3 Histopathological techniques

In short, mice were anesthetized, transcardially perfused with saline followed by 4% paraformaldehyde and the brains were removed from the skull. After postfixation (4h) and cryoprotection (24h in 30% sucrose), six series (1 in 6) of coronal sections were cut through the brain. The first series of sections was mounted unstained, and the second, third and fourth series were stained immunohistochemically according to published protocols (Kadish et al., 2002; Van Groen et al., 2006) the other two series were stored in at -20°C in antifreeze for future analysis. One half of the second series was stained for human Aβ using the W0-2 antibody (mouse anti-human $A\beta_{4-9}$; Ida et al., 1996), the other half of the second series was stained for mouse Aβ (rabbit anti-rodent Aβ; Covance; Van Groen et al, 2006). The first half of the third series was stained for Aβ40 (mouse anti-Aβ40, Covance) the other half for Aβ42 (mouse anti-Aβ42; Covance). In some animals, one half of the fourth series was stained for GFAP (mouse anti-GFAP; Sigma), whereas the other half was stained for CD11b (rat anti-mouseCD11b; Serotec), a marker of microglia, to analyze inflammation in the brain. Some of these sections were double stained with either Congo red, thioflavine S or thiazine red to visualise β sheets, i.e., Aβ plaque cores in our material, in a few animals methoxy-X04 (Klunk et al., 2002) was infused during the perfusion to label all Aβ in the brain. The sections destined for immunohistochemical Aβ staining were pretreated for 30 min with hot (85°C) citrate buffer. The series of sections were transferred to a solution containing the primary antibody (W0-2, mouse monoclonal), this solution consists of TBS with 0.5 % Triton X-100 added (TBS-T). Following incubation in this solution for 18 h on a shaker table at room temperature (20°C) in the dark, the sections were rinsed three times in TBS-T and transferred to the solution containing the appropriate secondary antibody (goat anti-mouse*biotin; Sigma). After two hours, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin® (Sigma), following rinsing the sections were incubated for approximately 3 min with Ni-enhanced DAB (Kadish et al., 2002). In a small number of sections, the Aβ deposits were double labeled for Aβ40 and Aβ42 using fluorescent secondary antibodies. All stained sections were mounted on slides and coverslipped.

Histochemical stains. Thioflavine-S staining (Guntern et al, 1982), sections are mounted on gelatinized slides, and air-dried. When dry the slides are immersed in distilled water for 5 min twice to rehydrate, then they are immersed in the Thioflavine-S solution (1g of Thioflavine-S in 100 ml distilled water) for 20 min. Then the slides are rinsed quickly twice in distilled water and air dried, when dry they are rinsed in xylene, and coverslipped with DPX. The staining procedure is performed in the dark, i.e., the solution of Thioflavine-S is kept in a opaque container, similarly the staining procedure is done in opaque containers. Thioflavine-T staining (Morimatsu et al, 1975), sections are mounted on gelatinized slides, and air-dried. When dry the slides are immersed in distilled water for 5 min twice to rehydrate, then they are immersed in the Thioflavine-T solution (1g of Thioflavine-T in 100 ml distilled water) for 20 min. Then the slides are rinsed quickly twice in distilled water and air dried, when dry they are coverslipped with DPX. The staining procedure is performed in the dark, i.e., the solution of Thioflavine-T is kept in a opaque container, similarly the staining procedure is done in opaque containers. Congo red staining (Glenner, 1981), slides with brain sections are put overnight in 4% paraformaldehyde solution, the next day slides are rinsed twice with distilled water for 5 min, then put in the pretreatment solution (a 80% ethanol saturated NaCl solution with 1% sodium hydroxide added [1ml per 100 ml) for 20 min. Then the slides are transferred to the Congo red staining solution (a 80% ethanol saturated NaCl solution with 0.2 g Congo red per 100 ml) for 25 min, the slides are rinsed quickly in distilled water and air dried. Thiazine red (Uchihara et al, 2000) slides with sections are rinsed in distilled water for 5 min, and put in the Thiazine red solution (0.1g Thiazine red in 300 ml 0.001 M Naphosphate buffer, pH 7.4) for 15 min. Then the slides are rinsed quickly twice in distilled water and air dried, when dry they are coverslipped with DPX. Methoxy-XO4 staining, slides with sections are rinsed in distilled water for 5 min, and put in the Methoxy-XO4 solution (3.3 mg Methoxy-XO4 in 100 ml 40% ethanol/60%distilled water at pH 10.00) for 10 min. Then the slides are rinsed quickly twice in distilled water and air dried, when dry they are coverslipped with DPX.

3. Results

3.1 In vitro staining

The staining of sections of paraformaldehyde fixed brains of **AP/PS** mice with histochemical methods revealed that all methods that are used for staining amyloid also stain amyloid β and stained all dense Aβ deposits, i.e., plaques (Figure 1). However, it should be noted that in the sections of the AP/PS mice that have large amounts of diffuse Aβ deposits, most these deposits were not stained. Staining intensity of the Aβ deposits was directly related to the method that was used, the solutions that we used contained the optimal concentrations of

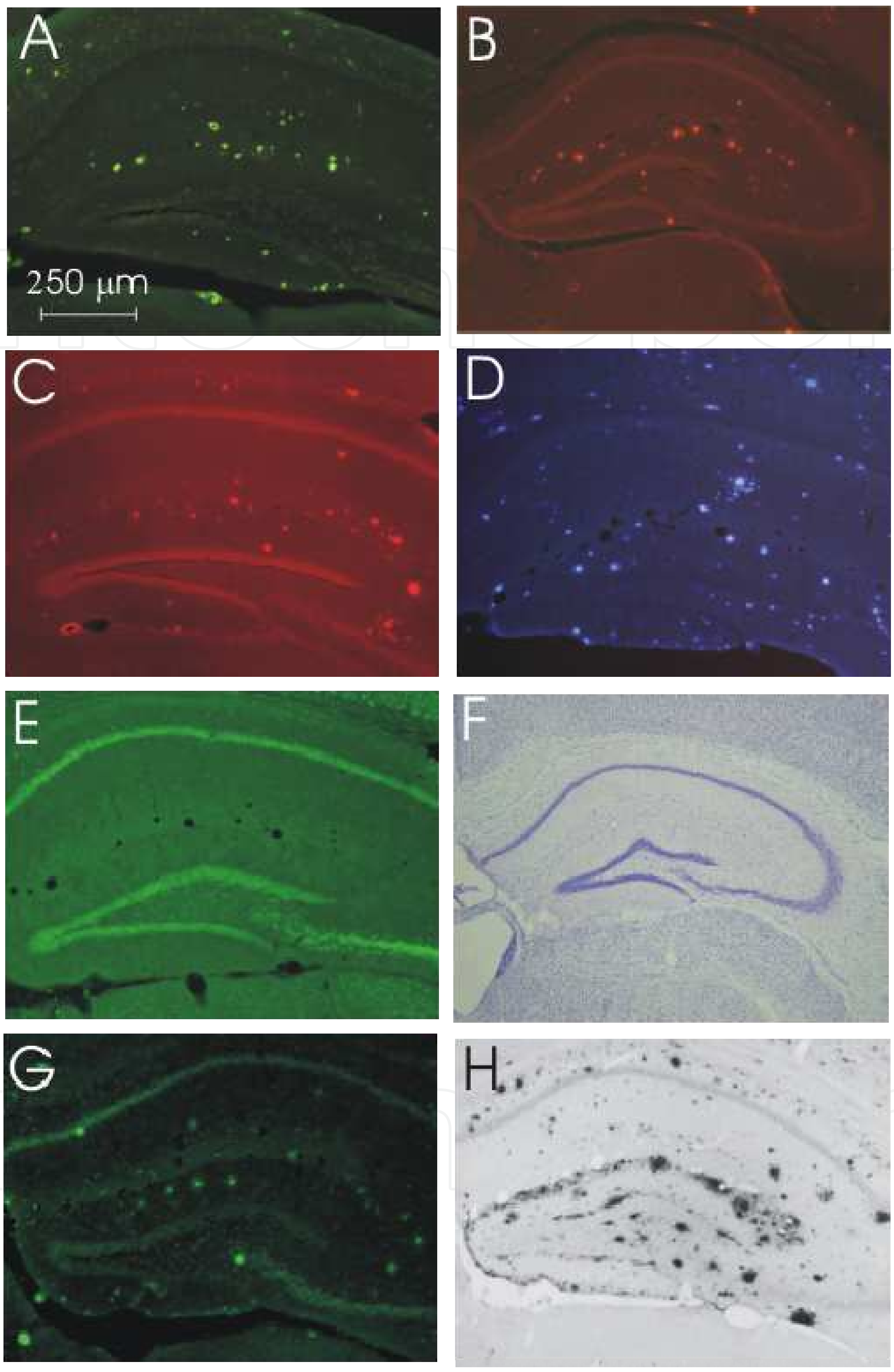

Fig. 1. Eight photomicrographs of coronal sections of the hippocampal formation of a Tg AD model mouse brain. **A**, section stained with thioflavine-S; **B**, section stained with Congo red; **C**, section stained with thiazine red; **D**, section stained with methoxy-X0; **E**, section stained with thioflavine-T; **F**, section stained with cresyl violet (Nissl stain), **G**, section stained with D3, and **H**, section stained for Aβ .

dye for these sections. They have the highest concentration that stains optimally in the shortest time. Longer time periods increased the background staining and did not improve staining quality (i.e., the signal/noise ratio; not illustrated).
The staining of sections of paraformaldehyde fixed brain sections of **AP/PS** mice with the three D-peptides revealed that all peptides (i.e., D1-D3) bound to all dense Aβ deposits, i.e., plaques (Figure 2). However, it should be noted that in the sections of the AP/PS mice that have large amounts of diffuse Aβ deposits, these deposits were not stained (Figure 1). Staining intensity of the Aβ deposits was directly related to both the D-peptide concentration that was tested (0.01, 0.001 and 0.0001 mg/ml), with the highest concentration staining optimally in the shortest time. At the highest concentration the optimal staining time was less than 5 min (i.e., with the best signal/noise ratio), whereas at the lowest

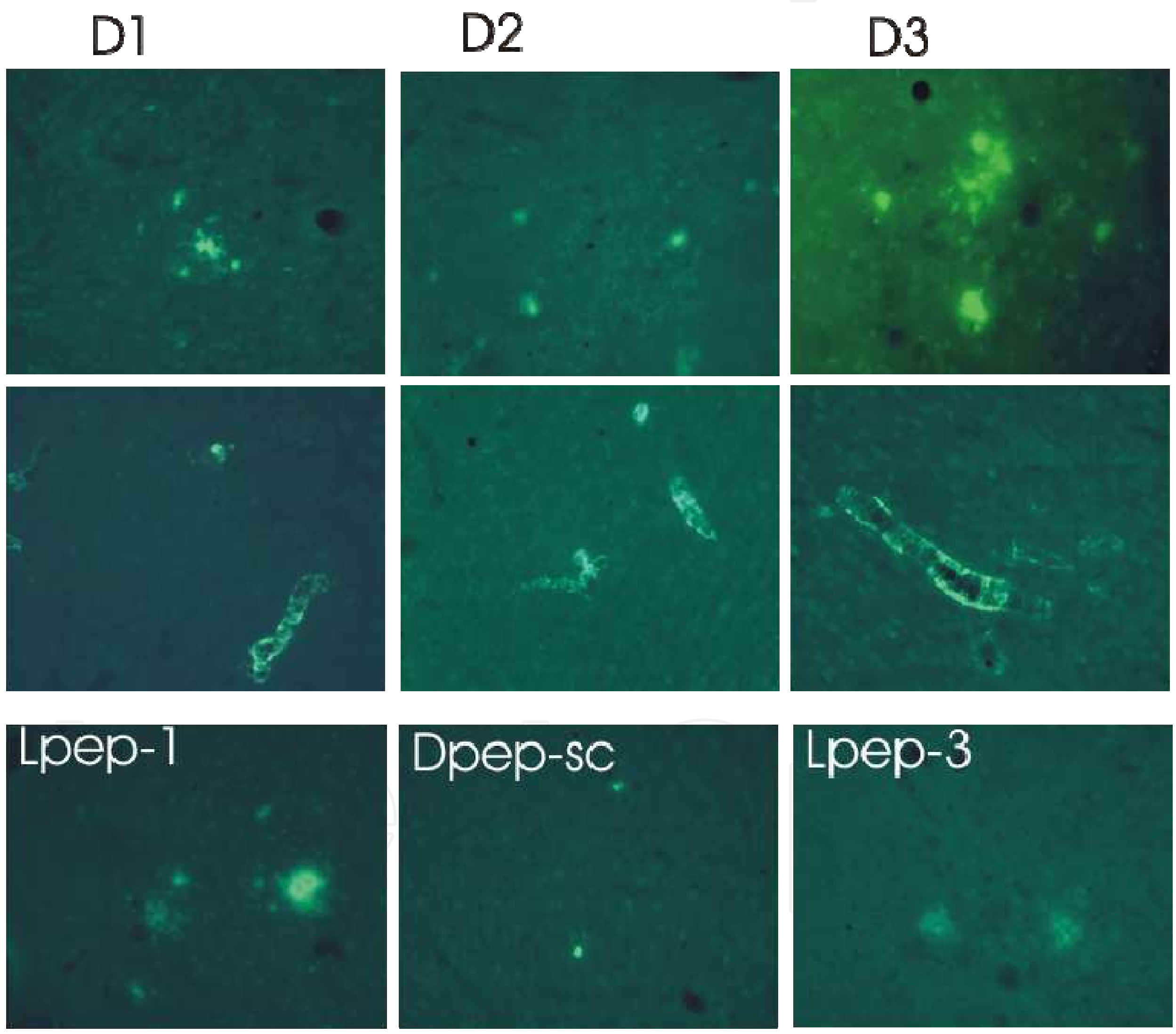

Fig. 2. Nine high power photomicrographs of adjacent coronal sections through the parietal cortex of an 18-month-old APP/PS1 mouse. The top six photomicrographs demonstrate the typical staining of plaques and blood vessels with respectively, D1, D2, and D3. The lower 3 photomicrographs show the typical staining of L-D1, sc-D1, and L3, respectively. Please note the lack of staining by the scrambled D-peptide (sc-D1), arrowhead indicates plaque core

concentration (0.0001 mg/ml) the time was more than 6 hours. Longer time periods increased the background staining and did not improve staining quality (i.e., the signal/noise ratio). It should be noted that with the lower concentrations, and appropriate longer staining time, the amount of non-specific staining (i.e., background) was significantly decreased. Similarly, post-staining rinsing of the stained sections in buffer decreased the amount of background staining, but even 24 h washing in buffer did not change the intensity of the specific binding.

Further, in general, the D3 peptide gave rise to slightly higher levels of specific staining than the D1 peptide. Further, very little Aβ was stained in the **AP** mouse brain sections, but the APP mutation in these mice is in the Aβ sequence and thus leads to Aβ proteins with a different amino acid sequence.

Comparison of the D-peptide stainings with the amyloid staining with Aβ40 and Aβ42 antibodies of sections of the **AP/PS** and **AP/PSΔ** mouse brains showed that there was nearly complete overlap between the location of Aβ42 staining (i.e., plaques) and the D-peptide binding (Figure 3; van Groen et al, 2009). Similarly, comparison of the immunohistochemical staining of the adjacent sections for human amyloid β (with the W0-2 antibody which is specific for human $A\beta_{4-10}$ sequence) showed that there was complete overlap between the location of dense Aβ staining (i.e., plaques) and the binding of the three D-peptides (D1-D3), but that the diffuse amyloid β deposits were not stained, neither in the hippocampus or in the cortex (Figure 1).

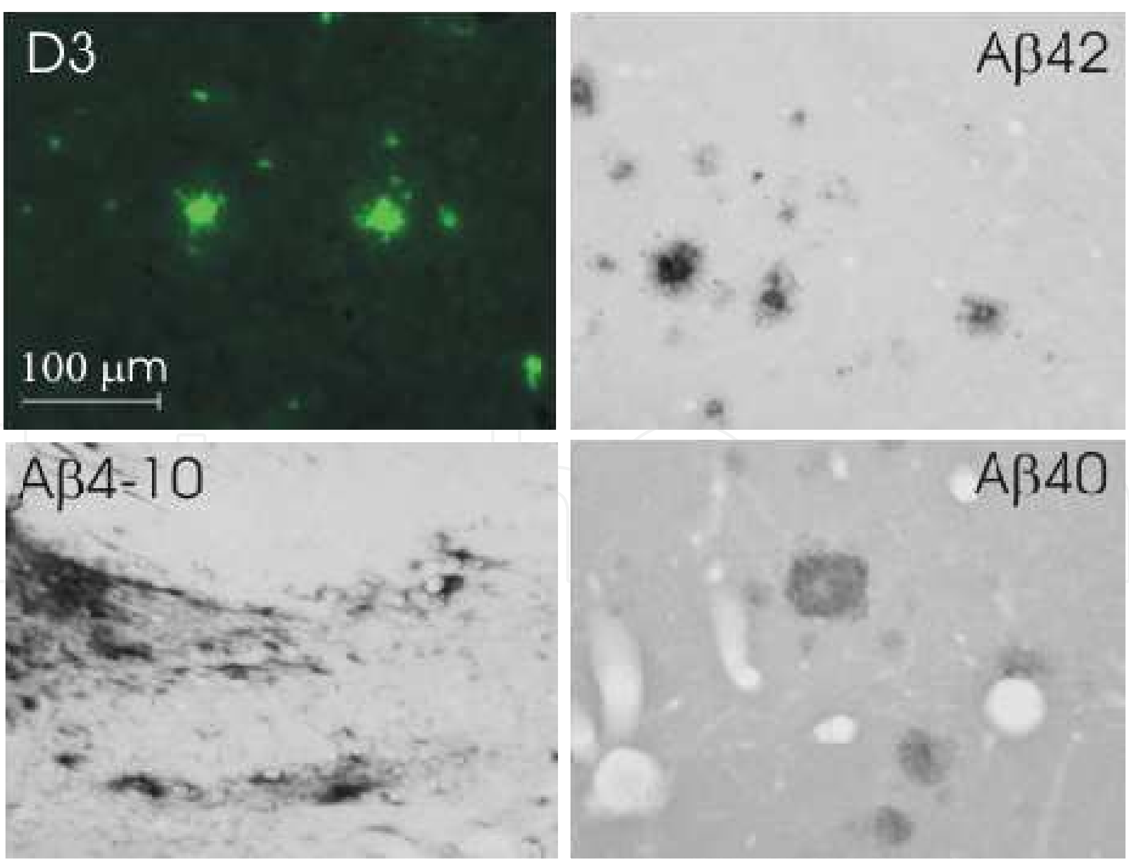

Fig. 3. Four high power photomicrographs of coronal sections through the hippocampus of a Tg AD model mouse. Sections were stained for D3, Aβ40, Aβ42, and Aβ, respectively. Please note the correspondence between D3 and Aβ42 stained sections.

Immunohistochemical staining for Aβ40 or Aβ42 of the sections that were adjacent to the sections stained with the D-peptides showed that the distribution of the D-peptides corresponded more closely to the distribution of Aβ42 than to the distribution of Aβ40 labeling (Figure 3). Both Aβ42 and D3 stain predominantly the core of the plaques, whereas Aβ40 stains mainly the outside of the plaques, i.e., the rim (Figure 3). Sections that were double-stained for both Aβ42 and the D-peptide demonstrated a total overlap between the site of dense Aβ42 deposits and the location of D1 and D3 (not illustrated). Furthermore, staining with β-sheet markers such as thioflavine-S, Congo red, or thiazine red revealed that all Aβ deposits with a β-sheet positive core also stained with the D-peptides (Figure 1). The staining of fixed brain sections of Tg AD model mice (APP-PS) from different ages revealed that in old mice (over 18 months of age), when blood vessel walls contain some Aβ42 deposits, they were stained by the D1 and D3 peptides (Figure 2), but not by the D2 peptide. It should be noted that at earlier ages only Aβ40 is found in the blood vessel wall, and that at that age no labeling with D-peptides is present. Labeling of sections of non-transgenic littermates or control animals did not show any staining at any place in the brain.

To analyze further the binding characteristics of the D-peptides in more detail, an L-peptide version of the D1-peptide (i.e., L-D1) was also tested, likewise a scrambled (sequence) peptide of similar amino acid length (i.e, sc-D1) was tested (Table 1). We used both 0.001 and 0.0001 mg/ml concentrations of L-D1 and sc-D1 on fixed brain sections of old (18 months of age) **AP/PS** mice. L-D1 bound quite similarly to Aβ deposits compared to its D-peptide analog, i.e., it labeled dense Aβ deposits, but not diffuse deposits, and it lightly labeled blood vessel walls with showed Aβ deposits. It should be noted that similar amounts of the L-peptide showed less labeling compared to the D1 peptide. Finally, in contrast to both the L- and D-peptide, the sc-D1 peptide showed significantly reduced binding to Aβ at any type of amyloid deposit (Figure 3). Further, we tested a peptide that was generated against the L-form of Aβ42, i.e., L3, this peptide showed very similar characteristics to the D3 peptide.

In these binding experiments all peptides that were used had been conjugated with a FITC molecule for visualization purposes, therefore we tested in a final set of experiments whether the D1 conjugated with different fluorophores would show distinct binding characteristics, i.e., study the interaction of the fluorescent moiety with the Aβ binding. The data show that no differences in specific binding are present at the 0.001 and 0.0001 mg/ml concentrations between these D-peptides (Figure 4). The D1 conjugated to Oregon green, which is similar in size and charge to FITC, bound Aβ42 similar to the D1*FITC, but the D1*Bodipy (Bodipy is smaller and more polar than FITC) showed significantly increased background staining (Figure 4).

Peptide	**Sequence**	**Description**
D1	qshyrhispaqv	Dominating sequence selection 1, Target: D-Aβ
L-D1	QSHYRHISPAQV	L-enantiomer of D1
sc-D1	hsspqivhqayr	Scrambled D1
D2	giswqqshhlva	Dominating sequence selection 2, Target: D- Aβ
D3	rprtrlhthrnr	Dominating sequence selection 3, Target: D- Aβ
L3	LRMMLQIKRIPR	Dominating sequence selection 3, Target: L- Aβ

Table 1. Showing the nomenclature and amino acid sequence of the peptides used in this study.

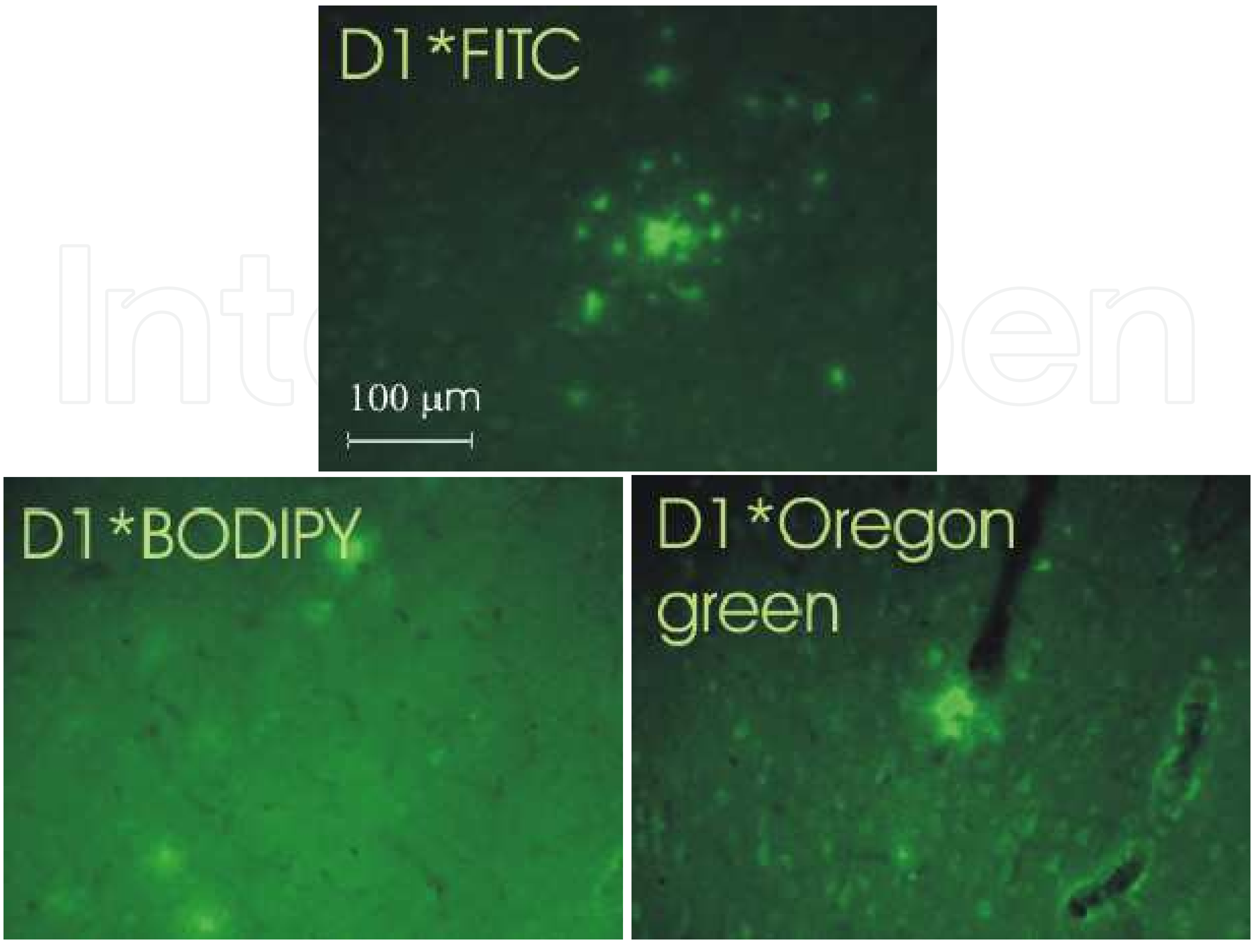

Fig. 4. Three photomicrographs of coronal sections of the parietal cortex of an 18-months-old APP/PS1 mouse stained with the D1 peptide conjugated with different fluorophores, showing staining with D1*FITC, D1*Bodipy, and D1*Oregon green, respectively. Please note the increased background staining with the D1*Bodipy, also note similarity of staining between D1*FITC and D1*Oregon green.

4. Discussion

In this study we compared the staining characteristics of three small D-amino acid peptides (i.e., D1, D2, and D3) that were designed to specifically bind Aβ42 (D1, Wiesehan and Willbold, 2003; Wiesehan et al., 2003; D3,) with the two traditional histochemical methods for amyloid (thioflavine-S and Congo red) and two newer techniques. We examined the labeling of Aβ deposits in Tg AD model mouse brain, in the hippocampus, cortex and in blood vessel walls. The data demonstrate that all dense Aβ deposits (plaques) are labeled with the D-peptides, but not diffuse deposits. This corresponds to the distribution of the Aβ staining in the brain when it is labeled with Aβ42 specific antibodies. Finally, the binding of the D-peptides corresponds closely to the localization of Aβ42 in the brain, more closely than to the localization of Aβ40.

Similarly, in brain tissue sections derived from AD patients, amyloid β plaques and leptomeningeal vessels containing Aβ are stained positively with the fluorescence-labeled derivative of D1 (Wiesehan et al, 2003). In contrast, fibrillar deposits derived from other amyloidosis are not labeled by D1 (Wiesehan et al, 2003). It should be noted that none of the D-peptides showed any binding to Aβ deposits in the brains of mice which express the APPswe/dutch/iowa mutation van Groen et al, 2009). This is to be expected since the structure of the Aβ peptide with these mutations (i.e., the Dutch and Iowa mutations) is predicted to be different from the "normal" Aβ peptide (Demeester et al., 2001; Kumar-Singh et al., 2002; Tsubuki et al., 2003; Watson et al., 1999). It should be noted that these mutations are in the Aβ peptide sequence of APP, in contrast to the Swedish mutation (Selkoe, 2001).

The data demonstrate that none of the three D-peptides binds to diffuse Aβ deposits, whereas they do bind to dense Aβ deposits, i.e., plaques. Earlier we have shown that the diffuse Aβ deposits do not stain with thioflavine S, Congo red or thiazine red, whereas the core of plaques does. Furthermore, the diffuse deposits consist primarily of N-terminal fragments of Aβ, they contain some Aβ40 but do not contain stainable amounts of Aβ42 (Van Groen et al., 2003), in contrast to plaques that consist of significant amounts of both Aβ40 and Aβ42. We have suggested earlier that the diffuse deposits consist of Aβ that has a different length (and structure) from the Aβ42 and Aβ40 that is present in plaques, even if Aβ fibrils are present in the diffuse deposits (Van Groen et al., 2003). Together these data indicate that the D-peptides bind very specifically to only Aβ42.

Furthermore, it has been shown that Aβ42 is actively taken up by astrocytes and microglia (Nagele et al., 2003; Rogers and Lue, 2001. In contrast, surprisingly, no D-peptides are visible in astrocytes and microglia, the phagocytosing cells in the brain (Rogers et al, 2002). Activated microglial cells are present in the brains of AD model mice but these cells never show any presence of intracellular Aβ (e.g., Stalder et al., 2003; but see Paresce et al., 1996).

We have used these peptides to treat AD model mice and we have shown that a brain infusion with D3 significantly reduces pathology and cognitive deficits in AD model mice (van Groen et al, 2009, Funke et al, 2011). In contrast D1 infusion does not improve cognition (van Groen et al, 2009). Similarly it has been demonstrated that Congo red (Inouye and Kirschner, 2005, Lee, 2002) and thioflavine-S improve pathology (Alavez et al, 2011).

Together, we have demonstrated that 1) D-peptides that specifically bind to Aβ42 and, 2) that the D-peptides staining is similar, but more specific, to most traditional histochemical amyloid staining methods. Thus, our data strongly suggest that these novel and highly specific Abeta42 ligands have potential application(s) in the diagnosis and therapy of Alzheimer's disease (Masters and Beyreuther, 2006; Monaco et al., 2006), especially since these D-peptides are much more resistant to proteolysis than natural L-peptides.

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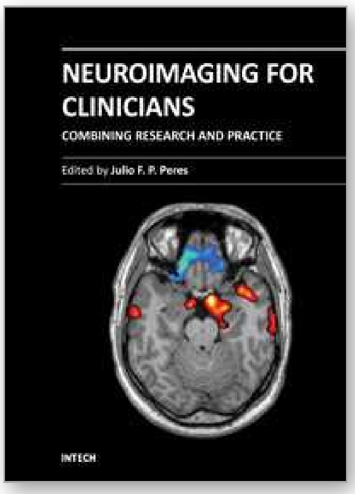

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Neuroimaging for clinicians sourced 19 chapters from some of the world's top brain-imaging researchers and clinicians to provide a timely review of the state of the art in neuroimaging, covering radiology, neurology, psychiatry, psychology, and geriatrics. Contributors from China, Brazil, France, Germany, Italy, Japan, Macedonia, Poland, Spain, South Africa, and the United States of America have collaborated enthusiastically and efficiently to create this reader-friendly but comprehensive work covering the diagnosis, pathophysiology, and effective treatment of several common health conditions, with many explanatory figures, tables and boxes to enhance legibility and make the book clinically useful. Countless hours have gone into writing these chapters, and our profound appreciation is in order for their consistent advice on the use of neuroimaging in diagnostic work-ups for conditions such as acute stroke, cell biology, ciliopathies, cognitive integration, dementia and other amnestic disorders, Post-Traumatic Stress Disorder, and many more