

The Fluorescent Congo Red Derivative, (*Trans, Trans*)–1-Bromo-2,5-Bis-(3-Hydroxycarbonyl-4-Hydroxy)Styrylbenzene (BSB), Labels Diverse β -Pleated Sheet Structures in Postmortem Human Neurodegenerative Disease Brains

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A novel Congo red-derived fluorescent probe (*trans, trans*), –1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (BSB) that binds to amyloid plaques of postmortem Alzheimer's disease brains and in transgenic mouse brains *in vivo* was designed as a prototype imaging agent for Alzheimer's disease. In the current study, we used BSB to probe postmortem tissues from patients with various neurodegenerative diseases with diagnostic lesions characterized by fibrillar intra- or extracellular lesions and compared these results with standard histochemical dyes such as thioflavin S and immunohistochemical stains specific for the same lesions. These data show that BSB binds not only to extracellular amyloid β protein, but also many intracellular lesions composed of abnormal tau and synuclein proteins and suggests that radioiodinated BSB derivatives or related ligands may be useful imaging agents to monitor diverse amyloids *in vivo*. (Am J Pathol 2001, 159:937–943)

Alzheimer's disease (AD) is the most frequent cause of dementia in the elderly. At this time there is no specific test available for a definite diagnosis at an early stage of the disease. Thus, efforts have been made to develop probes that bind AD amyloid plaques in brain for use as *in vivo* imaging agents.^{1–3} For example, a Congo red (CR)-derived fluorescent probe, X-34, was developed

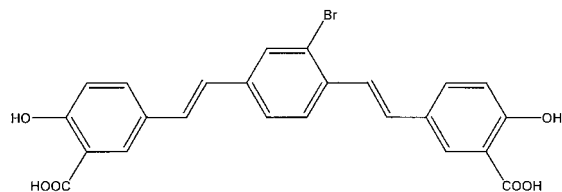
recently that binds to AD brain lesions in tissue sections and has several desirable characteristics required for an *in vivo* amyloid imaging agent.⁴ Another CR derived compound that binds to AD neurofibrillary tangles (NFTs), neuropil threads (NTs) and amyloid β -peptide ($A\beta$) deposits in plaques as well as to AD-like amyloid plaques in the brains of a transgenic mouse model of AD amyloidosis after *in vivo* injection.¹ However, this probe, (*trans, trans*), –1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene or BSB (Figure 1), is not a specific ligand for fibrillar $A\beta$ peptides, the major constituents of plaques in AD brains, since it also binds to NFTs and NTs both of which are composed of amyloid-like paired helical filaments (PHF) formed by hyperphosphorylated tau proteins.¹

Since there are two major species of $A\beta$ in AD amyloid plaques, i.e., peptides ending at amino acid 40 ($A\beta_{x-40}$) or 42 ($A\beta_{x-42}$), the current study investigated whether BSB binds to all or a subset of $A\beta$ plaques and whether it binds preferentially to plaques consisting mainly of either $A\beta_{x-40}$ or $A\beta_{x-42}$.⁵ Further, since NFTs in AD are composed of six tau isoforms in about equal proportions,^{6–10} but in other neurodegenerative diseases abnormal accumulations of tau proteins in neurons and/or glial cells are predominantly composed of either three or four microtubule (MT) binding repeat tau isoforms, we also asked if BSB binds all or a subset of tau inclusions. For instance, NFTs and glial inclusions in progressive supranuclear palsy (PSP), cortical basal degeneration (CBD), and certain tauopathies, i.e., frontotemporal dementia with parkinsonism (FTDP-17) are mainly composed of four repeat tau isoforms while neuronal inclusions in Pick's disease are mostly composed of three repeat tau.¹¹ Finally, we investigated whether BSB binds to amyloid-like lesions containing α -synuclein including

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1-Bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene

Figure 1. Schematic illustration of BSB.

Lewy bodies (LB) in dementia with LB (DLB), Parkinson's disease (PD), and neurodegeneration with brain iron accumulation type 1 (NBIA-1) as well as glial cytoplasmic inclusions (GCI) in multiple system atrophy (MSA).¹² We found that BSB labeled all of the above mentioned lesions although there were quantitative and qualitative differences when compared with thioflavin S (THIOS) and specific immunohistochemical stains for these lesions. Thus, BSB or related derivatives may be exploited as imaging agents for diverse amyloids including lesions formed by $A\beta$, tau, and α -synuclein.

Materials and Methods

Brain tissues from 23 patients were used in this study. Nineteen of these patients had neurodegenerative diseases and one control patient had no neurological disorder. Demographic data including the disease, age, and

gender of these patients are listed in Table 1 in addition to other information on the brain samples. Also listed are the postmortem interval and the fixative used to preserve the tissues. Tissue blocks of interest were removed at the time of autopsy and fixed in either 70% ethanol containing 150 mmol/L sodium chloride or 10% neutral buffered formalin overnight and subsequently embedded in paraffin. Six- μ m-thick serial sections were cut and adjacent sections were stained with BSB, THIOS, or antibodies to $A\beta$, tau, and α -synuclein (Table 2). Immunohistochemistry was performed using ABC Kits (Vector Laboratories, Burlingame, CA) and diaminobenzidine as chromogen according to previously described procedures.^{13;14}

Thioflavin S staining was carried out according to a protocol reported by Guntern et al.¹⁸ BSB staining was performed as described.¹ Briefly, deparaffinized and hydrated tissue sections were immersed in a 0.01% BSB dissolved in 50% ethanol for 30 minutes. Then the sections were rinsed in a saturated aqueous solution of lithium carbonate. Finally, the sections were differentiated in 50% ethanol under microscope control. This process was stopped by immersion in distilled water. The sections were then coated with a thin layer of Vectorshield (Vector Laboratories) before coverslipping. The THIOS and BSB stained sections were viewed in an epifluorescence microscope using a fluorescent filter cube with an excitation filter of 405 nm and an emission filter of 435 nm. A fluorescein isothiocyanate (FITC) filter cube with an excitation filter of 350–390 nm and an emission filter of 530 ± 15 was used for comparison. Electronic images were obtained using a Cool-Snap camera (Biovision, Exton, PA) mounted on a Nikon

Table 1. Demographic Data of Patients Used in this Study

Patient no.	Diagnosis	Age	Gender	PMI (hours)	Fixative	Brain area
1	AD	79	F	7	NBF	Frontal cortex
2	AD	80	F	7	E	Frontal cortex
3	FAD/PS2 (N141I) = Volga Germans	65	F	13.5	E	Frontal cortex
4	DS	33	M	8	E	Frontal cortex
5	DS	54	M	9	E	Frontal cortex
6	FTDP-17/(P301L)	62	M	NA	NBF	Basal ganglia
7	FTDP-17/(exon 10 + 16) = familial progressive subcortical gliosis	48	M	20	E	Cerebellum
8	FTDP-17/(N279) = pallido-ponto-nigral degeneration	50	M	6.5	NBF	Frontal cortex
9	AD/LBv	71	M	9	E	Frontal cortex
10	DLB/PD	85	F	6	E	Cingulate cortex
11	PSP	72	M	10	E	Midbrain
12	PSP	73	M	20.5	E	Midbrain
13	PSP	83	F	24	E	Midbrain
14	Pick's disease	72	M	6.5	NBF	Hippocampus
15	MSA	43	M	20	E	Cerebellum
16	MSA	79	M	16	E	Cerebellum
17	CBD	77	M	NA	NBF	Frontal cortex
18	CBD	64	F	NA	NBF	Frontal cortex
19	NBIA I	29	M	6	E	Hippocampus
20	PDC	63	M	NA	NBF	T, L Spin.c.
21	PDC	64	M	NA	NBF	T, L Spin.c.
22	PDC	72	M	NA	NBF	C Spin.c.
23	Normal control	76	F	13	E	Frontal cortex

Abbreviations: AD/LBv, Alzheimer's disease/Lewy body variant; C, cervical; CBD, cortico-basal degeneration; E, 80% ethanol with 150 mM sodium chloride; F, female; FAD/PS2, familial Alzheimer's disease with a mutation in the PS2 gene; FTDP-17, fronto-temporal dementia with parkinsonism; L, lumbar; M, male; NBF, neutral buffered formalin; PDC, parkinsonism-dementia complex of Guam; PMI, post mortem interval; PSP, progressive supranuclear palsy; T, thoracic.

Table 2. Antibodies and Immunostaining Conditions

Antibody designation	Specificity	Species raised in	Dilution	Pretreatment for tissues fixed in ethanol	Pretreatment for tissues fixed in formalin	Reference no.
17026	Full length tau	Rabbit	1:10000	None	None	15
4G8	A β aa17-24	Mouse	1:10000	None	Formic acid	16
BCO5	A β aa38-42	Mouse	1:20000	Formalin and formic acid	Formic acid	5
BA27	A β aa36-40	Mouse	1:20000	Formalin and formic acid	Formic acid	5
SYN303	Synuclein aa1-89	Mouse	1:1000	None	None	17

Abbreviations: aa, amino acid; A β , amyloid beta protein.

FXA microscope (Optical Apparatus, Inc., Ardmore, PA). These images were printed on a Fuji Pictography 3000 printer (Mid City Camera, Philadelphia, PA).

Results

A β -Positive Lesions

Monoclonal antibody (MAB) 4G8 demonstrated A β plaques in a variety of neurodegenerative diseases: AD,

Down's syndrome (DS), familial AD (FAD), and DLB. The brains of the AD, DS, and FAD patients had the largest amounts of A β plaques. Thus, we chose consecutive tissue sections from the brains of each of these three patients and probed them with MAB 4G8, as well as with two other MAB specific for the x-40 (BA27) and x-42 (BCO5) forms of A β and also with the fluorescent dyes BSB and THIOS. BCO5 immunostained the largest numbers of A β plaques and BA27 the least although there was considerable regional variability in the number of

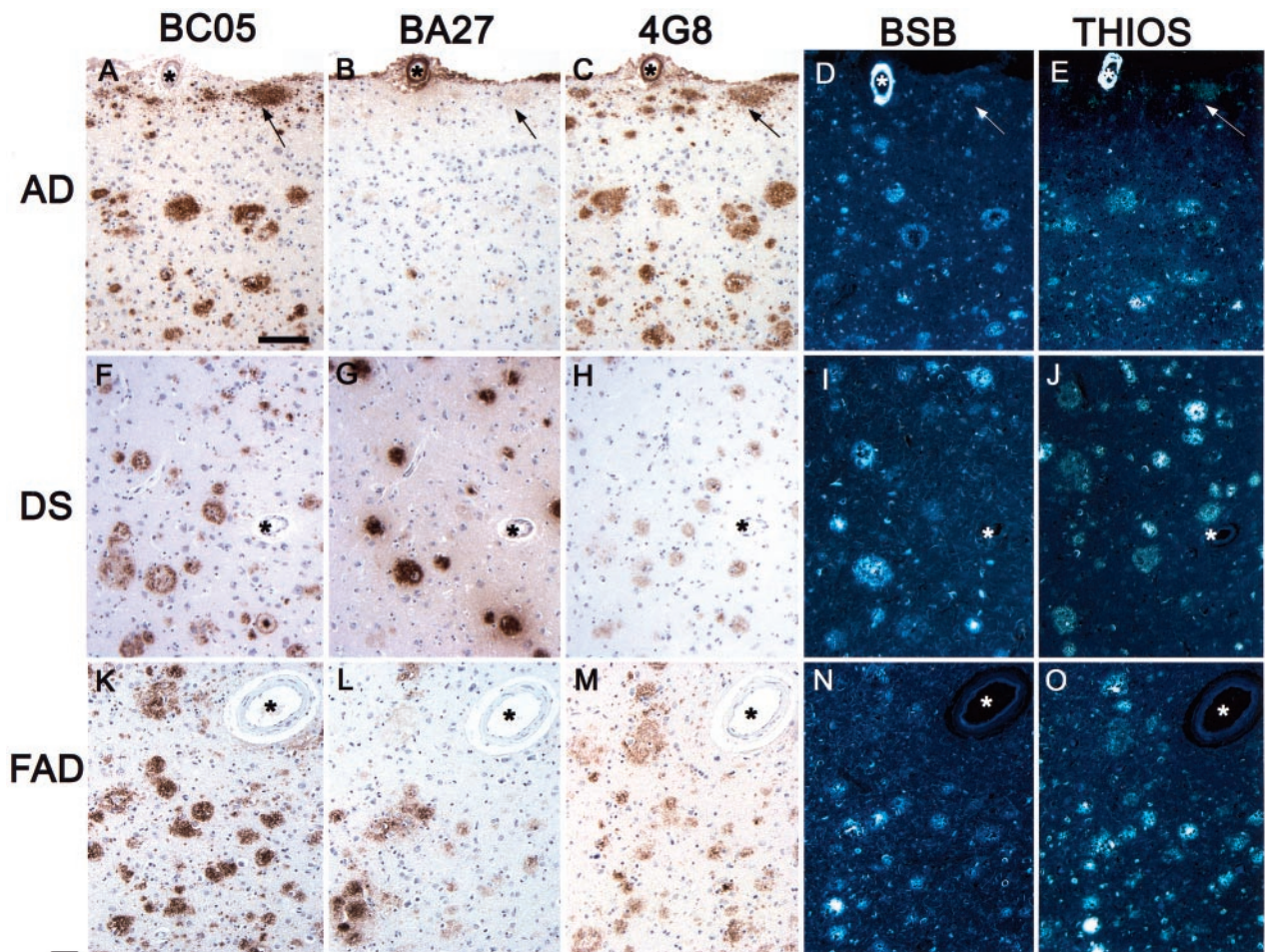


Figure 2. Neurodegenerative lesions composed of A β protein. This figure depicts tissue sections of frontal cortex immunostained with MAB. BCO5, **first column**; BA27, **second column**; 4G8, **third column**, or stained with BSB, **fourth column**; and THIOS, **fifth column**. The first row (A-E) shows an AD case, the second row (F-J) shows a DS case with AD, and the third row (K-M) shows a case of FAD with a mutation in PS2. The **asterisks** indicate the blood vessel that was used as landmark to identify the same area in each set of consecutive sections. The **arrows** point to the same subpial A β deposit. All images were taken at the same magnification. Scale bar, 100 μ m.

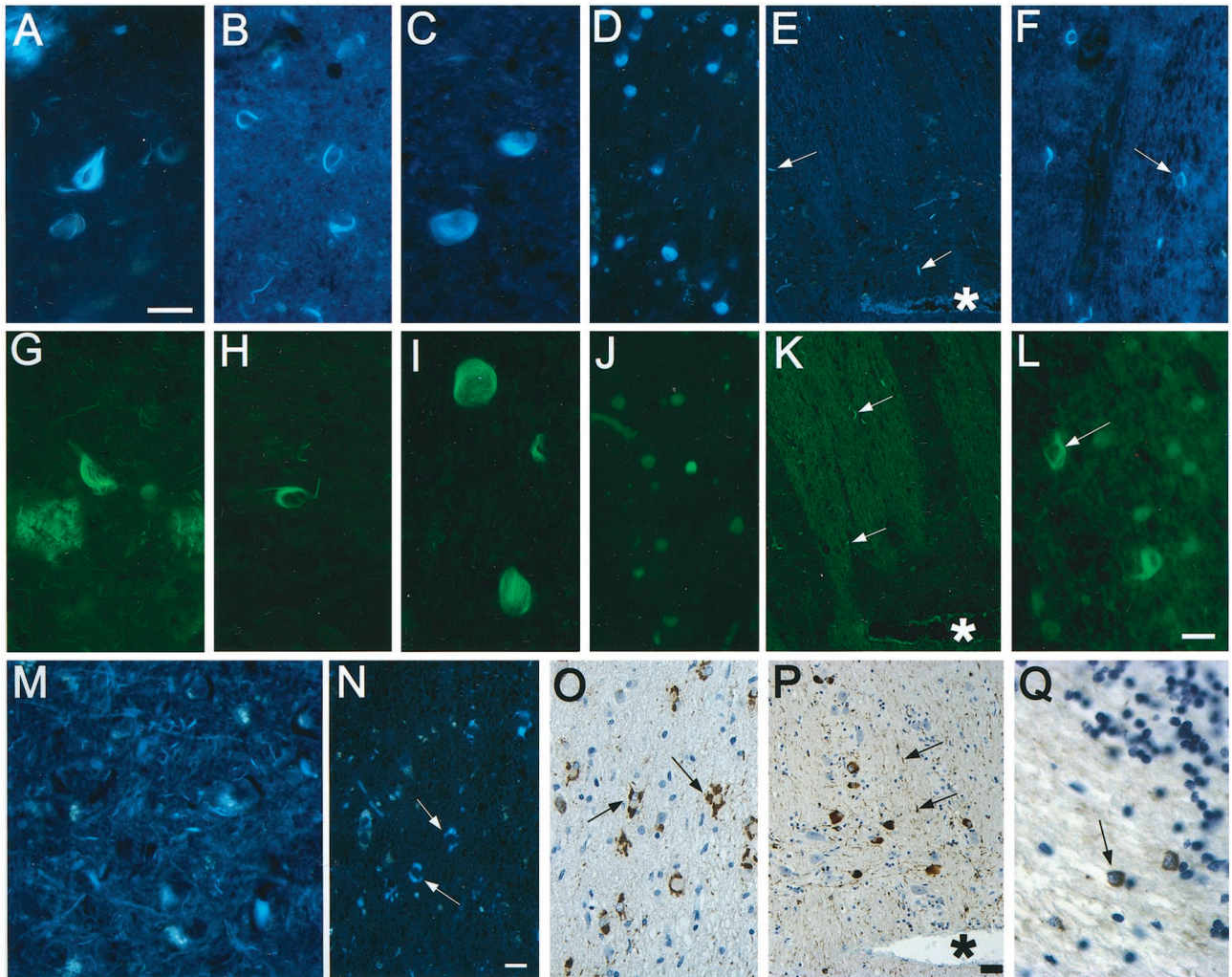


Figure 3. Neurodegenerative lesions composed of tau protein. This figure shows tau immunoreactive lesions in various neurodegenerative diseases stained with BSB and THIOS. All sections in the first row (A–F) were stained with BSB and all sections in the second row (G–L) were stained with THIOS. In the third row the sections depicted in panels M and N are stained with BSB and in panels O to Q are immunostained with antiserum 17026. **A** and **G**: NFTs and A β plaques from an AD patient. **B** and **H**: Coiled bodies in the midbrain of a patient with PSP. **C** and **I**: Globose tangles in the midbrain of a patient with PSP. **D** and **J**: Pick bodies in the dentate gyrus of a patient with Pick's disease. **E**, **K**, and **P**: Consecutive sections through the pons of a FTDP-17 (N279K) patient. **P** shows tau-positive neurons and neuropil threads. Both BSB and THIOS stain some neuropil threads in adjacent sections (**E** and **K**, respectively) but tau immunopositive neurons are not stained. **Asterisks** indicate the same blood vessel in these images and the **arrows** point to NTs. **F**, **L**, and **Q**: Coiled bodies in a patient with familial PSG (exon 10 + 16 mutation). **M**: NT pathology in a CBD patient. **N** and **O**: Tau and BSB stained glia (**arrows**) in the amygdala of a FTDP-17 (P301L) patient. **A**, **C**, **G**, **I**, and **M** are the same magnification; scale bar in **A** = 20 μ m. **B**, **H**, **L**, and **Q** are the same magnification; scale bar in **L** = 10 μ m. **D**, **F**, **J**, **N**, and **O** are the same magnification; scale bar in **N** = 10 μ m. **E**, **K**, and **P** are the same magnification; scale bar in **P** = 50 μ m.

BA27-positive plaques even within the same tissue section. These results are illustrated in Figure 2. It is apparent that both BSB and THIOS stain larger numbers of plaques than BA27. However, by comparing many large BCO5 positive plaques with adjacent tissue sections treated with BSB and THIOS, it is evident that these two fluorescent dyes stain nearly as many plaques as BCO5 although some of the fluorescently stained plaques show a signal that is barely above background (Figure 2, arrowheads in panels A, D, and E). Amyloid angiopathy was immunostained by BA27 as well as fluorescently labeled by BSB and THIOS.

Rare 4G8 positive A β plaques were observed in the brain of a 33-year-old DS patient, a Pick's disease patient, a FTDP-17(P301L) patient and an elderly non-demented control patient. BSB did not stain plaques in any of these patients although weakly stained plaques could

occasionally be demonstrated with THIOS (data not shown).

Tau-Positive Lesions

BSB stains NFTs and NTs in AD very prominently (Figure 3, A and G). In PSP both globose tangles and coiled bodies were stained by BSB (Figure 3 B, C, H, and I). Tau-positive oligodendrocytic inclusions in a case of familial progressive subcortical gliosis (PSG) were also stained by BSB and THIOS (Figure 3, F, L, and Q). However, of the large numbers of tau-immunoreactive coiled bodies present in FTDP-17 (N279K) only a fraction was stained by either BSB or THIOS (not shown). Also, most of the tau-immunoreactive neurons from the same case were not stained by BSB or THIOS (Figure 3, com-

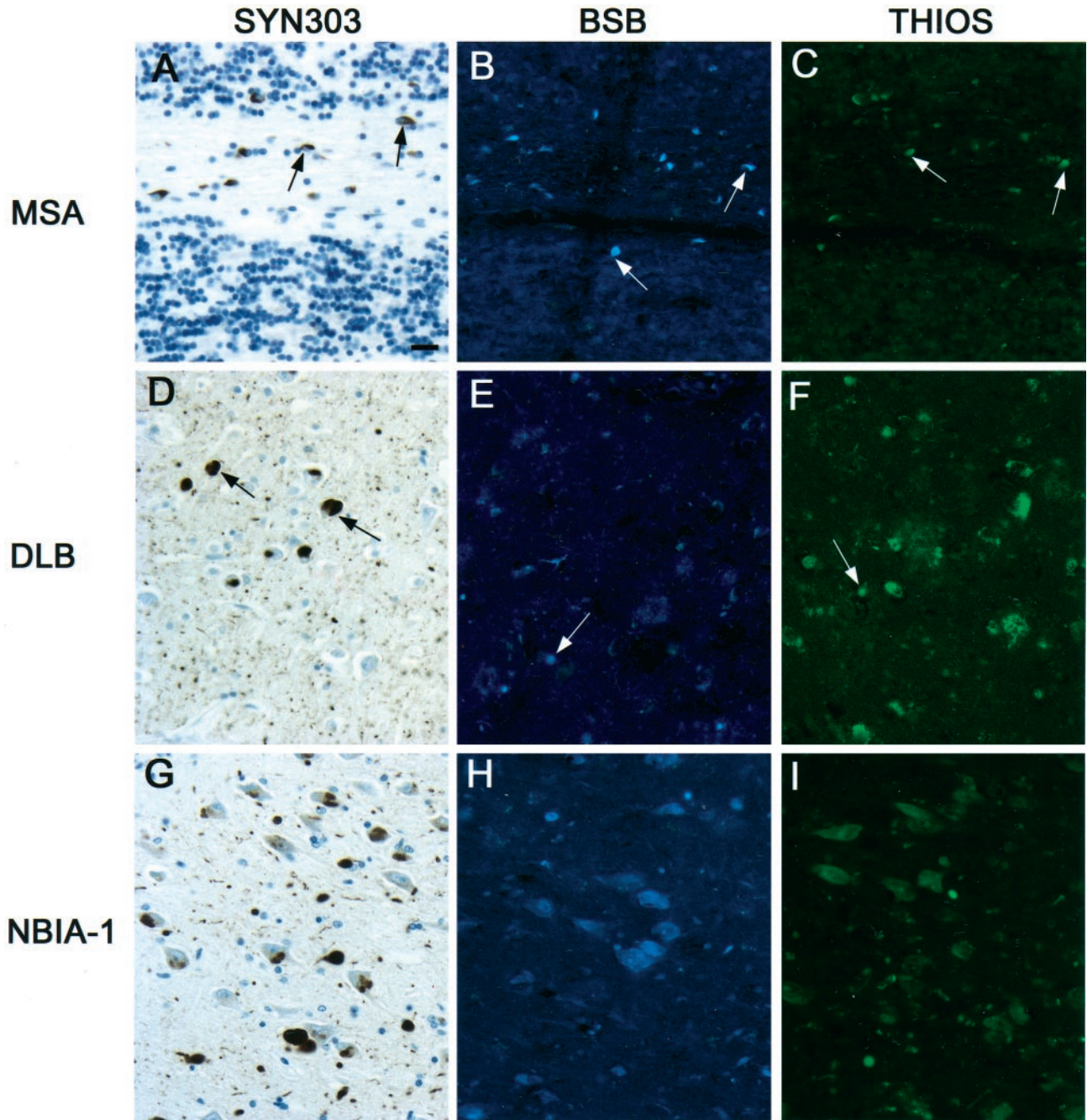


Figure 4. Neurodegenerative lesions composed of α -synuclein. The **first column** of this figure depicts tissue sections immunostained with Syn303. The **second and third columns** show tissue sections stained with BSB and THIOS, respectively. **A to C:** Cerebellar white matter flanked on the top and the bottom by granule cell layers of a patient with MSA. **Arrows** point to CGIs in each of the three panels. **D:** Several cortical LBs in the cingulate cortex of a patient with DLB/PD. In contrast to the α -synuclein antibody, BSB (**E**) and THIOS (**F**) stain only a few LBs in adjacent tissue sections. **Arrows** point to LBs in all three panels. **G:** A large number of α -synuclein-positive inclusions in the CA3 region of the hippocampus of a patient with NBIA-1. **H** and **I** were taken from adjacent tissue sections and show rare globules but no perikaryal inclusions. All panels are the same magnification; scale bar in **A** = 20 μ m.

pare E, K, with P). The most frequently stained BSB and THIOS positive lesions in this case were NTs (arrowheads). Similarly, tau-immunoreactive neurons in CBD cases were not positive for BSB or THIOS (data not shown). However, BSB demonstrated extensive NT pathology in CBD in gray as well as in white matter (Figure 3M). In another FTDP-17 case with the P301L mutation, perikaryal tau immunoreactivity was prominent in several neocortical areas and many large tau-positive cell bod-

ies were present in the basal forebrain. None of these lesions were stained with BSB or THIOS. In this case many tau positive astrocytes with a characteristic crenated appearance were observed and a subset of these stained weakly with BSB (Figure 3, N and O). The astrocytic nature of these lesions had been confirmed by double immunohistochemical staining with MABs to tau and glial fibrillary protein (unpublished observation). Pick bodies in Pick's disease were stained, although weakly,

Table 3. BSB and THIOS Stained Lesions in Various Neurodegenerative Diseases

Antibody specificity	Cases	Lesions	BSB	THIOS
4G8 (total A β)	AD, FAD, DS (54 yr)	Plaques	+	+
4G8 (total A β)	AD, FAD, DS (54 yr)	Vascular amyloid	+	+
BA27 (A β x-40)	AD, FAD, DS (54 yr)	Plaques	+	+
BA27 (A β x-40)	AD, FAD, DS (54 yr)	Vascular amyloid	+	-
BCO5	AD, FAD, DS (54 yr)	Plaques	+	+
4G8 (total A β)	DS (33 yr)	Plaques	-	+/-
4G8 (total A β)	Pick's disease	Plaques	-	+/-
4G8 (total A β)	FTDP-17 (P301L)	Plaques	-	+/-
4G8 (total A β)	Elderly non-demented control	Plaques	-	+/-
17026 (tau)	AD (all tau isoforms)	NFT	+	+
17026 (tau)	AD (all tau isoforms)	NT	+	+
17026 (tau)	PSP (4 repeat tau)	Globose tangles	+	+
17026 (tau)	PSP (4 repeat tau)	Coiled bodies	+	+
17026 (tau)	FTDP-17 cases (4 repeat tau):			
17026 (tau)	exon 10 + 16	Oligodendrocyte inclusions	+	+
17026 (tau)	N279K	Coiled bodies	+/-	+/-
17026 (tau)	N279K	Tau+ neurons	-	-
17026 (tau)	N279K	NT	+	+
17026 (tau)	P301L	Neocortical perikaryal tau+	-	-
17026 (tau)	P301L	Basal forebrain tau+ cell bodies	-	-
17026 (tau)	P301L	Tau+ astrocytes	+/-	-
17026 (tau)	CBD (4 repeat tau)	Tau+ neurons	-	-
17026 (tau)	CBD (4 repeat tau)	NT (gray and white matter)	+	-
17026 (tau)	Pick's disease (3 repeat tau)	Pick bodies	+/-	+/-
17026 (tau)	Guamanian PDC	Spinal cord NFT	+	+
SYN303 (a-synuclein)	MSA	Glial cytoplasmic inclusions	+	-
SYN303 (a-synuclein)	DLB	Cortical LB	+/-	+/-

with both BSB and THIOS (Figure 3, D and J). Finally, NFTs in the cervical, thoracic and lumbar regions of the spinal cord of three Guamanian PDC patients stained with antiserum 17026, BSB, and THIOS (data not shown).

α -Synuclein-Positive Lesions

Both BSB and THIOS stained α -synuclein immunoreactive GCIs in MSA (Figure 4, A-C). Cortical LBs in DLB stain strongly with anti- α -synuclein antibodies, but only occasional LBs are weakly stained with BSB and THIOS (Figure 4, D-F). In a case of NBIA-1, many α -synuclein positive intraneuronal inclusion bodies (LBs and GCIs) were seen. These were especially abundant in area CA3 of the hippocampus, however neither BSB nor THIOS stained these inclusions (Figure 4, G-I). The data of the BSB and THIOS staining of A β -, tau-, and α -synuclein-positive lesions are summarized in Table 3.

Discussion

The data presented here indicate that BSB binds to a variety of amyloid-like lesions in neurodegenerative diseases that are formed by very different building block peptides or proteins including: A β peptides (amyloid plaques), hyperphosphorylated tau proteins (NFTs, NTs), and α -synuclein (LBs, GCIs). Each of these three proteins forms fibrils that deposit into amyloid-like lesions. The results of this study show that the binding properties of BSB are very similar to those of THIOS, but only BSB, and not THIOS crosses the blood brain barrier to label amyloid plaques in mice that model AD amyloidosis as reported earlier.¹ Indeed, the entry of BSB into brain was

demonstrated further using a BSB derivative wherein the bromide was exchanged for iodine-125 and *in vivo* bio-distribution data indicated that 0.27% of the injected compound infiltrated the brain 6 minutes after intravenous delivery in mice.¹⁹ BSB binds not only to A β plaques in living tissue and A β aggregates in cultured cells but also in sections of frozen tissue.¹ While THIOS has been used in histology as an amyloid binding dye for several decades, the mechanisms whereby this and related histochemical stains bind to these fibrillar lesions is still under investigation.²⁰ Although the β -pleated sheet formation occurring in the fibrils formed by A β , hyperphosphorylated tau, as well as α -synuclein may be the substrate for BSB and THIOS binding, minor variations in the β -pleated sheet structures in the lesions described may account for the differences observed in the binding of BSB and THIOS to these lesions.^{12,21} In addition, since fibrils formed by three or four microtubule-binding repeat tau isoforms had different affinities for BSB, variations in β -pleated sheet structures formed by these different tau isoforms also might contribute to variable BSB binding to tangles. For example, Pick bodies composed of three MT binding repeat tau stained weakly with BSB and THIOS, while PSP lesions composed of four MT binding repeat tau stained strongly with BSB and THIOS the 4-repeat lesions in CBD and FTDP-17 stained to variable degrees or not at all (Table 3).

Although BSB binds to a number of fibrillar lesions occurring in neurodegenerative diseases tau- and α -synuclein positive lesions are much smaller and usually far fewer in numbers than A β plaques and would not pose a problem in prospective whole brain imaging of BSB bound to A β plaques. However, BSB did not stain

the plaques in a young DS patient who was in the beginning stages of AD. Similarly, BSB did not stain the diffuse plaques of an elderly non-demented patient with pathological aging²² which could be a preclinical stage of AD. Although BSB compound will not detect non-fibrillar A β accumulations that may be the earliest pre-clinical manifestations of AD, our data suggest that BSB or related compounds with the ability to readily cross the blood-brain barrier, such as the radioiodinated compound mentioned above, could be exploited to develop *in vivo* imaging agents to monitor the burden of diverse amyloid deposits in AD and other neurodegenerative disorders as well as the effects of novel therapeutic agents designed to reverse or ameliorate the accumulation of these lesions in the brains of living patients.

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