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Characterization and expression of presenilin 1 in mouse brain

Duong P. Huynh, Vinh V. Ho and Stefan-M. Pulst[1,CA]

Neurogenetics Laboratory, CSMC Burns and Allen Research Institute, and [1]Division of Neurology, Cedars-Sinai Medical Center, University of California, Los Angeles, School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA

[CA,1]Corresponding Author

THE expression pattern of presenilin 1 (PS-1) in adult mouse brain was investigated using antibodies to specific peptides of PS-1. One antibody, Alz14A, specifically detected a 53 kDa protein in retinoic acid-treated P19 cells and mouse brain protein extracts consistent with the predicted PS-1 molecular weight. Immunohistochemical staining revealed that PS-1 was localized predominantly in large neurons in areas that were known to be affected by Alzheimer disease (AD) such as the hippocampal formation, entorhinal cortex and the subiculum. Selected neurons in other regions not known to be directly affected by AD, such as thalamic nuclei, Purkinje cells, large neurons in the brainstem and the gray matter of the spinal cord, and the dorsal root ganglion, also expressed PS-1. These observations suggest that other as yet identified factors might interact with mutated presenilins to cause neurodegeneration in AD-affected areas.

Key words: Alzheimer's disease; Antibody; Hippocampus; Immunohistochemistry; Mouse brain; Presenilin 1; Spinal cord; Superoxide dismutase; Thalamus

Introduction

Early-onset familial Alzheimer's disease (AD) is a neurodegenerative disorder caused by the defect of at least three genes. The first gene identified was the amyloid β-protein precursor (APP) gene on chromosome 21.[1] Mutations of this gene account for only 5% of the affected Alzheimer families. The other two genes, presenilin 1 (PS-1)[2] and presenilin 2 (PS-2),[3] were recently identified and account for the majority of all early-onset AD. The PS-1 gene is located on chromosome 14 and PS-2 gene on chromosome 1. Both presenilins are highly homologous, with 67% identity at the amino acid level and show high homology to two *Caenorhabditis elegans* proteins, SPE-4[4] and SEL-12.[5,6] The PS-1 gene encodes a protein consisting of 467 amino acid residues with a predicted mol. wt of 54 kDa. The putative secondary structure of PS-1 consists of at least 7–10 transmembrane domains. In the N-terminal region, alternative splicing resulted in the deletion of the VRSQ sequence (codons 26–29).[7,8] The VRSQ sequence is the potential phosphorylation site for protein kinase C and casein kinase H, which suggests that the N-terminal domain is located intracellularly. Therefore, the presence of the VRSQ sequence and the absence of a membrane signal peptide suggested that presenilins proabably localize intracellularly.

Currently, there are no data on the localization of presenilins in the central nervous system (CNS). Such knowledge would contribute valuable information on the normal biological function of presenilins and their potential roles in the pathogenesis of familial AD. Here, we present a detailed analysis of the localization of PS-1 in adult mouse brain and spinal cord.

Materials and Methods

Antibody production: We raised rabbit antibodies against C-terminal and N-terminal peptides of human PS-1.[2] Peptide Alz14A is located at amino acids 55–68 (PQGNSRQVVEQDEE), and peptide Alz14B is located at residues 331–344 (NDDGGFSEEWEAQR). Each peptide was conjugated to keyhole limpet hemocyanin and injected into two rabbits. Collected antisera were affinity purified and used for Western blots and immunohistochemistry as previously described.[9]

Western blots and protein extraction: Mice, 3–4 months old, were perfused with phosphate buffered saline (PBS) and the brains were removed and homogenized in 5 ml triple detergent buffer (TDB) per gram of brain (TDB = 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% NP40, 0.5% deoxycolic acid, 2 μg ml^{-1} aprotinin, 500 μg ml^{-1} Pefabloc). P19 cells were grown in the presence of 5×10^{-7} M retinoic acid for 1 week to allow for > 90% differentiation to neuronal phenotype. Cells were then homogenized in 2 ml TDB per 10^7 cells and spun for 30 min at 10 000 r.p.m. at 4°C.

The supernatants were aliquoted and stored at –80°C. Protein concentrations were determined by the BioRad DC-Bradford Protein Assay Kit. Protein (100 μg per lane) was loaded in 4–20% gradient SDS–polyacrylamide gels (BioRad) and run at 100 V for 1 h at room temperature. The fractionated proteins were transferred to a nitrocellulose filter.[10] The filter was rinsed briefly with TBS (150 mM NaCl, 50 mM Tris, pH 8.0), blocked with 5% nonfat dried milk, and incubated with the desired primary antibody overnight at 4°C. The primary antibody was detected using the BioRad Immunoblot Assay Kit for alkaline phosphatase-conjugated anti-rabbit IgG.

Immunohistochemistry: Mice, 3- to 4 months old, were perfused first with 100 ml 100 mM PBS, pH 7.3 followed by 100 ml of 2% paraformaldehyde in 100 mM PBS. Brains were removed and placed in paraffin-embedded molds, incubated in 4% paraformaldehyde at 4°C overnight, processed using a Tissue-Tek VIP processor (Miles Scientific, Indiana) in 10% formalin fixative, and embedded with paraffin. Seven micron sections were cut and mounted onto Superplus Fisher microscopic slides and air-dried. The sections were rehydrated by rinsing three times at 5 min intervals in xylene, 100% ethanol, 95% ethanol and 70% ethanol. Prior to antibody treatment, rehydrated sections were treated as previously described.[9] Sections were incubated with 20 μg ml^{-1} affinity purified Alz14A antibody overnight at 4°C. The primary antibodies were detected using the Vector ABC elite peroxidase kit (Vector, CA), enhanced by diaminobenzidine (DAB) enhancer and visualized with (DAB) (Biomeda, CA). Sections were counterstained with aqueous hematoxylin (Xymed, CA). Controls consisted of antibodies preabsorbed with the respective peptide conjugated to Sepharose-4B or pre-immune sera of comparable concentrations (1/500).

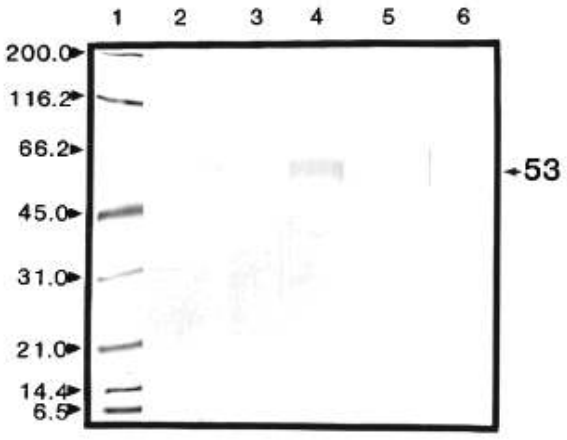

FIG. 1. Western blot of protein extracts from retinoic acid-treated mouse P19 cells (lanes 2,3) and adult mouse brain (lanes 4–6). The Alz14A-AB specifically detected a 53 kDa protein (lanes 2 and 4). Lane 1 contains a biotinylated mol. wt standard (broad range, BioRad). Lanes 3 and 5 were incubated with Alz14A-AB pre-absorbed with the peptide used as antigen. Lane 6 was incubated with 1/500 dilution of the pre-immune serum. The mouse brain protein extract (lane 4–6) was intentionally overloaded to visualize any other Alz14A-AB interacting proteins.

Cell culture: P19 cells are derived from mouse embryonal stem cells which can be differentiated into neuron-like and glial-like cells after treatment with retinoic acid.[11] P19 cells were grown in bacterial grade petri dishes with 5×10^{-7} M retinoic acid in α-MEM containing 2.5% FBS and 7.5% fetal calf serum for 5 days, then transferred to cell culture-coated flasks and grown in retinoic acid for 2–3 additional days to visualize development of neuronal morphology before protein extraction.

Results

Western blot analyses with antibodies to PS-1 peptides: To establish the specificity of the antibodies to PS-1 and determine the size of PS-1, we performed Western blot analyses of protein extracts from mouse P19 cells treated with 5×10^{-7} M retinoic acid (RA), and adult mouse brain. Figure 1 shows a Western blot of protein extracts from RA-treated P19 cells and adult mouse brain with affinity purified Alz14A antibody. The Alz14A antibody detected a single 53 kDa protein in protein extracts from RA-treated mouse P19 cells (Fig. 1, lane 2) and adult mouse brain (Fig. 1, lane 4). In contrast, the affinity purified Alz14B antibody detected multiple bands (including the 53 kDa protein) indicating potential non-specific interaction of this antibody with other proteins in mouse brain protein extracts (data not shown). The pre-absorbed Alz14A-AB did not detect any proteins in PC19 cells and brain homogenates (Fig. 1, lanes 3 and 5,). The 1/500 dilution of pre-immune serum of Alz14A did not detect the 53 kDa protein in mouse brain homogenate (Fig. 1, lane 6). Based on the specificity of the Alz14A antibody in Western blot analysis, we used this antibody for immunolocalization of PS-1 in adult mouse brain sections.

Localization of PS-1 in mouse brain: PS-1 was mainly expressed in selected neurons in the CA1, CA2, CA3, CA4 regions and the dentate gyrus of the hippocampal formation, basomedial amygdaloid nucleus, thalamic nuclei, subiculum, pretectal area, substantia nigra pars reticularis, entorhinal cortex, retrosplenial cortex, pontine nuclei, deep mesencephalic nuclei, occulomotor nuclei and caudate-putamen (Fig. 2). No immunoreactivity was observed

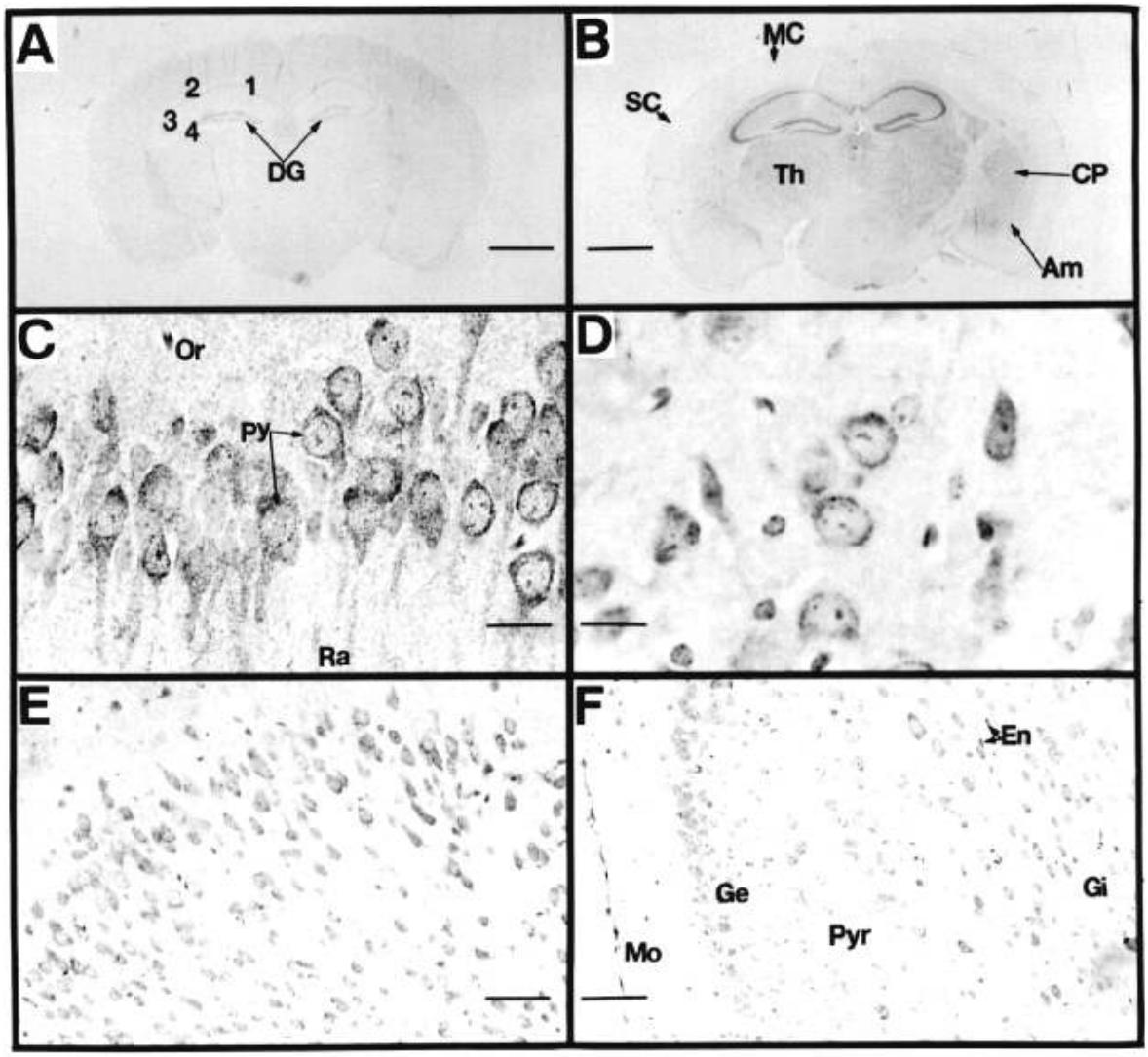

FIG. 2. Localization of presenilin 1 (PS-1) in adult mouse brain. Coronal sections of mouse brain were stained with pre-absorbed antibody (**A**) or with affinity purified Alz14A-AB (**B–F**) and counterstained with aqueous hematoxylin. (A) Whole coronal section stained with peptide adsorbed Alz14A-AB. Numbers 1, 2, 3, 4 denote CA1, CA2, CA3, and CA4. (**B**) Whole coronal section stained with 20 μg ml^{-1} Alz14A-AB. (**C**) High magnification view of CA1 area of the hippocampus showing strong staining of a subset of pyramidal neurons. (**D**) High magnification view of the thalamus showing cytoplasmic staining of thalamic neurons. (**E**) Pyramidal neuronal layer of the subiculum. (**F**) Differential localization of PS-1 in the retrosplenial cortex. Note the absence of PS-1 in the glial cells of the molecular layer (Mo) and granule cells of the internal granular layer (Gi). High levels of PS-1 are observed in pyramidal neurons of the pyramidal layer (Pyr) and low levels in granule neurons of the external granular layer (Ge). Bars = 1.5 mm (A,B), 60 μm (C,D), 200 μm (E), 230 μm (F). Abbreviations: DG, dentate gyrus; MC, motor cortex; SC, somatosensory cortex; Th, thalamic nuclei; Am, amygdaloid nuclei; CP, candate putamen; Or, stratum oriens; Ra, stratum radiatum; Py, projecting pyramidal neurons; En, endothelial cells.

in sections stained with pre-absorbed Alz14A antibody (Fig. 2A). Pre-immune sera at comparable IgG concentrations were also immunonegative (data not shown).

The majority of pyramidal neurons in the CA1 to CA4 regions were stained (Fig. 2B) while granule neurons in the dentate gyrus had weaker staining intensity. PS-1 staining was observed only in the cytoplasm. In the CA1-CA4 areas, only a subset of neurons were stained (Fig. 2C). Pyramidal neurons in the subiculum and the retrosplenial cortex were strongly stained (Fig. 2E,F). Other PS-1 immunoreactive areas in the mouse brain included the medial geniculate bodies, temporal auditory cortex and occipital cortex (data not shown). Light staining was observed in the motor or somatosensory cortex (Fig. 2B). No staining was observed in glial cells. In the brainstem, immunoreactivity was observed in

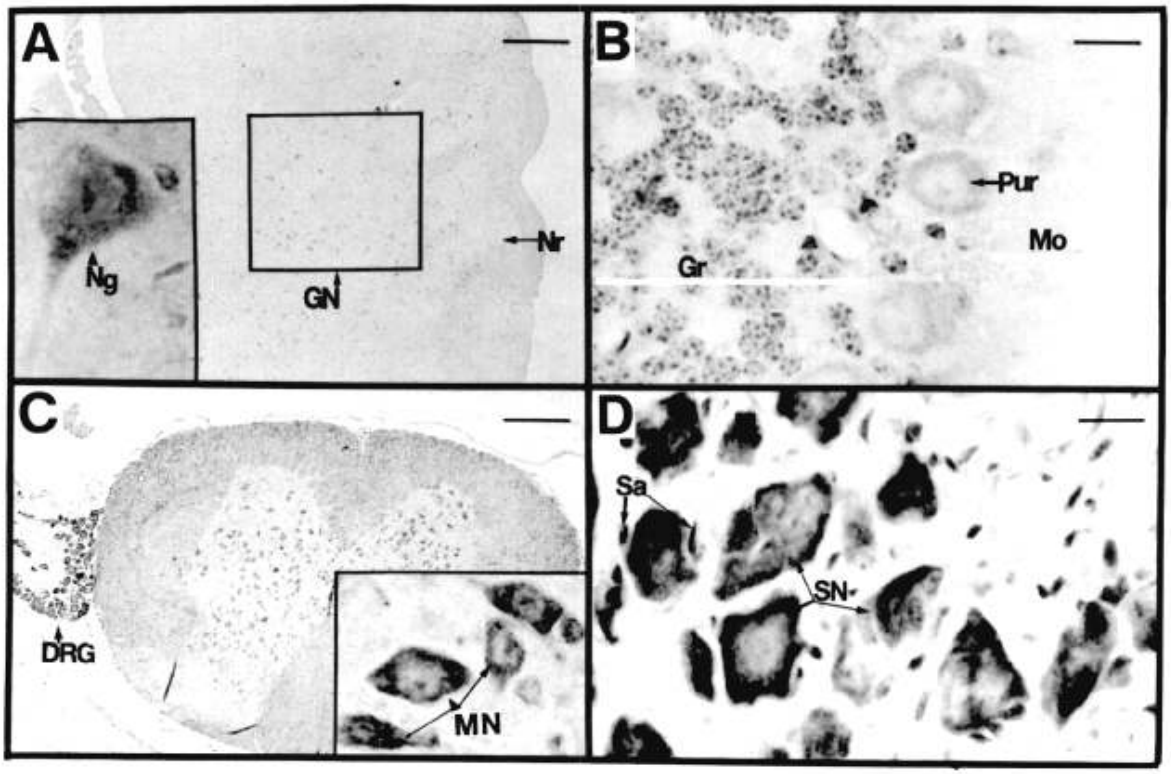

FIG. 3. Localization of PS-1 in other areas of the nervous system. (**A**) Coronal section of the pons stained with Alz14A antibody showing staining of subsets of nuclei. GN, gigantocellular reticular nuclei; Nr, nucleus raphe magnus. Insert shows high magnification of a gigantocellular neuron (Ng). (**B**) High magnification of cerebellum showing staining of Purkinje neurons (Pur) and lack of staining in the granular (Ge) and the molecular layer (Mo). (**C**) Cross-section of the spinal cord and dorsal root ganglion (DRG) stained with the Alz14A antibody showing staining of large neurons in the anterior and posterior horns, and the sensory neurons (SN) of the DRG. Insert shows high magnification of PS-1 positive motor neurons (MN). (**D**) High magnification view of the DRG showing intense staining of sensory neurons (SN). Bars = 1.5 mm (A,C), 60 μm (A insert, B, C insert, D).

the pons where large gigantocellular neurons were strongly stained (Fig. 3A and insert). In the cerebellum, only Purkinje neurons were stained, while neurons in the granular and molecular layers were not stained (Fig. 3B). In the spinal cord, neurons in the anterior and posterior horns were strongly immunoreactive (Fig. 3C). In the dorsal root ganglion, sensory neurons were strongly stained while Schwann cells and satellite cells were lightly stained (Fig. 3D).

Discussion

To date, the normal biological functions and cellular distribution of presenilins are unknown. Homology of presenilins to two *Caenorhabditis elegans* proteins, SPE-4[4] and SEL-12,[5,6] suggests that presenilins might play a role in intracellular protein transport and signalling.[12,13] As part of the attempt to elucidate the function of PS-1, we produced antibodies to peptides of different regions of PS-1 and used these antibodies to investigate the expression pattern of PS-1 in normal mouse brain sections. Our analyses showed that PS-1 was restricted to the cytoplasm of selected neurons within restricted regions of the CNS. This pattern of expression is unlike that of the amyloid β-protein precursor, which is ubiquitously expressed.[14,15]

Western blot analyses confirmed that PS-1 is a 53 kDa protein. The Alz14A antibody specifically detected a 53 kDa protein in protein extracts from retinoic acid-treated mouse P19 cells and mouse adult brain (Fig. 1, lanes 2 and 4). The single 53 kDa protein detected by the Alz14A antibody suggests that there are no major post-translational changes, and that alternative splicing resulting in the synthesis of PS-1 isoforms (containing the Alz14A peptide) with large differences in mol. wts are not abundant.

The Alz14A antibody is probably specific to PS-1 and does not recognize PS-2 based on the following observations. First, the amino acid sequence of the

Alz14A peptide is PQGNSRQVVEQDEE[2] whereas the sequence for the corresponding region in PS-2 is DRYVCSGVPGRPPG.[3] Thus, the sequence identity between peptide Alz14A and the corresponding region in PS-2 is only one amino acid. Second the calculated mol. wt for PS-2 is approximately 43 kDa, which is 10 kDa smaller than the mol. wt of PS-1. The 53 kDa and the 43 kDa proteins were easily resolved in a 4–20% gradient SDS–polyacrylamide gel in our study, and PS-2 can be identified as a 43 kDa protein using PS-2 antibodies (unpublished data).

Immunohistochemical staining showed that PS-1 was localized in the cytoplasm (Figs 2, 3). The intracellular localization of PS-1 is consistent with the absence of a signal peptide in the primary amino acid sequence and the presence of the VRSQ peptide in the N-terminal domain. Since structural analysis of the primary amino acid sequence of PS-1 predicted that PS-1 is a transmembrane protein,[3] observation of the PS-1 in the cytoplasm suggests that PS-1 is probably localized in the membrane of intracellular organelles. A recent report describing the expression of a fusion PS-1 protein suggested that PS-1 might be localized in the Golgi apparatus[13] and thus involved in the processing or transporting of newly synthesized proteins.

In the hippocampal formation, strong staining for PS-1 protein was found in a subset of neurons in the pyramidal layer of the cornu ammonis (CA) areas. Similar observations were made in previous immunohistochemical studies of the pyramidal layer using antibodies to a neuronal cell surface glycoprotein, Thy-1[16] and Cu, Zn superoxide dismutase (Cu,ZnSOD).[17] Both Thy-1 and Cu,ZnSOD were highly expressed in a subset of hippocampal neurons. It will be interesting to determine whether PS-1 and Thy-1 or SOD co-localize in these neurons.

The regional and neuronal distribution of PS-1 detected by Alz14A is consistent with PS-1 mRNA expression as recently reported by other investigators.[13,18] PS-1 mRNA levels were highest in neuronal cell populations in the rat hippocampal formation, dentate gyrus and the Purkinje cell layer.[13,18] No PS-1 mRNA was detected in glial cells. Although PS-1 mRNA appeared to be expressed in the granular cell layer of the cerebellar cortex,[18] there was no evidence that cerebellar granular cells produced PS-1 protein in this study (Fig. 3B). This observation suggests that PS-1 mRNAs might not be translated in cerebellar granular cells under normal circumstances, or that the resolution of *in situ* hybridization techniques was not sufficiently sensitive to detect differential expression of PS-1 in different neuronal cell types.

Neurons from the hippocampal formation receive input from the entorhinal cortex and project to the mammillary bodies and the limbic nuclei of the thalamus, which project to the retrosplenial region and subiculum. These in turn project back to the entorhinal cortex (for review, see Ref. 19). In AD, neurodegeneration begins in the entorhinal region and progresses from there to the hippocampal formation and finally to the destruction of neocortical areas.[19] Recent evidence showed that mutations of presenilins increased the synthesis of βA42 in fibroblasts derived from both carriers and affected familial AD individuals.[20] Furthermore, APP and its derivatives have been shown to be involved in the reduction of Cu(II) to Cu(I), resulting in increased levels of free hydroxyl radicals.[21] These observations led to the hypothesis that mutations of presenilins affect the processing of APP, enhance the production of βA42 and ultimately lead to neurodegeneration due to increased copper-mediated toxicity.[13,21] Our results showing high levels of PS-1 in pyramidal neurons of the entorhinal cortex, retrosplenial cortex and the hippocampus are consistent with anatomical observations that these neurons are vulnerable in AD.[19,22,23] Thus, alteration in the function of PS-1 might result in neuronal toxicity by altering the normal processing or transporting of proteins such as APP involved in regulating cellular homeostasis.

Presenilin expression was also found in other regions of the CNS that are not known pathologically to be severely affected by AD. High levels of PS-1 were found in large neurons of thalamic nuclei, gigantocellular nuclei of the brainstem, the motor and sensory neurons of the spinal cord, and the sensory neurons of the dorsal root ganglion. Thus, other as yet unidentified factors might act synergistically with mutated presenilins to cause neurodegeneration in the AD-affected area.

Conclusion

We have produced an antibody, Alz14A-AB, specific to PS-1 and identified the localization of PS-1 in mouse adult brain. High levels of PS-1 were observed in regions that are known to be vulnerable in AD such as neurons in the CA1, CA2, CA3, CA4 regions, dentate gyrus, subiculum, retrosplenial cortex, entorhinal cortex, caudate-putamen and amygdaloid nuclei. PS-1 was also observed in areas that were not known to be associated with AD such as thalamic neurons, the sensory neurons of the dorsal root ganglion and anterior and posterior horn neurons of the spinal cord, Purkinje cells of the cerebellum and gigantocellular neurons of the pons. These observations suggest that PS-1 may interact with other proteins in AD-affected neurons to cause neurodegeneration in those areas.

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