Early Neuronal Loss and Axonal/Presynaptic Damage is Associated with Accelerated Amyloid-β Accumulation in AβPP/PS1 Alzheimer’s Disease Mice Subiculum

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Abstract. The progressive cognitive decline leading to dementia in Alzheimer’s disease (AD) patients is the consequence of a severe loss of synapses and neurons affecting particular cell subpopulations in selected brain areas, with the subiculum being one of the earliest regions displaying severe atrophy and pathology. The lack of significant neuronal loss in most AD models is, in fact, the major shortcoming for the preclinical evaluation of drugs that could have greater potential in patients to alleviate or prevent this disease. In this study, using immunohistochemical and stereological approaches, we have analyzed the histopathological events in the subiculum of AβPP751SwedLondon/PS1M146L mice, a transgenic model that displays neuronal vulnerability at early ages in hippocampus and entorhinal cortex. Our results indicate that the subiculum is the earliest affected region in the hippocampus, showing a selective early loss of both principal neurons (28%) and SOM-positive interneurons (69%). In addition, our data demonstrate the existence of an early axonal and synaptic pathology, which may represent the beginning of the synaptic disruption and loss. These neurodegenerative processes occur in parallel, and closely related, with the onset and accelerated progression of the extracellular amyloid-β deposition, thus suggesting plaques as major contributors of neuronal/axonal damage. Data reported here indicate that this AD model displays a selective AD-like neurodegenerative phenotype in highly vulnerable regions, including the subiculum, and therefore can be a very useful model for testing the therapeutic ability of potential compounds to protect neurons and ameliorate disease symptoms.

Keywords: Alzheimer’s disease, amyloid-β plaques, axonal damage, hippocampus, neuronal loss, subiculum, transgenic mice

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INTRODUCTION

The progressive cognitive decline that ultimately leads to dementia in Alzheimer’s disease (AD) is consequence of a severe loss of synapses and neurons that selectively affects particular cell subpopulations in brain areas critical for learning and memory [1–6]. Although transgenic mice, based on the overexpression of proteins harboring one or several mutations found in familial AD, progressively develop amyloid-β (Aβ) deposits and tau hyperphosphorylation, along with dystrophic neurites and activated astrocytes and microglia, very limited or no neuronal death has been reported in vulnerable brain areas of these AD models [7–10]. This lack of significant neuronal loss is, in fact, the major shortcoming of AD models for the preclinical evaluation of drugs that could have greater potential in patients to alleviate or prevent this disease. Therefore, the characterization of the neurodegenerative phenotype of AD models is a critical step in ensuring success for translating therapeutic efficacy.

The hippocampal formation, a key structure of the medial temporal lobe memory system and one of the earliest regions to be affected in AD, consists of a number of subdivisions including the dentate gyrus, the hippocampus proper (areas CA1 and CA3), the entorhinal cortex, and the subiculum [11–13]. Marked neuronal reduction occurs in the hippocampal formation of AD patients [14–21]. Even in mild AD, a marked neuronal loss can be found in the entorhinal cortex and hippocampus [2, 4], and these changes result in decreased volume of these brain areas [22, 23]. Unlike most transgenic animal models, which do not exhibit the neurodegenerative spectrum of disease observed in the patient population, in the APP/PS1 model by immunohistochemistry and stereological approaches to detect changes in the number of neurons. Our data indicate that the subiculum is the earliest affected hippocampal region showing a selective loss of both principal cells and SOM-positive interneurons at an early age (4–6 months) in parallel with an early onset of extracellular amyloid deposits and prominent axonal damage. The most relevant feature of this model is the selective AD-like neurodegenerative phenotype in highly AD-vulnerable regions. Therefore, this model can be very useful for testing the therapeutic ability of potential compounds to protect neurons and ameliorate disease symptoms due to this neurodegenerative phenotype.

MATERIALS AND METHODS

Animals

Male transgenic mice expressing familial AD-causing mutations in the AβPP and PS1 genes were used in this study [40]. The bigenic mice were obtained by crossing homozygous mice expressing human mutant PS1M146L (under HMG-CoA reductase promoter) to hemizygous mice expressing human mutant AβPP751 carrying the Swedish (KM670/671NL) and London (V717I) mutations (under the control of the Thy1 promoter). Mice represented F6-F10 offspring of heterozygous transgenic mice. Non-transgenic mice (WT) of the same genetic background (C57BL/6) and age were also used. All animal experiments were carried out in accordance with the European Union regulations (Council Directive 86/609/EEC of November 24th, 1986) and approved by the committee of
animal use for research at Malaga University, Spain (RD 1201/2005 of October 10th, 2005).

**Tissue preparation**

After deep anesthesia with sodium pentobarbital (60 mg/kg), 2, 4, 6, 12 and 18-month-old, AβPP/PS1, and WT mice (n=6/age/genotype) were perfused transcardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4 followed by 4% paraformaldehyde, 75 mM lysine, 10 mM sodium metaperiodate in 0.1 M phosphate buffer (PB), pH 7.4. Brains were then removed, post-fixed overnight in the same fixative solution at 4°C, cryoprotected in 30% sucrose, sectioned at 40 μm thickness in the coronal plane on a freezing microtome, and serially collected in wells containing cold PBS and 0.02% sodium azide (each series contained sections that represented 1/7th of the total brain).

**Immunohistochemistry**

Serial sections from AβPP/PS1, PS1, and WT mice were assayed simultaneously for light and confocal microscopy immunohistochemistry using same batches of solutions to minimize variability in immunolabeling conditions as previously reported [24–26, 41]. Free-floating sections were first pre-treated with 3% H2O2/3% methanol in PBS pH 7.4 for 20 min to inhibit endogenous peroxidase, and then with avidin-biotin blocking kit (Vector Labs, Burlingame, CA, USA) for 30 min to block endogenous avidin, biotin and biotin-binding proteins. For single immunolabeling, sections were incubated overnight at room temperature with one of the following primary antibodies: anti-somatostatin (SOM) (1/5000 dilution, Swant); anti-Aβ mouse monoclonal 6E10 (1:1500 dilution, Swant); anti-human amyloid β protein precursor (hAβPP) rabbit polyclonal (1/20000; Sigma) or goat polyclonal (1/20000; Meridian life sciences); anti-Aj mouse monoclonal 6E10 (1:1500 dilution; Sigma); anti-Aj rabbit polyclonal (1/5000; Millipore); anti-oligomeric Aβ (OC) rabbit polyclonal (1/5000; Millipore); anti-synaptophysin (Syn) rabbit polyclonal (1:1000 dilution; Abcam); anti-MAP-2 rabbit polyclonal (1/5000 dilution; Chemicon); anti-neurofilament rabbit polyclonal (1/5000 dilution; Chemicon); anti-ubiquitin rabbit polyclonal (1/5000 dilution; Dako); anti-phospho-tau (AT8) mouse monoclonal (1/250 dilution; Pierce); anti-cathepsin D (Cat-D) goat polyclonal (1/100 dilution; Santa Cruz Biotechnology); anti-choline acetyltransferase (ChAT) goat polyclonal (1/1000 dilution; Millipore) over 24, 48, or 72 h at room temperature. To retrieve intracellular Aβ, sections were pre-treated for 7 min with 85% formic acid before incubation with the anti-Aβ antibodies. For general antigen retrieval method sections were previously heated at 80°C for 20 min in 50 mM citrate buffer pH 6.0. The tissue-bound primary antibody was detected by incubating with the corresponding biotinylated secondary antibody (1:500 dilution, Vector Laboratories), and then followed by 1:2000 streptavidin-conjugated horseradish peroxidase (Sigma Aldrich). The peroxidase reaction was visualized with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB), 0.03% nickel ammonium sulphate, and 0.01% hydrogen peroxide in PBS. Specificity of the immune reactions was controlled by omitting the primary antisera. After DAB, some immunolabeled sections were incubated 3 min in a solution of 20% of Congo red. Sections were then mounted onto gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene and coverslipped with DPX (BDH) mounting medium.

For double AβPP/PS1 Labeling, we used a 5× immunohistochemical approach previously reported [26]. Sections were first and sequentially incubated with the following interneuron markers: anti-SOM goat polyclonal (1-1000 dilution; Santa Cruz Biotechnology); anti-parvalbumin (PV) rabbit polyclonal (1/5000 dilution; Swant); anti-human amyloid β protein precursor (hAβPP) rabbit polyclonal (1/20000; Sigma) or goat polyclonal (1/20000; Meridian life sciences); anti-Aj mouse monoclonal 6E10 (1:1500 dilution; Sigma); anti-Aj rabbit polyclonal (1/5000; Millipore); anti-oligomeric Aβ (OC) rabbit polyclonal (1/5000; Millipore); anti-synaptophysin (Syn) rabbit polyclonal (1:1000 dilution; Abcam); anti-MAP-2 rabbit polyclonal (1/5000 dilution; Chemicon); anti-neurofilament rabbit polyclonal (1/5000 dilution; Chemicon); anti-ubiquitin rabbit polyclonal (1/5000 dilution; Dako); anti-phospho-tau (AT8) mouse monoclonal (1/250 dilution; Pierce); anti-cathepsin D (Cat-D) goat polyclonal (1/100 dilution; Santa Cruz Biotechnology); anti-choline acetyltransferase (ChAT) goat polyclonal (1/1000 dilution; Millipore) over 24, 48, or 72 h at room temperature. To retrieve intracellular Aβ, sections were pre-treated for 7 min with 85% formic acid before incubation with the anti-Aβ antibodies. For general antigen retrieval method sections were previously heated at 80°C for 20 min in 50 mM citrate buffer pH 6.0. The tissue-bound primary antibody was detected by incubating with the corresponding biotinylated secondary antibody (1:500 dilution, Vector Laboratories), and then followed by 1:2000 streptavidin-conjugated horseradish peroxidase (Sigma Aldrich). The peroxidase reaction was visualized with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB), 0.03% nickel ammonium sulphate, and 0.01% hydrogen peroxide in PBS. Specificity of the immune reactions was controlled by omitting the primary antisera. After DAB, some immunolabeled sections were incubated 3 min in a solution of 20% of Congo red. Sections were then mounted onto gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene and coverslipped with DPX (BDH) mounting medium.

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peroxidase reactions, the first one (for interneurons) was always developed with DAB-nickel (dark blue) solution whereas the second one (NeuN) only with DAB (light brown). Moreover, the different compartment localization of interneuron (cytoplasm) and NeuN (nuclei) epitopes completely guarantee the correct non-overlapped visualization of both reactions and the interpretation of the results.

**Thioflavin-S staining**

Free-floating sections were incubated for 5 min with 0.015% Thio-S (Sigma) in 50% ethanol, and then washed in 50% ethanol, in PBS, mounted onto gelatin coated slides and coverslipped with 0.1 M PBS containing 50% glycerin and 3% triethylenediamine.

**Plaque loading quantification**

Plaque loading was defined as percentage of total subicular area stained for Aβ. Quantification of extracellular Aβ content was performed as previously reported [26]. Thioflavin-S staining was examined under an Olympus BX-61 epifluorescent microscope using FITC filter and 4× objective. Images were acquired with an Olympus DP71 high-resolution digital camera using the Cell-A program (Olympus). The camera settings were adjusted at the start of the experiment and maintained for uniformity. Digital images (4 sections/mouse) from 2, 4, 6, and 12-month-old AβPP/PS1 mice (n = 4/age) were analyzed using Visilog 6.3 analysis program (Noesis, France). The plaque area (Thioflavin-S positive) within the subiculum was identified by level threshold which was maintained throughout the experiment for uniformity. Digital images were converted to binary images with plaques. The subicular area in each 4× image was manually outlined. The plaque loading (%) for each transgenic mouse was estimated and defined as (sum plaque area measured/sum subicular area analyzed)×100. The sums were taken over all slides sampled and a single plaque burden was computed for each mouse. The mean and standard deviation (SD) of the plaque loading were determined using all the available data. Quantitative comparisons were carried out on sections processed at the same time with same batches of solutions.

**Plaque size morphometric analysis**

Four coronal sections stained with Thioflavin-S from 2 (n = 5), 4 (n = 5), 6 (n = 5), and 12-month-old (n = 5) AβPP/PS1 mice were analyzed using the nucleator method with isotropic probes by the NewCAST software package from Olympus stereological system. Subiculum was analyzed using a counting frame of 6022.8 μm² and step length of 173.5 μm. For individual plaque measurement, a 40× objective was used. Number of plaques/mm² falling into four surface categories (ranging from <200 μm² to >2000 μm²) was calculated. Each analysis was done by a single examiner blinded to sample identities.

**Stereological analysis**

Immunopositive cells for SOM, PV, or NeuN belonging to the different animal groups (WT, PS1, and AβPP/PS1) and ages (2, 4, 6, 12, or 18 months) were quantified (n = 5-6/age/group). Briefly, the quantitative analyses were performed using an Olympus BX61 microscope interfaced with a computer and a Olympus DP71 digital camera, and the NewCAST (Computer Assisted Stereological Toolbox) software package (Olympus, Denmark). The number of neurons was quantified in every 7th section (with a distance of 280 μm between sections) through the rostrocaudal extent of the subiculum (between −2.46 mm anterior and −4.60 mm posterior to Bregman coordinates, according to the atlas of Franklin and Paxinos [42]). An average of 6-7 sections was measured in each animal. The subicular area was defined using a 4× objective and the number of neurons was counted using a 100×/1.35 objective. We used a counting frame of 902.52 μm² with step lengths of 46.98 μm for SOM and 95 μm for NeuN counting. The numerical density (ND; cells/mm³) was estimated using the following formula: ND = Q/(Σ Qi × h), where ‘Q’ is the number of dissector-counted somatic profiles, ‘Σ Qi’ is the area of the counting frame, and ‘h’ is the height of the optical dissector (10 μm). The precision of the individual estimations is expressed by the coefficient of error (CE) [43] calculated using the following formula: CE = 1/[Q × (3A−4B + C]/2), where A = Σ Qi, B = Σ Qi × Qi+1, C = Σ Qi × Qi+2. The CEs ranged between 0.07 and 0.1. An investigator who was blind to the experimental conditions (age, genotype, and marker) performed neuronal profile counting.

**Co-localization analysis**

Double immunopositive subicular cells for Aβ42 and Cat-D or for Aβ42 and hAβF were analyzed to determine the extent of colocalization between both couple of markers in 2 month-old AβPP/PS1 Subiculum.
animals. Confocal images of 1,024 × 1,024 pixels were acquired by using a Leica S 5 II confocal microscope and a 40× objective. A total of 30 (Aβ42/Cat-D) to 50 (Aβ42/hAβPP) cells were randomly photographed (n = 3). Laser settings were adjusted at the start of the experiment and maintained for uniformity. Images were analyzed using LAS AF Lite program (Leica).

The experiment and maintained for uniformity. Images were first washed with PBS and incubated in a 50 mM glycine solution 5 min in order to increase the antibody binding efficiency. Following the standard immunohistochemical protocol, the tissue was incubated 48 h in cold PB and 0.02% sodium azide. For standard electron microscopy, the 250 μm thick sections were postfixed with 1% osmium tetroxide in 0.1 M PB, block stained with uranyl acetate, dehydrated in acetone, and flat embedded in Araldite (EMS, USA). Selected areas with uranyl acetate, dehydrated in acetone, and flat embedded in Araldite (EMS, USA). Selected areas were cut in ultrathin sections and examined with an electron microscope (JEOL JEM1400).

For the immunogold labeling, the 50 μm sections were first washed with PBS and incubated in a 50 mM glycine solution 5 min in order to increase the antibody binding efficiency. Following the standard immunohistochemical protocol, the tissue was incubated 48 h in primary rabbit polyclonal antibody anti-Aβ (1/5000; Millipore) in a PBS 0.1M/0.02%Tx-100/1% BSA solution at 22°C. Then, sections were washed in PBS, and incubated with 1.4 nm gold-conjugated goat anti-rabbit IgG (1-100; Nanoprobes), and gold-tomed. Finally, the immunolabeled sections were processed as above by the osmium fixation, dehydration and embedding steps. In negative control experiments, the primary antibody was omitted.

**Statistical analysis**

Data was expressed as mean ± SD. The comparison between two mice groups (WT and AβPP/PS1 mice or PS1 and AβPP/PS1 transgenic mice) was done by two-tailed t-test, and for comparing several groups (WT, PS1, and AβPP/PS1 mice) and ages, we used one-way ANOVA, followed by Tukey post-hoc multiple comparison test (SigmaStat® 2.03, SPSS Inc).

In both cases, the significance was set at 95% of confidence.

**RESULTS**

**Selective loss of subicular interneurons at early ages**

We have first determined the numerical density of SOM-immunostained neurons in the subiculum (including pro-subiculum) of AβPP/PS1 at 2, 4, 6 and 12 months of age and compared to age-matched PS1 and WT mice. The initial immunohistochemical analysis showed that the majority of subicular SOM-containing cells were located in the deep pyramidal cell layer and in the polymorphic layer (Fig. IA1–A3). These interneurons (see inset in Fig. IA2), corresponding to O-LM cells of hippocampal sector CA1, innervate the distal apical dendrites of pyramidal neurons in the outer molecular layer which receives the main excitatory input from layer III of the medial entorhinal cortex. WT and PS1 animals displayed a similar pattern of SOM-immunolabeling.

However, AβPP/PS1 mice (Fig. IB1–IB3) showed a reduced number of labeled somata since early ages (4–6 months) and the presence of numerous SOM-positive dystrophic neurites (see inset in Fig. IB2), mostly located around amyloid plaques.

The stereological study (Fig. 1C) demonstrated a significant decrease (27.17 ± 8.53%, Tukey p < 0.05) in the numerical density (neurons/mm2) of SOM-positive cells in AβPP/PS1 mice at 4 months of age compared to age-matched WT group. This decrease was much more marked at 6 months of age (69.37 ± 8.53%, Tukey p < 0.05). No further decrease was detected at 12 month-old (69.15 ± 3.05%, Tukey p < 0.05). PS1 group did not show changes respect to WT group at any age tested. These data were in line with our previous reports showing a significant loss of SOM interneurons in the hippocampus proper (CA1-CA3 and dentate gyrus) and entorhinal cortex of our AβPP/PS1 model at 6 months of age [25, 26].

We have also analyzed whether another major interneuron population, the cells expressing the calcium binding protein parvalbumin (PV) which include basket and axo-axonic GABAergic neurons, was also early affected in the subiculum of our AD model. PV-positive interneurons were present throughout the principal cell layer of the subiculum (Fig. 2A-B).

We have not found significant differences in the numerical density (Fig. 2C) of this GABA population at 6 months of age between AβPP/PS1 (8415.58 ± 1429.79) and
WT (10721.93 ± 3057.86) mice and neither at the advanced age of 18 month-old (7556.79 ± 1033.90 versus 9136.28 ± 1205.44) for Aβ/PP/PS1 and WT mice, respectively). Moreover, and unlike SOM-cells, the neuronal population positive for PV did not develop dystrophic neurites with the progression of age. In fact, even the PV-immunopositive processes and somata that were located in the very near proximity of amyloid plaques displayed a normal morphology (see Fig. 2B2).

These findings indicated that in the subiculum of this AD model the SOM interneurons, but not the PV-cells, were highly vulnerable at the initial stages of the disease and that the degenerative process of the SOM population preceded the observed in the hippocampus and entorhinal cortex.

**Loss of subicular principal neurons at early ages**

Though in this AD model the loss of hippocampal principal neurons is a late event (17–18 months of age) [27, 44], in the entorhinal cortex pyramidal neurodegeneration begins at an early age (6 months) [26]. Therefore, we next examined whether subicular principal neurons were also affected early by determining their numerical density at 2 and 6 months of age in comparison with age-matched PS1 and WT animals. To specifically distinguish principal cells from interneurons, we have performed a multiple immunoperoxidase labeling approach as previously reported (5× immunolabeling) [26]. Principal cells were discriminated by a single NeuN-nuclei labeling in light brown color whereas interneurons (those expressing SOM/PV/CR/VIP alone or in combination) displayed also a dark-blue cytoplasmic labeling (see Fig. 3 and for details see insets).

As shown in Fig. 3, the microscopic observation of the 5× immunolabeled sections at 2 and 6 months of age revealed no qualitative differences in the immunostaining pattern and cell distribution between Aβ/PP/PS1 and PS1 or WT animals. Small rounded areas devoid of cells and characterized by the presence of dystrophic neurites (in this case only GABAergic dystrophies), which corresponded to areas occupied by amyloid plaques, were easily detected in Aβ/PP/PS1 animals, few plaques at 2 months (Fig. 3 A3) and more numerous at 6 months of age (Fig. 3B3), as expected. The quantitative stereological study (Fig. 3C) revealed a significant (−28.04 ± 11.76%; p < 0.05) principal cell loss in Aβ/PP/PS1 mice (compared to PS1 and non-transgenic littermates) at 6 months of age. No differences were detected at 2 months of age. These data demonstrated that principal subicular neurons were vulnerable at early ages and the first hippocampal pyramidal neurons to be affected by the course of the disease.

**Accelerated intra- and extracellular Aβ accumulation in the subiculum**

Considering the toxic effect of Aβ accumulation on neuronal survival we next investigated the temporal intra/extracellular expression of Aβ in the subiculum of the double transgenic model from 2 to 12 months of age by Aβ1-42 immunohistochemistry (Fig. 4). Similar results were obtained with the OC antibody for the oligomeric forms of Aβ (results not shown). As shown in the panoramic images (Fig. 4 A–C), the subiculum is one of the earliest forebrain regions to express and accumulate Aβ. At 2 months of age the presence of intracellular Aβ was clearly seen in subicular neurons which presented a strong punctuate immunolabeling (Fig. 4A, D, and for a detail see inset in D). We did not check in younger animals, but most probably the intracellular Aβ accumulation in subiculum started before 2 months of age. The formation of extracellular Aβ deposits was also first observed in this area at 2 months of age, however the number of plaques was really low indicating that the onset of extracellular amyloid pathology was at the initial stage. At 4 months, numerous plaques were already formed in the subiculum (Fig. 4E) and further on the number and size of these deposits significantly increased, as shown here for 6 and 12 months of age (Fig. 4F and G, respectively). As presented (5× immunolabeling) [26]. Principal cells were discriminated by a single NeuN-nuclei labeling in light brown color whereas interneurons (those expressing SOM/PV/CR/VIP alone or in combination) displayed also a dark-blue cytoplasmic labeling (see Fig. 3 and for details see insets).

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rapidly increased the extracellular accumulation of Aβ and the presence of intraneuronal Aβ in the somata tended to decrease. In fact, it was really difficult to distinguish Aβ-positive somata at light microscopy from 6 months onward due to the high amount of extracellular amyloid deposits occupying the subiculum.

To quantitatively compare the extracellular amyloid progression with age in the subiculum with other...
Fig. 3. Loss of principal neurons in the AβPP/PS1 subiculum at early ages. Multiple 5× (SOM, PV, CR, VIP and NeuN)-immunolabeling in the subiculum, of WT (A1 and B1), PS1 (A2 and B2), and AβPP/PS1 (A3 and B3) mice at 2 and 6 months of age. Principal neurons (single NeuN-labeled cells in brown color) were immunohistochemically differentiated from interneurons (SOM/PV/CR/VIP-labeled cells in dark blue color) as seen in the higher magnification images of the insets. Stereological counts (C) of principal cells revealed a significant (two-tailed t-test, p<0.05) decrease in the density (neurons/mm³) of this cell population in the AβPP/PS1 subiculum compared to age-matched WT or PS1 mice at 6 months of age. No differences were found at 2 months of age. Data are given as mean ± SD. Open white circles indicated amyloid plaques location in the AβPP/PS1 subiculum. Scale bars, A1–A3 and B1–B3, 200 μm; insets in A1 and A3, 100 μm; inset in B3, 50 μm.
Fig. 4. Early accelerated intra- and extracellular Aβ accumulation in the AβPP/PS1 subiculum. AβPP immunohistochemistry at 2 (A and D), 4 (B and E), 6 (C and F), and 12 (G) months of age. A–C, panoramic views of the caudal telencephalon showing the AβPP immunoreactivity in the subiculum (dashed area pointed with a black arrow) compared to other hippocampal and cortical areas from 2 to 6 months. Intracellular Aβ appears as early as 2 months and neurons show a punctate labeling suggestive of a vesicular location (inset in D). Abundant Aβ plaques were already seen at 4 months of age and plaques progressively increased in number and size, with age. CA1, CA3, hippocampal subfields; DG, dentate gyrus; Ent, entorhinal cortex; Per, perirhinal cortex; Au, auditory cortex. Scale bars, A–C, 500 μm; D–G, 200 μm; inset in D 10 μm.

Highly vulnerable brain areas, such as CA1 and entorhinal cortex, we have measured the area occupied by the Aβ deposits (plaque loading) in these brain regions using Thioflavin-S stained sections (images not shown). Thioflavin-S labeled only extracellular Aβ and allowed better image analysis quantification of plaques since intracellular Aβ pool was excluded. As shown, the subiculum (Fig. 5A) is the earliest and most severely affected area by the extracellular amyloid pathology. The subicular amyloid load was 0.06 ± 0.1% and
Aβ load and plaque progression with age in AβPP/PS1 subiculum. A) Aβ load in subiculum rapidly increased with age and it was significantly higher than in CA1 and entorhinal cortex at all ages analyzed. B, C) The number (plaques/mm²) and the size (µm²) of the Aβ plaques in the subiculum exhibited a marked increase with age (two tailed t-test, *p<0.05, **p<0.01, ***p<0.001).

At 4 months of age, the subicular amyloid deposition was markedly accelerated and reached up to 5 times higher than in CA1 and 7 times than in entorhinal cortex. Therefore, the subiculum showed the greatest age-related Aβ load and also exhibited the earliest neuronal loss.

The age-dependent increase in the total amyloid load in the subiculum appeared to be associated with both the number and size of the plaques. To support this observation, we next determined the plaque density...
Fig. 6. Intracellular Aβ is mostly localized in lysosomal vesicles of principal cells. Double confocal immunofluorescence labelings show the presence of Aβ in AβPP-positive (principal) neurons (A1–A3) but not in SOM-interneurons (B1–B3). Most Aβ42 was localized in lysosomal vesicles as shown by double Aβ42/Cathepsin-D labeling and confocal microscopy (C1–C3). Aβ immunogold electron microscopy reveals the restricted subcellular location of Aβ within multilysosomal organelles of principal neuronal cell bodies. Scale bars: A–C, 10 μm; D, 2 μm; E–G, 0.2 μm.
Fig. 7. Aβ plaques are closely surrounded by axonal/synaptic dystrophies containing phospho-tau and autophagy vesicles. A) Aβ plaque (Congo red-stained) surrounded by numerous dystrophic neurites immunopositive for hAβPP antibody. B) Dystrophic neurites were not immunopositive for MAP-2 (dendritic marker). C) Immunoreactivity for neurofilament (axonal marker) was found in dystrophic neurites surrounding plaques. D) Plaque-associated dystrophies were immunoreactive for synaptophysin (synaptic marker). E1–E3) Confocal double immunofluorescence labeling for hAβPP (red) and VGLUT1 (green) shows extensive co-localization (arrows) of the two markers indicating the axonal/synaptic glutamatergic nature of hAβPP-positive dystrophic neurites around plaques. F–H) Congo red-stained plaques were surrounded by axonal/synaptic dystrophies immunopositive for VGAT (marker for GABAergic terminals), somatostatin (marker for a GABAergic subpopulation) and ChAT (cholinergic marker). I–K) Dystrophies around plaques were immunolabeled for the autophagy marker LC3, ubiquitin and phospho-tau (AT8). Asterisks indicate Aβ plaques. Scale bars: A–D and F–K, 25 μm; E1–E3, 10 μm.

(plaque/mm²) dissected into four size categories ranging from <200 μm² to those >2000 μm² (Fig. 5B), as well as the percent of each plaque category (Fig. 5C), at 2, 4, 6 and 12 months of age. The appearance of plaques in this region began at 2 months of age and they were mostly under 200 μm², and then progressively increased in number at 4 months with the formation also of bigger plaques. However, the most significant
increase in the number of plaques/mm² was at the age of 6 months for each size category (5, 4.6, 3.4, and 14.5 times higher than at 4 months for those plaques <200, 200–500, 500–2000, and >2000 μm², respectively, n = 5, two tailed t-test, p < 0.05), with the most abundant being those at <500 μm². Interestingly, at 12 months the plaque distribution switched and the predominant plaque size was >500 μm². Then our data demonstrated that the number of plaques significantly build-up with age, but most remarkable, plaque size also displayed a striking increment.

Since intraneuronal Aβ accumulation precedes amyloid plaque formation in the subiculum, we further investigated the Aβ expression in subicular neurons as a potential toxic agent to induce the neurodegeneration. The early presence of intraneuronal Aβ in AβPP-positive cells was confirmed by double Aβ42/hAβPP immunofluorescence labeling (Fig. 6A1–A3) in 2-month-old AβPP/PS1 mice. These AβPP-positive cells corresponded to principal neurons since the mutated human AβPP transgene is expressed only by this population in the transgenic mice used. It can be argued then that this early accumulation of Aβ within principal cells could be responsible of their vulnerability. However, SOM-positive interneurons were also highly affected at early ages, and these
cells did not accumulate intracellular Aβ (Fig. 6B–B3), since they do not express the mutated human AβPP. Therefore, it is very unlikely that the neuronal loss in the subiculum, at least for interneurons, was induced by the intracerebral Aβ.

As shown here, the labeling of Aβ1–42 and hAβPP just marginally overlaps in the same subcellular compartments. In fact, only 3.36 ± 1.79% (n = 50 cells) of Aβ1–42 co-localized with hAβPP. This demonstrated the specificity of the Aβ antibody, since it does not cross-react with the AβPP antibody and that hAβPP processing and Aβ accumulation should take place in different intracellular compartments. In this sense, the punctuate labeling of the Aβ1–42 antibody was suggestive of vesicular location. Several studies have reported the preferential location of Aβ in vesicles of the endosome-lysosome system [38] including autophagy vesicles [41, 45]. Confocal images of double Aβ1–42/cathepsin-D labeling (Fig. 6C1–C3) indicated that most of the Aβ was in fact associated with lysosomal structures in the soma of the principal subicular neurons. Quantitative analysis demonstrated that 8.9 ± 18.2% (n = 50 cells) of the intracellular Aβ1–42 labeling co-localized with cathepsin-D. Thus, Aβ is accumulated preferentially in lysosomal vesicles. Furthermore, immunogold electron microscopy experiments demonstrated a restricted location of Aβ within endolysosomal subcellular compartments of the principal neuronal somata (Fig. 6D–G). Immunogold labeled organelles displayed an electron-dense granular content typical of endolysosomes (see higher magnification images in Fig. 6E–G).

Aβ plaques as inducers of early axonal/synaptic pathology in the subiculum

The pathogenic mechanisms leading to neuron loss in AD have not been completely elucidated yet, and the extracellular amyloid deposits could be major contributors for neuronal damage/loss. In our model, the hippocampal plaques are surrounded by numerous dystrophic neurites of axonal/synaptic origin [41, 45]. Then, we next examined the dystrophy pathology in the subiculum and its association with amyloid plaques using light and electron microscopy approaches. The formation of plaques in the subiculum was paralleled with the appearance of dystrophic neurites in their very close periphery. The hAβPP antibody is a well-established marker for dystrophies (Fig. 7A), and in our model labeled dystrophies belonging to glutamatergic cells as we show here with the double labeling AβPP/VGLUT1 (see Fig. 7E1–E3).

no dystrophic dendrites around plaques were detected with MAP2 immunostaining (Fig. 7B), numerous dystrophic neurites were labeled with different axonal/synaptic markers such as NF (Fig. 7C), synaptophysin (Fig. 7D), VLGUT1 (Fig. 7E2), and VGAT (Fig. 7F). In addition, we observed SOM-positive (7G) or ChAT-positive (7H) axonal dystrophies surrounding subicular plaques. Altogether, these data confirmed the axonal origin of the dystrophies that surround plaques in the subiculum.

We and others have reported the accumulation of autophagy vesicles within dystrophies [41, 45–47]. This abnormal collection of vesicles belonging to the autophagy-lysosome degradation system might result from a defective cytoskeleton-mediated transport. We have next checked whether subicular dystrophies displayed immunoreactivity for LC3 (autophagy marker), ubiquitin (marker for protein degradation), and AT8 (phosphorylated tau). As shown in Fig. 7 (I–K), these axonal dystrophies were strongly labeled with all these markers suggesting a focalized altered microtubule vesicular transport that compromise protein degradation with the subsequent accumulation of vesicles and un-degraded proteins that disrupt axonal structure. A direct toxic effect of the Aβ plaques on surrounding axons might be the cause of dystrophic formation. In fact, the confocal microscopy evaluation of double Aβ1–42/synaptophysin labeling (Fig. 8A) revealed a very close spatial association between both markers with the extracellular Aβ encircling, almost wrapping, the synaptophysin-positive dystrophies. For a better resolution, we next evaluated this tight association between plaque and dystrophies at the transmission electron microscopy (Fig. 8B–D). Figure 8B shows a typical amyloid plaque in this model (neuritic plaque), completely surrounded by numerous dystrophic neurites. In their periphery, plaques gave off many long branches coming in close contact with the dystrophies (Fig. 8C, D). Dystrophies were morphologically characterized by the presence of multiple heterogeneous autophagy-like vesicles, as expected.

The early axonal defects in the subiculum of this AD model could directly affect presynaptic terminals. Though the plaque-associated dystrophies were labeled with the synaptic marker synaptophysin, this labeling could be due to the abnormal accumulation of this protein along the axon due to transport failure, and not to be present in synaptic boutons. However, electron microscopic examination revealed that presynaptic terminals were indeed dystrophic (Fig. 8D) and displayed abnormal morphology with huge collection of autophagy/lysosomal vesicles. Though these
neuronal death [39, 48–51], however this was not quantitatively minor and/or occurred at very early ages. Subicular neurodegeneration has been previously reported in the subiculum of this AD transgenic model that may represent the beginning of synaptic disruption and loss.

DISCUSSION

Neuron loss is the best anatomopathological substrate that correlates with cortical atrophy and dementia during disease progression in AD. However, most transgenic animals do not display this fundamental degenerative feature of patients. To evaluate the effectiveness of potential neuroprotective therapies for AD, it is essential that animal models exhibit neuronal damage/dysfunction, and even most importantly neuronal death, relevant to the disease in vulnerable brain regions and cell populations. The present work provides new evidence and further support our previous studies [24–27, 30, 41, 45] on the selective AD-like neurodegenerative phenotype of the AβPP/PS1 model used in this study. In fact, Aβ pathology in the subiculum preceded the loss of neurons. Furthermore, we have also compared the severity of the amyloid pathology in the subiculum with other highly AD vulnerable brain areas, such as CA1 of hippocampus and entorhinal cortex. Interestingly, the subiculum displayed up to 5 and 7 fold increased Aβ load than CA1 and entorhinal cortex, respectively, at the age of 4 months. Therefore, subiculum is the earliest and most severely affected region by the amyloid pathology. In consequence, we also observed an extensive neuronal (pyramidal and GABAergic) degeneration in this particular brain region.

Among the potential causative agents for this neuronal loss, Aβ is the leading candidate in amyloidogenic models bearing familial mutations, as the AβPP/PS1 model used in this study. In fact, Aβ pathology in the subiculum preceded the loss of neurons. Furthermore, we have also compared the severity of the amyloid pathology in the subiculum with other highly vulnerable brain areas, such as CA1 of hippocampus and entorhinal cortex. Interestingly, the subiculum displayed up to 5 and 7 fold increased Aβ load than CA1 and entorhinal cortex, respectively, at the age of 4 months. Therefore, subiculum is the earliest and most severely affected region by the amyloid pathology. In consequence, we also observed an extensive neuronal (pyramidal and GABAergic) degeneration in this particular brain region.

Abundant intraneuronal accumulation of Aβ at 2 months of age mostly located in the endolysosomes and immediately after a rapid onset of amyloid plaques was manifested. As the plaque load increased with age the intracellular Aβ labeling was less evident. In fact, at 6 months of age it was really difficult to visualize Aβ-immunolabeled neuronal somata at light microscopy suggesting a possible transient localization of Aβ in the neuronal compartments (soma versus axonal/synaptic?). However, immunogold labeling demonstrated the presence of some intracellular Aβ within the cell bodies at least in 6-month-old AβPP/PS1 mice. We cannot rule out the possibility that plaques bind most Aβ antibodies thus preventing the intracellular Aβ labeling. Our results are in agreement with others since Aβ pathology (intra preceding extra) was reported to be early present in the subiculum of other transgenic models [38, 39, 52].

As to which Aβ (intra or extra) mainly contributes to the neuronal death in this region, our data point to the extracellular pool as the most likely toxic agent. Interneurons do not express the human mutant AβPP transgene in our AβPP/PS1 model (see Fig. 6).
therefore these cells do not produce and accumulate Aβ, ruling out the possibility of the intraneuronal source as the causative agent for the death in the SOM-population. On the other hand, principal subicular neurons contain intracellular Aβ at an early age (2 months or even before), and though the present results do not allow us to completely discard the toxic role of this intracellular stock, the loss of this population (approximately 30%) is delayed until the age of 6 months. Thus, it is most likely to be associated with the accelerated extracellular Aβ accumulation. This aseveration was also based on: 1) early (2 months) intracellular Aβ accumulation is seen in CA1 principal neurons of our model (see Fig. 4 A,D), however no neuronal loss in the CA1 pyramidal layer is detected until 17-18 months of age [27, 44]; 2) principal cell loss in the entorhinal cortex is also an early event (6 months) [26, 27]; 3) principal neuronal loss in the CA1 pyramidal layer is detected [27, 44], however no neuronal loss is usually absent or occurred at late ages [7–10]; 4) Aβ plaques are a potent source of neurotoxic damage as many axonal dystrophies developed in their close periphery (neuritic plaques); 5) Aβ plaques induced a strong glial activation in subiculum (data not shown) as seen in hippocampus and entorhinal cortex [26, 27]. A cytotoxic profile of this intracellular Aβ accumulation. This accumulation was also based on: 1) early (2 months) intracellular Aβ accumulation is seen in CA1 principal neurons of our model (see Fig. 4 A,D), however no neuronal loss in the CA1 pyramidal layer is detected until 17-18 months of age [27, 44]; 2) principal cell loss in the entorhinal cortex is also an early event (6 months) [26, 27]; 3) principal neuronal loss in the CA1 pyramidal layer is detected [27, 44], however no neuronal loss is usually absent or occurred at late ages [7–10]; 4) Aβ plaques are a potent source of neurotoxic damage as many axonal dystrophies developed in their close periphery (neuritic plaques); 5) Aβ plaques induced a strong glial activation in subiculum (data not shown) as seen in hippocampus and entorhinal cortex [26, 27]. A cytotoxic profile of this intracellular Aβ accumulation. This accumulation was also based on: 1) early (2 months) intracellular Aβ accumulation is seen in CA1 principal neurons of our model (see Fig. 4 A,D), however no neuronal loss in the CA1 pyramidal layer is detected until 17-18 months of age [27, 44]; 2) principal cell loss in the entorhinal cortex is also an early event (6 months) [26, 27]; 3) principal neuronal loss in the CA1 pyramidal layer is detected [27, 44], however no neuronal loss is usually absent or occurred at late ages [7–10]; 4) Aβ plaques are a potent source of neurotoxic damage as many axonal dystrophies developed in their close periphery (neuritic plaques); 5) Aβ plaques induced a strong glial activation in subiculum (data not shown) as seen in hippocampus and entorhinal cortex [26, 27].

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- Summary of the major neuropathological findings in the AβPP/PS1 London/PS1M146L transgenic model

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axonal/synaptic damage could trigger a neurodegenerative process toward the cell bodies. In line with this, we and others have reported a relationship between extracellular Aβ accumulation in axonal fields and the progressive degeneration of their away located projecting neurons in AD models [24, 57]. This suggestion is also supported by the apparent resistance of the PV positive GABAergic cells. As shown in this and previous work, the number of subicular, hippocampal, and entorhinal PV positive cells was not modified in the AβPP/PS1 model. These data are in line with the relative resistance of these cells observed in AD patients [58, 59]. Furthermore, at the age tested, the relative resistance of these cells observed in AD models [24, 57]. This suggestion of neuronal/axonal damage might contribute to the altered neuronal network in the hippocampus and entorhinal cortex seen in AD.

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