Short Communication

Peripheral Amyloid Levels Present Gender Differences Associated with Aging in A β PP/PS1 Mice

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Abstract. The accumulation of amyloid- β (A β) peptide is one of the major neuropathological hallmarks of Alzheimer's disease (AD). We have analyzed whether the progression of amyloidosis differentially affects males and females along aging in A β PP/PS1 transgenic mice. The levels of peripheral amyloid, A β ₄₀ and A β ₄₂, are not modified in either sex until 9 months of age. After that, however, there is an increase in amyloid levels in plasma among females and a decrease among males. These findings could be essential to design gender-specific strategies in other *in vivo* experiments or even in AD treatments.

Keywords: Aging, Alzheimer's disease models, amyloid- β , A β PP/PS1, peripheral amyloid, transgenic mice

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by two pathological features in the brain, extracellular amyloid plaques and intracellular neurofibrillary tangles [1, 2]. Most of AD cases are sporadic (unknown cause); however a minor population of AD is from genetic origin, known as familial AD and related to any mutation in the amyloid- β protein precursor (A β PP) [3], Presenilin 1 [4], or Presenilin 2 [5]. In the study of AD and in order to find therapies, different transgenic mouse models expressing A β PP and/or PS1/2 human mutations have been reported. These transgenic mice accumulate A β as diffuse neuritic plaques [6]. These models are a useful tool for evaluating the effects of potential therapeutic drugs designed to target A β , even

though the tau pathology is not completely reflected in these models [7–9].

The initial mechanisms of pathology in sporadic AD remain unclear, however, some risk factors have been reported with age a strong one (see i.e., [10, 11]). Epidemiological studies show an increased risk of AD with the age-related loss of sex steroid hormones. Clinical studies have shown that females present an increased risk of developing AD when compared with males [12–14].

Our aim in the present study is to evaluate whether a double-transgenic (A β PP/PS1) mouse model presented differences associated with aging and/or gender. Thus, we have analyzed A β production and accumulation; and some additional brain markers over the life-span of A β PP/PS1 mice [15], evaluating males and females separately.

A β ₄₀ and A β ₄₂ levels have been analyzed in brain tissue and in peripheral circulation (blood/plasma). It has been suggested that A β is kept in equilibrium

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between brain and blood through the blood-brain barrier, and that peripheral sequestration of A β may shift this equilibrium from the brain, which can reduce A β in the brain following a reduction in the levels in peripheral blood ("sink" effect) [7, 8]. As a general procedure, one hemisphere was snap frozen for subsequent homogenization either by ELISA or Western blot and the other fixed for histology studies (more detail in Supplementary Material) [16]. Blood samples were taken by submandibular vein puncture [17] to isolate plasma fraction in EDTA.

The levels of A β_{40} and A β_{42} in plasma showed important differences between males and females (Fig. 1A, B). The concentration of A β_{40} in female plasma from 3 to 15 months increased by more than 80%, while it decreased by 40% in males. The oldest mice tested, 15 months, showed that the levels of A β_{40} in females were more than 4-fold higher than in males. In the analysis of A β_{42} , slighter differences were found; A β_{42} levels in female plasma were 25% more abundant at 15 months than at 3 months. Meanwhile, male levels decreased by nearly 20% from 3 months to 15 months. Older mice showed that females' plasma levels were 1.3 times higher than in males ($p=0.007$) at 15 months. Some previous reports had found that A β PP23 transgenic mice didn't show any differences in the concentration of A β in plasma with age [18]. However, other studies with A β PP2576 strain males confirmed our findings [19]. Nevertheless, this is the first time age-associated differences in plasma A β levels are described between males and females.

The A β_{42} /A β_{40} ratio is another important marker that may reflect cognitive decline and neurodegeneration [20, 21]. This value showed gender differences in mice older than 12 months. The A β_{42} /A β_{40} ratio changes with age from 3 to 15 months: it is nearly 30% higher in males and more than 50% lower in females (Fig. 1C). Higher toxicity has been described at higher A β_{42} /A β_{40} ratios in cultured neurons [21], as has a relationship between lower levels of A β_{42} and A β_{42} /A β_{40} ratio in plasma and the progression of AD [22]. The difference between A β_{42} /A β_{40} has been seen by some authors as confirmation that A β_{42} deposition precedes A β_{40} and that all A β -positive plaques include A β_{42} but do not necessarily contain A β_{40} [23].

All these data are really important and must be taken into account in all experiments designed with these transgenic mice in any therapeutic assay designed or to be designed. Of course, these results make it impracticable to mix sexes in any assay with mice older than 10–12 months, particularly if the studies are testing the level of A β in plasma.

A β PP/PS1 mice showed A β plaques in the brain from 3 months of age (diffuse plaques) and levels were highest at 12–15 months of age [24]. We have checked the levels of A β_{40} in a large number of brain samples (37 males and 33 females), from one to 15 months with the same ELISA kit used in plasma analysis. There were no statistically significant differences (gender related) along the life-span analyzed. However, the increase in the concentration seemed to be faster in females, reaching the plateau before males (Fig. 1D). These data contrast with previous published figures that found differences between males and females at all age points studied [24]. Our levels may show higher deviations between individuals.

In parallel we have analyzed the level of brain amyloid deposition at different age by immunohistochemistry as described in the Supplementary Material. The quantification of plaques showed that males reached the plateau before females, at 9 months ($p=0.009$), just the opposite of what we found in the ELISA tests (Fig. 2A–C). Although A β concentrations increased more in females, the number of plaques was smaller than in males. This observation confirms that the deposition kinetic varies greatly between the sexes.

We can conclude that A β PP/PS1 transgenic mice show slight gender differences in the kinetic of accumulation but not in the final amount of amyloid accumulated at older age. The number and complexity of the plaques increase exponentially with age, being surrounded by *reactive* glia (Fig. 2B). These mature plaques finally occupied a significant portion of the brain tissue; the quantification showed no significant differences; males *versus* females. Surprisingly this final accumulation, at least in our colony, doesn't imply any significant reduction in the survival rate, as reported in some A β PP/PS1 colonies [1, 16]. In addition, some mice reach surprisingly high ages of 24 months in overall good health. However, we have observed some bladder malfunction presented as a swollen bladder that affects differentially both sexes (Supplementary Fig. 1B). Males showed these problems from the very beginning of their lives, while females do not present any before 10 months of age (Supplementary Fig. 1A). This event could be related to unknown deposits in the urine that are not A β -positive unless by ELISA (data not shown). Although changes in bladder innervation have been described in A β PP/PS1 mice [25], we can confirm that this event shows gender differences too.

Finally, we have checked some biochemical parameters following molecular markers by Western blot in brain samples (described in Supplementary Material).

Fig. 1. A β levels in plasma and brain samples of males (blue square) and females (pink triangle) transgenic mice. Levels of plasma A β_{40} (A) and A β_{42} (B) in mice (from 3 to 15 months) were obtained by ELISA. C) Ratio A β_{42} /A β_{40} in plasma from 3 to 15 months. D) A β_{40} burden in brain samples of transgenic mice from 1 to 15 months. When statistically significant $**p \leq 0.01$ (t -test). Western blot analysis of some biochemical markers in the mice brains; 6 and 15 months, male, female, transgenic and wild type. A β and β -secretase levels (E), synaptic markers (F) and kinases (G).

Fig. 2. Histology analysis of males and females transgenic mice brains from 3 to 15 months. A) Representative images of immunohistochemistry studies (6e10 antibody). B) Comparison of the increase in A β plaques deposition and glial reaction between 3- and 23-months-old mice. A β plaques (in red) marked with 6e10 antibody and glial (in green) marked with GFAP antibody, DAPI in blue was used to stain nuclei. C) Graph of the percentage of area occupied by plaques obtained by Image J software. When statistically significant $**p \leq 0.01$ (*t*-test).

Levels of β -secretase (BACE), PHF1, GFAP and A β (monomer and oligomer) have been checked in all the groups analyzed; 6 and 15 months, transgenic and wild-type (wt), males and females (Supplementary Fig. 1C). We have compared the levels of some relevant kinases for tau phosphorylation, such as Akt, GSK3, and Erk (Fig. 1G); and some synaptic proteins such as PSD95, p120, p-Synapsin and N-Catenin (Fig. 1F).

It is very relevant that the levels of p-synapsin, p120 and PSD95 are increased in old transgenic females being higher than transgenic males. Moreover, 15 months transgenic females showed higher levels of p120 and PSD95 than wt at the same age. Levels of N-catenin were higher in transgenic females than transgenic males at 6 months of age but no differences were found in older mice with this marker. In general, we can observe that 6 months mice did not show any modification in the synaptic markers; however we can see very increased levels in old transgenic females.

Kinases determination showed different results. Wt females showed higher levels of pAKT than transgenic females at 6 months; however pAKT levels were not modified at 15 months. When we analyze p-Erk we have found increased levels in old transgenic males while females showed no differences. Finally, pGSK3 was not modified in any group.

In conclusion, both genders accumulate plaques in the brain exponentially with age and they both have detectable levels of A β in plasma. However, the evolution of A β levels is divergent, so it is tempting to propose that an "aging factor" differentially affects male and female specimens in this AD model. These gender differences may be related to sex hormones; being even though some hypotheses point to oestrogen as the main party responsible [26, 27].

Finally, considering the differences shown in this mice model, we would like to emphasize that these differences should be taken into account not only for previously published data but also for future experiments because mixing male and female will result in confusing or counter-balanced results [8]. In addition, this opens up the possibility of performing a wide-ranging analysis of peripheral A β in the human population to try to correlate the amount of A β ₄₂/A β ₄₀ with normal aging and AD evolution in both genders.

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SUPPLEMENTARY MATERIAL

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