Estudi de la relació existent entre les alteracions de la barrera hematoencefàlica i la β-amiloïdosi en el model murí de senescència accelerada i malaltia d'Alzheimer SAMP8.

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(Summarized version of the thesis “Estudi de la relació existent entre les alteracions de la barrera hematoencefàlica i la β-amiloïdosi en el model murí de senescència accelerada i malaltia d’Alzheimer SAMP8”)

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2010

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Programa de doctorat:

Recerca, desenvolupament i control de medicaments

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Memòria presentada per Jaume del Valle i Macià per optar al títol de doctor per la universitat de Barcelona

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2010

1. Introduction

1.1 Alzheimer's disease

More than a hundred years ago, Alzheimer's disease (AD) was first described in a patient, Auguste D., as a rare case of dementia (Alzheimer, 1907). Nowadays, AD is a neurodegenerative disorder that is the most common cause of dementia in the aged population, affecting one in ten people over 65 years of age (Smith, 1998). What's more, the number of people with the disease doubles for every 5 year age interval beyond age 65 (Katzman and Fox, 1999), and by the year 2050 it will affect more than a hundred million people worldwide. Due to the increasing age of the population, AD has become one of the biggest healthcare challenges of the 21st century (Avramopoulos, 2009).

Alzheimer's disease is a slowly progressive disorder, with insidious onset and progressive impairment of episodic memory; instrumental signs include aphasia, apraxia, and agnosia, together with general cognitive symptoms, such as impaired judgment, decision-making, and orientation (Blennow et al., 2006). Late-onset AD is the most common form of AD (nearly 90%), appearing after 65 years of age (Newman et al., 2007), and susceptibility to late-onset AD shows no apparent familial aggregation (Bertram and Tanzi, 2008). On the other hand, early-onset AD is the rarest form of the disease with almost half of the cases being due to familial AD.

The Clinical Dementia Rating scale (CDR) can be used to outline three ordinal stages of AD as mild (CDR 1), moderate (CDR 2), or severe (CDR 3). Therefore, a conceptual framework can be provided for the clinical classification of AD along the cognitive decline after the evolution of the dementia (Hughes et al., 1982). In addition, the concept of mild cognitive impairment (MCI) as a phase between normal aging, early dementia, and AD has recently appeared and can be used as an early detection of the disease (Markesbery et al., 2006). Therefore, in the clinical progression from MCI to AD the subject may still remain in a CDR 0,5 category (Petersen, 2004).

The two main hallmarks of AD are the progressive extracellular accumulation of amyloid deposits of amyloid-β peptides (Aβ), often known as senile plaques, and intracellular neurofibrillary (NFT) tangles of hyperphosphorylated tau (Selkoe, 1991).

These lesions are mainly located in the neocortex and the hippocampus of AD patients (Taguchi et al., 2005). Although the etiology of AD remains unknown the senile plaques and the NFT are still the only established diagnoses of the disease presently available that rely on post-mortem verification (McKhann et al., 1984; Epis et al., 2010). However, how these two pathological hallmarks are connected and the influence they have in the onset and progression of the disease is still unclear and various hypotheses have been suggested to explain the molecular pathogenesis of AD (Mudher and Lovestone, 2005).

Among the different hypotheses, one can find the cholinergic hypothesis (Bartus et al., 1982; Francis et al., 1999), the amyloid cascade hypothesis (Hardy and Higgins, 1992; Hardy, 2006; Pimplikar, 2009), the tau and tangle hypothesis (Mudher and Lovestone, 2002), the oxidative stress hypothesis (Markesbery, 1997) or the two hit hypothesis (Zhu et al., 2004). In 1989, it was suggested that the possible importance of a failing blood brain barrier (BBB) and the vascular defects present in AD might be important in the development of the disease (Scheibel et al., 1989) and several years later the neurovascular hypothesis was proposed (Zlokovic, 2005) (Fig 1). Briefly, this hypothesis claims that in AD, a defective clearance of Aβ across the blood-brain barrier (BBB), the senescence of the cerebrovascular system and an altered angiogenesis could lead to Aβ accumulation, neurovascular uncoupling, vessel regression, brain hypoperfusion and neuro-vascular inflammation. These facts would lead to BBB disruption, chemical imbalance in the neuronal environment and to synaptic and neuronal dysfunction, injury and loss (Zlokovic, 2005).

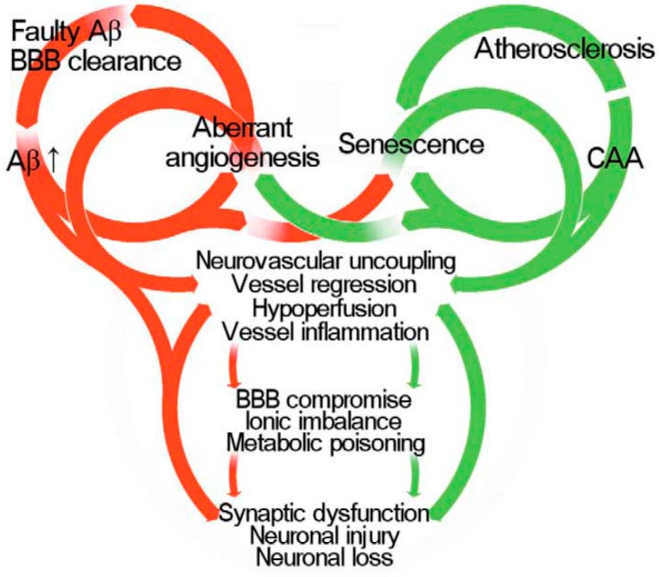

Fig 1 Neurovascular model of Alzheimer's disease. Multiple pathogenic cascades originating from altered cerebral arteries (green) or altered brain capillaries (red) can initiate disintegration of the neurovascular unit, including aberrant angiogenesis, cerebral amyloid angiopathy (CAA), senescence and faulty clearance of Aβ across the BBB, resulting in increased Aβ levels. This leads to BBB compromise which can result in, or amplify, synaptic and/or neuronal and oligodendroglial dysfunction, neuronal injury and loss. Adapted from Zlokovic, 2005.

1.2 The blood-brain barrier

The barrier function of the BBB is a combination of the physical barrier, made of tight junctions (TJ) between adjacent brain endothelial cells (BEC) that force most molecular traffic to take a transcellular route across the BBB, the transport barrier that can permit or facilitate the entry of required nutrients and is also able to exclude or efflux potentially harmful compounds, and the metabolic barrier, a combination of intracellular and extracellular enzymes capable of metabolizing and inactivating many neuroactive and toxic compounds (Abbott *et al*., 2006).

The BEC and the TJ, altogether with neurons, and nonneuronal cells (e.g., pericytes, astrocytes, and microglia) together form a functional unit, often referred to as the "neurovascular unit" (fig 2A) (Zlokovic, 2005). The close proximity of different nonneuronal cell types with each other and with neurons allows for effective paracrine regulations that are critical for normal CNS functioning and disease processes (Zlokovic, 2008).

If a brain disease has its origin in a primary neuronal disorder, the neurons can send signals to the neurovascular unit and activate it, contributing to the progression of the disease. If the disease has a primary vascular origin, the activated endothelium, microglia and astrocytes can send signals to neurons, exacerbating in most cases the neuronal injury. In addition, circulating neurotoxins will be able to cross the BBB and reach their neuronal targets, and proinflammatory signals in BEC or reduction of the brain blood flow can disrupt normal synaptic transmission and cause neuronal injury. Moreover, microglia recruited from the blood or brain and the vessel wall can detect signals from neurons (Zlokovic, 2008).

A large number of risk factors for AD and vascular dementia are shared, such as advanced age, atherosclerosis, stroke and transient ischemic attacks, hypertension, hyperlipidemia, head injury, elevated serum viscosity, thrombogenic factors, heart disease, diabetes and apoE4 (Iadecola, 2004; Zlokovic, 2005; de la Torre, 2004; Luchsinger et al., 2007). Hypoperfusion and hypoxia are also present in the brains of AD patients. In addition, several changes have been described in brain microvessels of patients with AD, such as reduced microvascular density, an increased number of fragmented vessels, atrophic string vessels, an increased irregularity of capillary surfaces, capillary basement membrane thickening, and collagen accumulation in the basement membrane (Farkas and Luiten, 2001, Bailey *et al*., 2004). In addition,

increased levels of collagen IV (Kalaria and Pax, 1995), deposits in the basement membrane of heparan sulfate proteoglycans (HPSG) and laminin (Verbeek *et al*., 1999; Berzin *et al*., 2000), degeneration of the BEC (Kalaria and Hedera, 1995) as well as edema in astrocyte foot processes and an increase in the number of pinocytic vesicles in BEC have also been described (Farkas and Luiten, 2001, Bailey *et al*., 2004).

In AD, Aβ accumulation in the brain and vessels lead to the development of cerebral amyloid angiopathy (CAA) (Greenberg et al., 2004). On the other hand, there is a reduction in the expression of GLUT1 in brain capillaries, although no changes occur in the structure of its mRNA (Mooradian et al., 1997) or alterations in its levels (Wu et al., 2005). In addition, the available area for glucose transport is significantly decreased (Bailey et al., 2004), suggesting that in AD the brain undergoes a reduction of energy intake due to alterations in the BBB. It has also been described that in AD, degeneration of BEC may reflect aberrant angiogenesis (Zlokovic, 2008). In addition, it has been observed that Aβ accumulation in the outer membrane of blood vessels has antiangiogenic effects and this could contribute to the reduction of capillary density observed in AD (Paris et al., 2004).

With regard to Aβ transport across the BBB, it has been observed that RAGE transporters, which capture and internalize circulating Aβ to the brain through the BBB, show increased levels in the cerebral vessels of patients with AD (Deane et al., 2003). Moreover, the LRP1 is a major efflux transporter of Aβ across the BBB (Shibata et al., 2000) and a reduced expression of LRP has been found during normal aging in rodents, nonhuman primates, and in AD individuals associated with positive staining of cerebral vessels for $A\beta_{40}$ and $A\beta_{42}$ (Shibata et al., 2000, Deane et al. , 2004, Donahue et at., 2006.)

1.3 The SAMP8 mice

Although some other species are used, the mouse is the major species used in AD research. The finding that in the familial forms of AD the genes that encode the proteins that are deposited in plaques and NFTs are mutated suggested a causal role for these proteins in disease and led to the generation of transgenic animal models (Götz et al., 2007), but they account for less than 5% of all AD cases (Tanzi and Bertram, 2005). However, there are non-transgenic models available such as the SAM (senescence-accelerated mice) mice that have shed some light on the AD research.

The senescence-accelerated mouse prone 8 (SAMP8) mice is one of the SAM strains that were developed by selective breeding of AKR/J mice in the Kyoto university by the group of Dr. Takeda (1981). These mice show an accelerated aging process and have been used in several behavioral, neuropathological and neurochemical studies as a model of AD and neurodegeneration (Pallàs et al., 2008; Takeda, 2009). SAMP8 mice share similar characteristics with aged humans such as a reduced lifespan, lordosis, hair loss and reduced physical activity (Takeda et al., 1994). Moreover, they exhibit impairments in learning tasks, as well as altered emotions and abnormality of the circadian rhythm (Miyamoto, 1997).

In addition, they present several neuropathological alterations that can be seen with advancing age in humans and rodents such as spongy degeneration (Yagi et al., 1989), neuronal cell loss (Kawamata et al., 1994), and gliosis (Nomura and Okuma, 1999) in the brain. Notably, SAMP8 also show similar changes to those described in AD in humans, such as learning and memory deficits (Nomura and Okuma, 1999), brain microvessels defects (Ueno et al., 2001), blood-brain barrier dysfunction (Ueno et al., 1993; Hosokawa and Ueno, 1999), alteration of the cholinergic system (Onozuka et al., 2002) and other neurotransmitter changes (Flood et al., 1998; Nomura et al., 1996).

Aβ presence and levels in SAMP8 mice have been studied by various authors. However, due to variations in data reported, in a recent review of the SAMP8 mice it was claimed to have confirmation of the Aβ deposition, and particularly of senile plaque-like structures, in these animals (Takeda, 2009). For example, it has been described an increased level of amyloid burden in the brain parenchyma, based on reports of brain overproduction of APP and amyloid-β (Morley et al., 2000; Takemura et al., 1993) and an increase in the brain cortex and hippocampus of APP mRNA expression in SAMP8 at 6 months of age (Tha et al., 2000). On the other hand, some

reports describe Aβ deposition no sooner than at 16 months (Morley et al., 2000) or at 18 months (Pallàs et al., 2008) but also as early as at 2 months of age (Takemura et al., 1993).

On the other hand, several authors have analysed the BBB status in SAMP8 mice and some controversy has arisen in the reported results. Some studies claim a BBB disruption in SAMP8 mice due to changes in the BBB permeability and consequent extravasation of different endogenous plasmatic substances and exogenous dyes (Ueno et al., 1993, 2001; Hosokawa and Ueno 1999). On the contrary, some other studies question the BBB disruption and report no brain retention and no brain presence of plasmatic molecules and an unaltered brain transport of different compounds (Banks et al., 2000, 2001; Moinuddin et al., 2000).

2. Aims and scope

The overall objective of this thesis is to characterize the time course of the alterations in the BBB of SAMP8 mice and to study the presence of amyloidosis in these animals as well as to determine the relationship between these two variables.

Specifically, the next aims are formulated:

1. To determine the existence and temporal progression of any changes in the permeability of the BBB in the hippocampus and the cerebral cortex of SAMP8 mice.
   1.1. To establish different methods to determine the BBB permeability to endogen plasmatic substances and/or exogenous dyes.
   1.2. To put into practice the different methods, previously established, in SAMP8 mice and compare the results with control strains.

2. To characterize the Aβ presence and its temporal progression in the hippocampus and brain cortex of SAMP8 mice.
   2.1. To describe the Aβ and APP staining with immunohystochemistry and microscopic analysis.
   2.2. To quantify and compare the different Aβ and APP stainings formerly described in SAMP8 mice and control strains.
   2.3. To characterize the temporal evolution of the number of amyloid positive vessels in SAMP8 and control strains.

3. To study the possible spatial and temporal relationship between the increase in Aβ and the BBB disruption in SAMP8 mice.
   3.1. To analyze whether amyloid cluster localization is associated with brain capillaries.
   3.2. To determine if the amyloid clusters are placed in brain areas with BBB disruption.
   3.3. To establish the possible relationship between amyloid clusters and amyloid positive vessels.
   3.4. To establish the relationship between amyloid positive vessels and the vessels with a disrupted BBB.

3. Results

3.1 Article 1

Increased permeability of blood-brain barrier on the hippocampus of a murine model of senescence

Mechanisms of Ageing and Development 2007; 128: 522-528.

Aim: To determine and quantify the time course of BBB disruption by analyzing blood to brain IgG permeability in the hippocampus of SAMP8 mice.

Materials & methods: Male SAMP8 and SAMR1 mice, 3, 7 and 12-month-old were used. The brain was obtained after anesthesia and perfusion with saline. Thereafter, frozen brains were cut into 20 μm-thick sections on a cryostat. Immunohystochemistry was performed with anti-PECAM antibodies to detect blood vessels and anti-IgG antibodies. A new image analysis methodology was designed to objectively determine the magnitude of the IgG extravasation in the hippocampus.

Results: SAMP8 mice of 12 months of age showed significantly higher levels of IgG extravasation in the hippocampus than age-matched SAMR1 animals. Neither SAMP8 nor SAMR1 mice aged 3 and 7 months showed positive staining for IgG. A marked IgG staining was observed in choroid plexus of both studied strains as expected due to the characteristic fenestrated capillaries of this zone.

Conclusions: The BBB is more disrupted in the hippocampus of 12-month-old SAMP8 mice than in age-matched SAMR1. SAMP8 of younger ages show no BBB alteration in the IgG permeability.

3.2 Article 2

A new method for determining blood–brain barrier integrity based on intracardiac perfusion of an Evans Blue–Hoechst cocktail

Journal of Neuroscience Methods 2008; 174: 42–49.

Aim: To describe a new method to determine the BBB permeability to exogenous dyes which would allow the application of immunohistochemistry to characterize different cerebral structures.

Materials & methods: In order to generate a brain region with a control positive BBB disruption, a cone-shaped pellet of dry ice with a 5-mm diameter tip was placed in direct contact with the exposed skull. Then, the animals were perfused with the cocktail solution, derived of a standard fixative solution of p-formaldehyde 4% with the addition of Evans blue (EB) and Hoechst as tracers. In order to compare the results, a group of animals received bovine serum albumin in the cocktail solution and another group did not receive the cryolesion. The results were also compared with the intravenous and intraperitoneal administration of the tracers prior to intracardiac perfusion.

Results: The cocktail method allows localization of the cryo-injured region by EB and Hoechst fluorescence. Both dyes also stained the choroid plexus and the circumventricular organs where there is no BBB and the capillaries are fenestrated. The EB staining obtained with the cocktail method is stronger than the obtained with previously described methods, i.e. intravenous and intraperitoneal administration. The Hoechst staining is only suitable to characterize BBB disruption when it is administered following the cocktail method because systemic administration of this dye stains all the nuclei of the brain parenchyma. Immunohistochemistry is suitable to be performed in brain sections after cocktail administration.

Conclusions: The cocktail method allows determining BBB integrity with two different tracers and also performing immunohistochemistry techniques to characterize and localize different cells and structures involved in the BBB disruption.

3.3 Article 3

Time-course of blood–brain barrier disruption in senescence-accelerated mouse prone 8 (SAMP8) mice

International Journal of Developmental Neuroscience 2009; 27: 47–52.

Aim: To characterize the time course of BBB permeability to exogenous dyes in SAMP8.

Materials & methods: Three, 6, 9, 12 and 15 month-old male SAMP8, SAMR1 (a strain genetically related to SAMP8) and ICR-CD1 (a control strain not genetically related to the other two) mice were studied. The animals were perfused with the cocktail solution following the cocktail method. The brains were obtained and coronal sections were made in the cryostat. The sections were observed in the fluorescence microscope and several images were obtained. The CA1 hippocampal region, the hippocampal fissure and the brain cortex were analyzed and the mean EB fluorescence was quantified by ImageJ software.

Results: The BBB integrity remains stable throughout the lifespan of ICR-CD1 mice. On the other hand, both SAMP8 and SAMR1 show a progressive increase of EB fluorescence from 6 to 15 months of age in all studied regions. During this period, EB extravasation was higher in the CA1 and cortex regions of the SAMP8 mice than in the SAMR1 mice. At 15 months of age both SAM strains showed higher extravasation values than ICR mice in CA1 and hippocampal fissure.

Conclusions: SAMP8 and SAMR1 mice show an increasing disruption of BBB with age. These alterations could contribute to the brain pathology described in these mice.

3.4 Article 4

Early amyloid accumulation in the hippocampus of SAMP8 mice

Journal of Alzheimer's Disease 2010; 19: 1303–1315.

Aim: To characterize Aβ deposition and its temporal progression in the hippocampus of SAMP8 mice

Materials & methods: Male SAMP8 aged 3, 6, 9, 12 and 15 months were used. The animals were perfused, the brains were dissected and cryostatic sections were obtained. Immunohistochemistry was performed to characterize APP, and Aβ, $A\beta_{40}$, and $A\beta_{42}$. The brain sections were observed and the amyloid burden was quantified by three blinded observers using a laser microscope. As positive granules were clustered, all the observers quantified the number of clusters in each hippocampus. Same procedures were applied to SAMR1 and ICR-CD1 control strains.

Results: SAMP8 mice present hippocampal amyloid deposits which increase from 3 months of age to 15 months. The deposits are constituted by clustered granules essentially containing $A\beta_{42}$ peptide but also $A\beta_{40}$ and APP fragments. Some basal levels of amyloid accumulation were found from 3 to 12 months of age in the two control strains, with increased values at 15 months of age although the levels did not reach those of SAMP8 animals.

Conclusions: SAMP8 mice show a marked amyloid deposition from 6 months onwards compared with SAMR1 and ICR-CD1 control strains. Therefore, SAMP8 mice become a very useful tool to study neurodegeneration and to understand the mechanisms involved in the formation of Aβ deposition in Alzheimer's disease.

3.5 Article 5

Cerebral amyloid angiopathy, blood-brain barrier disruption and amyloid accumulation in SAMP8 mice.

Submission pending

Aim: To characterize the temporal evolution of Aβ deposition in blood vessels and to study the spatial relationship between an increase in Aβ and the BBB disruption in SAMP8 mice.

Materials & methods: Male SAMP8 and ICR aged 3, 6, 9 and 12 months were used. The animals were perfused, the brains were dissected and cryostatic sections were obtained. Immunohistochemistry was performed to characterize blood vessels, clusters of $A\beta_{40}$, and $A\beta_{40}$ and IgG accumulation in blood vessels. The brain sections were observed and the number of amyloid positive vessels, positive IgG vessels and $A\beta_{40}$ clusters in the CA1 subzone of the hippocampus were quantified by two blinded observers using a laser microscope.

Results: In the CA1 subzone of the hippocampus, the SAMP8 strain of mice show more amyloid positive vessels than age-matched ICR mice. Aging increases this deposition in both strains. As expected, amyloid clusters increased with aging in SAMP8 but not in ICR mice. Moreover, an increase in the number of IgG positive vessels can be observed at 12 months of age in both strains, being the levels higher for SAMP8 than for ICR. Moreover, amyloid cluster deposition shows no direct spatial association with the amyloid positive vessels and with the BBB disrupted vessels. In addition, at 12 months of age, all the vessels with amyloid deposition also showed IgG deposition, but several capillaries with BBB disruption showed no amyloid within their walls.

Conclusions: As expected, SAMP8 mice show a marked amyloid deposition. BBB disruption and CAA increase with age. Amyloid aggregates are not directly associated with neither blood vessels, BBB disrupted vessels or amyloid positive vessels. At 12

months of age, all the amyloid positive vessels are IgG positive but not all the IgG vessels show amyloid deposition.

4. Discussion

The experiments performed in this thesis have allowed to characterize the time course of BBB disruption and the amyloid deposition in the vessels and in the parenchyma of the hippocampus of SAMP8 mice, a strain of mice with an accelerated senescence and a model of neurodegeneration (Takeda, 2009) and AD (Morley et al., 2004; Pallàs et al., 2008).

Manifold methods have been applied to investigate BBB integrity in different animal models, being the most popular the analysis of tracer extravasation or plasmatic presence in the brain parenchyma. These substances can only be found in the brain when the BBB integrity is compromised. Some authors claim that the pathologic responses to different brain insults can be heterogenic and different BBB alterations can occur at the same time in different vascular segments or brain regions (Nitsch and Klatzo, 1983; Ge et al., 2005). These arguments led us to develop new methods where different brain areas with different BBB integrity could be characterized unlike some other established methods where mechanical disgregation does not allow localizing BBB disturbances.

Several studies have used the visualization of IgG extravasation as a variable to identify BBB disturbances in murine models of a variety of diseases (Fullerton et al., 2001; Natah et al., 2005; Sugimura et al., 2005; Tomás-Camardiel et al., 2005). In the first study, an imaging methodology was specially designed to quantify IgG extravasation. This novel strategy allows for the quantification of extravasation extension to objectively analyze the existence of significant differences between both mice strains. As expected, the vessels in the hippocampal fissure and the choroid plexus exhibited positive staining to IgG in all the animals due to the fenestrated capillaries characteristic of this zone.

The presence of IgG in the parenchyma of the hippocampus from SAMP8 mice can be explained by three different mechanisms: 1) local synthesis of IgG in the brain tissue, which would involve some kind of immune or autoimmune response, 2) disturbances on the carriers situated on the plasmatic membranes of endothelial cells and responsible of the transcellular exchange of IgG, and 3) disturbances in the intercellular junctions of the vascular endothelium that constitute the BBB, which would allow the

paracellular flux of IgG. To this end, the first explanation seems to be the least plausible because it would imply IgG staining not only around the blood vessel but also where the microglial cells would be activated. In addition, provided the IgG around the blood vessels was produced from IgG secretor cells there would be an accumulation of these cells adjacent to the vessels and Hoechst staining would have showed cellular aggregates or non-endothelial extravascular cells responsible of the generation of this IgG. For the second option, it should be taken into account that the BBB has several IgG transporters that can efflux the IgG from the brain to the blood (Schlachetzki et al., 2002), if IgG could not be eliminated due to alterations in its transporters it would imply that IgG could be found throughout the brain parenchyma and not only near the blood vessels. The third option seems the most plausible because IgG appears as an aureole around the blood vessel and it loses intensity when the distance from the blood vessel increases. The aureole around the vessels can be easily explained by an extravasation of IgG and its slow expansion towards the cerebral parenchyma. For all these reasons, the presence of IgG around the blood vessels in the hippocampus is related to its extravasation from functionally altered BBB.

In the second article, we developed a new method to characterize BBB integrity with two exogenous molecules, EB and Hoechst. Traditionally, EB and other tracers, such as trypan blue or horseradish peroxidase (HRP), have been administered intravenously or intraperitoneally (Broman, 1944; Dobbing, 1961; Broman et al., 1966; Reese and Karnovsky, 1967) to study BBB status. However, these methods require *in vivo* treatment of the animals which can affect the results. Moreover, as some tracers can be retained by lipid deposits, which may be modified in some pathological states, tracer extravasation quantification might be biased, especially when tracers are intraperitoneally administered. The addition of the tracers within the fixative solution administered by the intracardiac perfusion avoids having to wait for the recirculation of tracers and minimizes the variability caused by the method itself.

Some authors claim that responses of the BBB to pathological insults might be heterogeneous, varying along different segments of the brain vasculature and among different brain regions (Nitsch and Klatzo, 1983; Ge et al., 2005). Such claims support methods that use brain sections to study BBB, such as ours, in contrast with those that process the tissue by mechanical disaggregation. Additionally, microscopic examination of perfused brain tissue may enable visualization of discrete alterations in permeability.

In our case, EB fluoresces in the regions with altered BBB when it extravasates and binds to previously extravasated albumin or other proteins. In the case of Hoechst, the tracer fluoresces when it is linked to DNA and its diffusion produces staining of all the brain nuclei if given sufficient time to recirculate, as occurs when the dose is administered intravenously. When the cocktail method is adopted, the tracer only stains the nuclei of those zones in which the BBB is impaired and paracellular extravasation is possible. Both tracers stain the lesion and the surrounding areas when a cryolesion is provoked, although in this latter zone, Hoechst stains all the nuclei while EB only stains some of the cells. It is conceivable that Hoechst stains all the nuclei in the periphery of the cryolesion because of its greater capacity to diffuse from the injured area. The EB staining of the EB^+ cells in the perilesional rim is probably due to the presence of neurons whose damaged axons or axon collaterals cross through or terminate in the lesion and take up plasma proteins almost immediately after the lesion (Loberg and Torvik, 1991).

Moreover, as EB-protein and Hoechst-DNA complexes fluoresce at different emission wavelengths, this method allows subsequent immunofluorescence staining to be performed, thereby enabling us to characterize and analyze structural and cellular changes in regions where BBB disturbances are present. Thus, the cocktail method described here is particularly useful for studying the processes involved in BBB disruption and can be applied in a wide variety of BBB disruption models.

In the first article, neither SAMP8 nor SAMR1 mice aged 3 and 7 months showed positive staining for IgG. On the other hand, a clear IgG positive staining was observed on the hippocampus of 12-month-old SAMP8 mice, while only a weak extravasation appeared in age-matched SAMR1 animals. When IgG extravasation was quantified by an image analysis strategy, 12-month-old SAMP8 mice showed significantly higher levels of IgG extravasation in the hippocampus than SAMR1 animals.

Ueno et al. (1993) reported that the transference of radioactive serum albumin in the hippocampus of 13-month-old SAMP8 mice is increased in relation to that of 3-month-old SAMP8 and 13-month-old SAMR1 mice. However, other studies performed by administering radioactive albumin intravenously and measuring the radioactive albumin in cerebrospinal fluid/serum ratio (Banks et al., 2000), reported no disturbances of BBB permeability. Our results support those obtained by Ueno et al. (1993) since SAMP8 mice, as they age, would present increasing permeability not only for albumin but also for IgG. As permeability increases are observed for albumin and IgG extravasation, we

suggest that these changes might be explained by paracellular permeability disturbances. Although the functionality of the endothelial cells carriers for IgG in the BBB of SAMP8 mice is not known, modifications just on the carriers' activity would not explain changes in albumin permeability; while changes in paracellular or parajunctional flux can explain changes in permeability for both serum albumin and IgG.

In the third article we examined the BBB integrity during the aging process in the three strains of mice SAMP8, SAMR1 and ICR-CD1. Three different cerebral regions, the hippocampus, the hippocampal fissure and the cerebral cortex, were analyzed quantitatively for BBB permeability after using the cocktail method. We found that while BBB integrity remained stable throughout the lifespan of ICR-CD1 mice, SAMP8 and SAMR1 showed a progressive increase of EB fluorescence from 6 to 15 months of age in all studied regions.

Results obtained at 3 months of age were unexpected, as all strains showed higher EB extravasation values than those obtained at 6 months of age. Although we did not find an explanation for that fact, we performed the statistical analysis with or without including 3-months data and we obtained similar statistical results, indicating the robustness of the analysis and corroborating the increase of EB extravasation with age in SAMP8 and SAMR1 animals. With regard to the results obtained in the cortex, it should be considered that a cortical atrophy has been described in SAMP8 mice (Kawamata et al., 1994) and alterations in the cortex layers II and III in some SAM strains (Shimada, 1999) have also been reported. Therefore, these alterations could explain the lower values obtained in the cortex in SAMP8 and SAMR1 compared with ICR mice at young ages.

On the other hand, it is worth mentioning that SAMR1 show several alterations such as a marked loss of photoreceptor cells and ganglion cells late in life (Shoji et al., 1998), age-related hearing impairment (Takeda et al., 1997), nonthymic lymphoma, histiocytic sarcoma and ovarian cysts (Takeda et al., 1999). A disruption in BBB to small molecules such as EB could be another pathological alteration of this strain.

HRP has also been used by many authors as a tracer to evaluate BBB permeability. Ueno et al. (1997) administered HRP to young and aged SAMP8 and SAMR1 mice (3 and 13 months old). They reported a high HRP staining that spread diffusely in the hippocampus in old SAMP8 mice, but they observed no staining in young SAMP8

animals, nor in aged or young SAMR1 animals. Although we found that EB permeability increased in aged SAMP8 and SAMR1 mice, it should be taken into account that EB tracer molecule (mol. wt. 961 D) is much smaller than HRP (mol. wt. 40 kD). Thus, these results are not in contradiction with ours, as we detected permeability of the BBB to smaller molecules than those used by Ueno et al. (1997).

To finish with the discussion of BBB status in SAMP8, it should be borne in mind that although various studies have questioned the BBB integrity in SAMP8 mice, they have used different dyes, substances and techniques to assess BBB integrity, which may explain the differences in the results obtained. We describe BBB disturbances in the hippocampus of SAMP8 mice at 6 months of age and we also report that these disruptions become more accentuated at 12 months of age. The alterations observed in the hippocampus of SAMP8 mice could suggest a link between these alterations and the learning and memory deficits described in this strain (Takeda, 2009). On the other hand, it could also be possible that these two facts are linked with a common ethiopathogenic factor such as an increase in the amyloid burden. Therefore, an increase in the toxic levels of Aβ could lead to BBB compromise which could modify the chemical balance in the brain parenchyma and induce, or amplify, the pathological features described in SAMP8.

Several studies looking at Aβ accumulation have been conducted in mice to gain a better understanding of AD, however, in most cases, these studies have used transgenic mice, which mimic early-onset AD (Codita et al., 2006). Therefore, the need still exists for a reliable model of age-related AD, which constitutes nearly 90% of AD cases (Morrissette et al., 2009). Notably, SAMP8 show some other characteristics seen in AD patients, such as learning and memory deficits (Nomura and Okuma, 1999; Spangler et al., 2002), brain microvessels defects (Ueno et al., 2001), BBB dysfunction (Ueno et al., 1993), alteration of the cholinergic system (Onozuka et al., 2002) and other neurotransmitter changes (Flood et al., 1998; Kondziella et al., 2002; Nomura et al., 1996). Given such changes, it is perhaps not surprising that SAMP8 have also been proposed as an animal model of AD (Morley et al., 2004; Pallàs et al., 2008).

However, there have been very few articles describing Aβ deposition in SAMP8 from 1993 onwards (Fukunari et al., 1994; Kato et al., 1997; Morley et al., 2000). Surprisingly, the Aβ deposition has been reported to appear at 2 months (Takemura et al., 1993), but also at no earlier than 16 months (Morley et al., 2000). Due to the variations in data reported up to now, in a recent review of the SAMP strain some

authors claimed to have confirmed the presence of Aβ deposition, and particularly of senile plaque-like structures, in SAMP8 mice (Takeda, 2009).

Our findings clearly show the presence of an age-dependent amyloid deposition in the hippocampus of SAMP8 mice. Staining with the anti-amyloid antibody 4G8 permitted observation of clustered amyloid granules, which were mainly located in the *stratum radiatum* of the hippocampus. In the course of aging, they extended to other hippocampal regions and their number increased. Moreover, the clustered amyloid granules seen in SAMP8 are essentially formed by $A\beta_{42}$. In addition, our results also indicated some $A\beta_{40}$ and APP deposition in the granules, which is in agreement with previous studies which reported that Aβ plaques begin as diffuse plaques consisting mainly of $A\beta_{42}$ peptide and thereafter incorporate $A\beta_{40}$ (Tahara et al., 2006) and other APP and amyloid fragments. Of note, similar granules have been reported in APP transgenic mice (Siedlak et al., 2009).

The present research also found some basal levels of amyloid accumulation from 3 to 12 months of age in the two control strains, with increased values at 15 months of age. This is consistent with the described deposition of fibrillar material in the brains of control mice, which occurred only occasionally in aged individuals (Jucker et al., 1994). Therefore, the levels of amyloid accumulation described here are not inconsistent with previously reported data, as SAMR1 and ICR-CD1 mice could be either healthy or diseased subjects within the ages studied.

Ultimately, we conclude that SAMP8 mice show a marked amyloid deposition from 6 months onwards compared with SAMR1 and ICR-CD1 control strains. SAMP8 mice have been used in several behavioral, neuropathological and neurochemical studies as a model of AD and neurodegeneration (Pallàs et al., 2008; Takeda, 2009). The SAMP8 strain develops hippocampal cognitive deficits, learning, memory and emotional alterations, neurodegeneration, DNA damage and oxidative stress. Such AD-like features, added to the distinguished amyloid deposition, reveal the SAMP8 strain to be a very useful and accessible tool for researchers, enabling them to gain a better understanding of the different phenomena that occur in AD and particularly the relationship of the mechanisms involved in the formation of Aβ deposition in late-onset AD.

Aβ deposition within the walls of the leptomeninges and parenchymal arteries, arterioles, and capillaries is defined as CAA and can produce degeneration of smooth muscle cells, ischemic white matter damage, fibrinoid necrosis, and dementia (Jellinger 2007). Moreover, cerebrovascular dysfunction might be an early event in the mechanisms of AD (Iadecola 2004) and it has been suggested that this dysfunction may precede cognitive decline and onset of neurodegenerative changes in AD and AD models (Bell & Zlokovic, 2009). In the last study, we reported that as early as at 3 months of age higher levels of β_{40} amyloid positive vessels were found in the CA1 subzone of the hippocampus of SAMP8 than age-matched ICR mice. In addition, there have been described age-related increases in protein and mRNA levels of APP in the hippocampus of SAMP8 mice (Morley et al., 2000). Taking into account that the origin of Aβ in blood vessel walls is still poorly understood (Jellinger, 2007), and assuming that in SAMP8 mice a marked amyloid deposition do not appear until 6 months of age, it would be interesting to investigate whether the amyloid vessel deposition can contribute to the onset and progression of AD in these mice or it is just a consequence of the increased Aβ cerebral levels in SAMP8.

It is important to consider that at 12 months of age, both strains ICR and SAMR1 show a disrupted BBB, another pathological feature that has been described in AD, but this disruption seems more severe in SAMP8 mice. In AD, aging is the principal risk factor, although it's neither necessary nor sufficient to develop the disease. In addition, aging is a significant risk factor in the effect of the Aβ on endothelium-dependent function of cerebral and peripheral vessels (Price et al., 2004). In other words, although old ICR mice show some pathological features that can be associated with AD, it is normal as the animals are becoming old and ageing favors these alterations. On the other hand, SAMP8 mice show several very early AD-like pathological features that deteriorate with age and are aggravated by their accelerated ageing and an increased Aβ deposition.

With regard to the localization of the amyloid clusters, amyloid positive vessels and IgG disrupted vessels, the results showed that in SAMP8 mice some amyloid clusters are adjacent to some vessels but there are also some other clusters without close proximity to blood vessels, pointing that the amyloid deposition in clusters is not necessary placed near to or related to blood vessels. Similarly, the comparison of the amyloid cluster deposition with the amyloid positive vessels and with the BBB disrupted vessels localization exhibited no direct association between these features. Although some authors have suggested that amyloid deposits accumulate near and around cerebral capillaries (Vinters et al., 1994; Kumar-Singh et al., 2005), our results are in

consonance with some other authors, who claim that capillaries play only a limited direct role, if any, in amyloid plaque formation, and that the apparent association of amyloid plaques and capillaries is no more than a chance contact (Kawai et al., 1990) and with those who claim that amyloid deposition blood vessel walls may not be instrumental in the formation of senile plaques (Lippa et al., 1993).

Finally, the neurovascular unit and the BBB are severely affected by CAA (Bell & Zlokovic, 2009) and a disrupted BBB can favor the amyloid accumulation in blood vessels (Zlokovic 2005). In fact, it has been described that Aβ fibrils can increase the permeability of endothelial cells and induce stress fiber formation, disruption and aggregation of actin filaments and cellular gap formation (Nagababu et al., 2009). Moreover, Aβ has been demonstrated to produce proinflammatory, proapoptotic and proangiogenic responses in the endothelial cells that make up the BBB (Dickstein et al., 2006). Here, we found that all the vessels with amyloid deposition showed BBB disruption with IgG deposition. Nevertheless, we also found several capillaries with IgG deposition and no amyloid accumulation within their walls. This fact could be explained because at 12 months of age there is a marked increase in the number of IgG positive vessels and we have not analyzed all the amyloid fragments that can be deposited in the vessel walls. Another possible explanation could be because the Aβ toxicity produced by soluble oligomers and the amyloid vessel deposition process takes place after the disruption of the BBB in the vessel. It could also be possible that while Aβ deposition disrupts the BBB, there may be another silent mechanism that produces BBB alterations with no direct association with the amyloid aggregates.

To sum up, in all the articles discussed here, we have reported that SAMP8 strain of mice show BBB alterations, an early amyloid deposition and an increased CAA. In addition, we have showed that amyloid aggregates are not directly associated with neither blood vessels, BBB disrupted vessels or amyloid positive vessels. Whether or not BBB disruption and amyloid pathology are cause, consequence or epiphenomenon, needs further investigation. To finish with, taking advantage of the fact that SAMP8 mice show several characteristics of AD, and is a non genetically modified strain of mice with non genetically induced Aβ formation, they are a very useful tool to investigate the different pathogenic mechanisms of late-onset AD.

To finish with, in all the articles that include this thesis, we have reported that SAMP8 strain of mice show BBB alterations, an early amyloid deposition and an increased CAA. In addition, we have showed that amyloid aggregates are not directly associated with neither blood vessels, BBB disrupted vessels or amyloid positive vessels. Whether or not BBB disruption and amyloid pathology are cause, consequence or epiphenomenon, needs further investigation.

5. Conclusions

- The imaging methodology specially designed to measure BBB disturbances by anti-IgG staining allows the quantification of IgG extravasation and its extension. Therefore, this method permits to objectively analyze and quantify the existence of disturbances in the BBB.

- When using the cocktail method, the red EB fluorescence can be seen in brain areas with a disrupted BBB while the Hoechst fluorescence can be seen in the nuclei in that same region. EB and Hoechst fluorescence can also be observed in the choroid plexus and circumventricular organs, where there is no functional BBB. Thus, the cocktail method is suitable to be used to find current BBB disturbances.

- Neither SAMP8 nor SAMR1 mice aged 3 or 7 months showed positive staining for IgG extravasation. On the other hand, a clear IgG staining was observed in the hippocampus of 12-month-old SAMP8 mice, while only a weak extravasation appeared in age-matched SAMR1 animals.

- Evans blue permeability remains stable throughout the lifespan of ICR-CD1 mice, meaning there are no BBB alterations in this strain of mice. On the other hand, SAMP8 and SAMR1 show a progressive increase of BBB permeability to EB from 6 to 15 months of age in the cortex, the hippocampus and the hippocampal fissure.

- The effect that age has in the integrity of BBB is different in SAMP8 than in SAMR1 and ICR-CD1 mice. SAMP8 mice exhibit higher BBB disturbances due to the aging process than the other two studied strains.

- Highly clustered extracellular amyloid granular structures up to 3μm in size can be found in the hippocampus of some animals. No amyloid clusters have been found outside the hippocampus.

- The composition of the amyloid granules is complex and variable. There is a greater prevalence of $A\beta_{42}$ in granular structures than other amyloid fragments. However, $A\beta_{40}$ and some other APP fragments can be found in the granules. In

addition, there are some amyloid granules with no $A\beta_{42}$, $A\beta_{40}$ or APP fragments presence.

- SAMP8 mice show higher amyloid levels in the hippocampus than both ICR and SAMR1 mice.

- In SAMP8 mice, there is an increase in the amyloid deposits from 3 to 15 months of age. On the other hand, SAMR1 and ICR exhibit some basal levels of amyloid accumulation from 3 to 12 months of age, but significant values are not reached until 15 months of age.

- The SAMP8 strain of mice present higher levels of β_{40} amyloid positive vessels in the CA1 subzone of the hippocampus than age-matched ICR mice. This increased degree of CAA appears as early as at 3 months of age and is maintained throughout the lifespan of the animals.

- Both SAMP8 and ICR strains show an increase of CAA at 12 months of age compared to young and adult mice, indicating that aging increases the risk of CAA.

- There is no direct association between the location of blood vessels in the hippocampus and where the amyloid clusters are formed.

- The comparison of the amyloid cluster placement with the BBB disrupted vessels localization exhibit no direct relationship.

- Amyloid clusters show no direct association with the localization of the amyloid positive vessels.

- At 12 months of age, all the vessels with amyloid deposition show a disruption in the BBB with IgG deposition. In addition, several capillaries with IgG deposition can be found with no amyloid accumulation within their walls.

- Taking advantage of the fact that SAMP8 is a non genetically modified strain of mice, they are a very useful tool to investigate the different pathogenic mechanisms of late-onset AD.

- It has not been possible to establish a direct relationship between BBB alterations, CAA and amyloid deposition in SAMP8 mice.

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