Presence of a neo-epitope and absence of amyloid beta and tau protein in degenerative hippocampal granules of aged mice

Gemma Manich · Jaume del Valle · Itsaso Cabezón ·
Antoni Camins · Mercè Pallàs · Carme Pelegrí ·
Jordi Vilaplana

Received: 4 April 2013 / Accepted: 1 July 2013 / Published online: 19 July 2013
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Abstract Clustered pathological granules related to a degenerative process appear and increase progressively with age in the hippocampus of numerous mouse strains. We describe herein the presence of a neo-epitope of carbohydrate nature in these granules, which is not present in other brain areas and thus constitutes a new marker of these degenerative structures. We also found that this epitope is recognised by a contaminant IgM present in several antibodies obtained from mouse ascites and from both mouse and rabbit sera. These findings entail the need to revise the high number of components that are thought to be present in the granules, such as the controversial β -amyloid peptides described in the granules of senescence-accelerated mouse prone-8 (SAMP8) mice. Characterisation of the composition of SAMP8 granules, taking into ac-

count the presence of the neo-epitope and the contaminant IgM, showed that granules do not contain either β -amyloid peptides or tau protein. The presence of the neo-epitope in the granules but not in other brain areas opens up a new direction in the study of the neurodegenerative processes associated with age. The SAMP8 strain, in which the progression of the granules is enhanced, may be a useful model for this purpose.

Keywords Aging · SAMP8 · Hippocampus · Mouse ascites Golgi · IgM · Periodic acid-Schiff · β -Amyloid

Introduction

Aging in mice involves several associated changes, some of which might be considered to result from a pathological process. One such change is the appearance of clustered pathological granular structures that are mainly located in the hippocampus. These clustered granules have been extensively described in the brain of several strains of aged mice, including C57BL/6 (Lamar et al. 1976; Jucker et al. 1994; Soontornniyomkij et al. 2012), ICR-CD1 (Del Valle et al. 2010), AKR (Mitsuno et al. 1999) and senescence-accelerated mouse prone mouse 8 (SAMP8; Akiyama et al. 1986; Kuo et al. 1996; Del Valle et al. 2010). In all strains, these granules start their development in the stratum radiatum of CA1, and thereafter, the number of clusters increases and they spread throughout the hippocampus (Akiyama et al. 1986; Del Valle et al. 2010). These granules, measuring up to 3 μ m

Carme Pelegrí and Jordi Vilaplana contributed equally to this study.

G. Manich · J. del Valle · I. Cabezón · C. Pelegrí ·
J. Vilaplana (✉)
Departament de Fisiologia, Facultat de Farmàcia,
Universitat de Barcelona, Av. Joan XXIII s/n, 08028
Barcelona, Spain
e-mail: vilaplana@ub.edu

A. Camins · M. Pallàs
Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia,
Institut de Biomedicina (IBUB), Universitat de Barcelona, Av.
Joan XXIII s/n, 08028 Barcelona, Spain

J. del Valle · A. Camins · M. Pallàs · C. Pelegrí · J. Vilaplana
CIBERNED, Centros de Biomedicina en Red de
Enfermedades Neurodegenerativas, Barcelona, Spain

in size, are clustered in groups of 40–50 (Akiyama et al. 1986; Del Valle et al. 2010; Kuo et al. 1996).

The main histochemical feature of the granules is their positive reaction for periodic acid-Schiff staining, which indicates a high proportion of carbohydrate macromolecules. In fact, they have been found to contain glycogen, glycoproteins and proteoglycans (Akiyama et al. 1986; Kuo et al. 1996; Manich et al. 2011; Takemura et al. 1993). Extracellular matrix-related proteins such as heparan sulphate proteoglycan, laminin (Jucker et al. 1994; Kuo et al. 1996) and reelin (Knuesel et al. 2009) have been detected in C57BL/6 granules, and polyglucosans have been described as the major components of granules in the AKR strain (Mitsuno et al. 1999).

Despite extensive studies of granule composition, several contradictions have emerged from the results, especially from those obtained by immunohistochemical procedures. As early as 1992, Jucker et al. observed that both normal rabbit sera and polyclonal sera antibodies, even preadsorbed ones, occasionally stained the C57BL/6 granules in a non-specific manner, and that this staining could lead to misinterpretation. The observations regarding rabbit pre-immune sera were later confirmed by Takemura et al. (1993).

False-positive stainings related to antibodies have been widely observed in several cases of immunohistochemistry procedures. In 1982, Gooi and Feizi detected natural antibodies against glycoproteins in mouse hybridoma-induced ascites (Gooi and Feizi 1982). Later, an anti-mouse ascites Golgi (MAG) antibody that stained Golgi apparatus was described as a contaminant in antibodies produced in mouse ascites fluid or sera and rabbit sera (Kliman et al. 1995; Shaw 1986; Spicer et al. 1994). The anti-MAG antibody stains are structures that contain large amounts of mucins or glycoproteins (Finstad et al. 1991; Kliman et al. 1995; Spicer et al. 1994). One important feature of anti-MAG antibodies is the haemagglutination of human blood group A erythrocytes (Finstad et al. 1991; Kliman et al. 1995). In nervous tissue, slight staining with anti-MAG antibody was encountered in perivascular astrocytes from human samples (Kliman et al. 1995), and A1 blood group individuals presented staining in the Golgi zone of some neurons (Ouwendijk et al. 2012). In experimental animals, MAG reactivity has only been described in rat pancreatic acinar cells, several rat glandular epithelial tissues and in gerbil intestine, oviduct and prostate (Spicer et al. 1994).

In the present work on the specificity of the immunostainings of granules, we mainly used the

senescence-accelerated SAMP8 strain. These mice present granules as early as 3 months of age, earlier than other mouse strains, and the number of clusters and granules increases more rapidly than in other strains, thus facilitating their study.

The SAMP8 mouse strain was developed spontaneously from selective breeding of AKR/J mice (Takeda et al. 1981). These animals exhibit early onset and irreversible advance of senescence, as demonstrated by the loss of normal behaviour, the appearance of skin lesions, increased lordokyphosis and a shortened lifespan (Takeda, 2009). Moreover, the SAMP8 strain manifests learning and memory deficits (Miyamoto 1997), neuronal cell loss (Kawamata et al. 1997), gliosis (Nomura and Okuma 1999), a reduction in the release of neurotransmitters in the brain (Kitamura et al. 1992) and increased oxidative stress (Zhang et al. 2008). Several studies have described an increase in both amyloid β precursor protein (A β PP) and derived β -amyloid (A β) peptides in the brain of SAMP8 mice, as well as the presence of A β plaques or deposits (Kumar et al. 2000; Morley et al. 2000; Takemura et al. 1993). The presence of A β has also been described in the granular structures of the hippocampus (Del Valle et al. 2010; Fukunari et al. 1994; Currais et al. 2012; Porquet et al. 2013; Yamaguchi et al. 2012). Furthermore, increased levels of hyperphosphorylated tau protein and tau deposits have also been described (Canudas et al. 2005; Manich et al. 2011; Wei et al. 1999). SAMP8 mice are thus used as a model of senescence but have also been proposed as a suitable model for studying Alzheimer's disease.

The present study of the specificity of the stainings of the pathological hippocampal granules revealed the presence of an epitope that is a neo-epitope of carbohydrate nature with characteristics similar to the MAG antigen. We also found that several commercial antibodies persistently contained an IgM contaminant antibody directed against this neo-epitope. Taking into account this possible contamination, the granule composition of SAMP8 mice was re-characterised, focusing in particular on the presence of β -amyloid peptide and tau protein.

Materials and methods

Mice

Male SAMP8 mice (9–14 months) and APPswe/PS1 δ E9 (9–17 months) were used. They were kept in standard

temperature conditions (22 ± 2 °C) and 12:12-h light–dark cycles (300/0 lx). Throughout the study, they had access to food and water ad libitum. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (DAAM 6459).

Brain processing, immunohistochemistry and histochemical staining

Animals were anaesthetised i.p. with sodium pentobarbital (80 mg/kg). A first group of animals received an intracardiac gravity-dependent perfusion of 50 mL of saline solution. Brains were dissected and frozen by immersion in isopentane and chilled in dry ice. Then, frozen brains were cut into 20- μ m-thick sections on a cryostat (Leica Microsystems, Germany) at -22 °C and placed on slides. Sections were fixed with acetone for 10 min at 4 °C and frozen at -20 °C. A second group of animals was perfused with 50 mL of phosphate-buffered saline (PBS) followed by 50 mL of paraformaldehyde (PF, Scharlab, Barcelona) 4 % in PBS. Brains were post-fixed in 4 % PF in PBS for 4 h, cryoprotected in PBS with 30 % sucrose (Sigma-Aldrich, Madrid, Spain) for 24 h and frozen in isopentane. Brain sections were cut and collected on Superfrost slides (Thermo Scientific, Alcobendas, Spain).

Immunohistochemistry for acetone and PF-fixed sections was performed as follows. Sections were rehydrated with PBS and then blocked and permeabilised with 1 % bovine serum albumin (Sigma-Aldrich) and 0.1 % Triton X-100 (Sigma-Aldrich) in PBS for 20 min. They were washed with PBS and incubated with the primary antibody (see Table 1) either overnight or for 1 h and 30 min. Slides were washed and incubated for 1 h at room temperature (RT) with the secondary antibody. Nuclear staining was performed with Hoechst (H-33258, Fluka, Madrid, Spain) and slides were washed and coverslipped with Prolong Gold antifade reagent (Life Technologies, Carlsbad, CA). Staining controls were performed by incubating with PBS instead of the primary antibody or both primary and secondary antibodies. In double stainings, antibody cross-reactivity controls were also performed.

Some antibody stainings required an antigen retrieval procedure for PF slices. Thus, slides were immersed in sodium citrate buffer (10 mM, pH=6.0) heated to 95 °C for 40 min and then cooled to RT for 20 min. The slides were washed before the permeabilisation step.

The following antibodies were used as secondary antibodies: Alexa Fluor 555 donkey anti-goat IgG, AF488 donkey anti-mouse IgG, AF546 goat anti-mouse IgG, AF555 goat anti-mouse IgM, AF555 donkey anti-rabbit IgG, AF555 donkey anti-rat IgG and IgM (Life Technologies), goat anti-rabbit IgM-fluorescein isothiocyanate (FITC) and rabbit anti-goat IgM-FITC (Abcam, Cambridge, UK).

Various lectins were used on brain slices for histochemical procedures: *Helix pomatia*-AF488, *Dolichos biflorus* agglutinin-FITC, soybean agglutinin-FITC and *Vicia villosa*-FITC, which selectively bind to *N*-acetylgalactosamine (GalNAc) residues; peanut agglutinin-FITC, which binds to β -galactose-3-GalNAc; concanavalin A-FITC, which binds to α -glucose and α -mannose; *Ricinus communis* agglutinin I-FITC, which binds to D-galactose (D-Gal); *Ulex europaeus* agglutinin I-FITC, which binds to α -fucose; and wheat germ agglutinin-FITC, which binds to *N*-acetylglucosamine. *H. pomatia*-AF488 was purchased from Life Technologies; the rest were obtained from Vector Laboratories (Burlingame, CA). All lectin stainings were performed on acetone-fixed brain slices.

Thioflavin S staining was used to detect A β in mouse brain tissue. Acetone-fixed slides were rehydrated in PBS for 5 min and then incubated in 1 % Thioflavin S (T-1892, Sigma-Aldrich) for 10 min while protected from light. Brain slices were washed twice in 80 % ethanol for 3 min and a third time in 95 % ethanol for 3 min. Slides were washed three times with PBS and mounted in Prolong Gold (Life Technologies).

Detection of A β in mouse brain tissue was also attempted with HiLyte-AF488 (Anaspec). This staining was combined with the immunohistochemical procedures and was performed by adding HiLyte-AF488 (at 400 ng/mL) to the secondary antibodies.

Purification of antibodies obtained from ascites fluid

The JJ319 antibody obtained from ascitic fluid was purified using a protein A column in order to separate this IgG from other proteins and Ig, and its concentration was quantified spectrophotometrically.

Tau5A antibody preadsorption with tau protein

Preadsorption controls for the Tau5A immunostaining were performed by incubating the antibody overnight at 4 °C with mild agitation with a 10 molar-fold

Table 1 Antibodies used for the experimental procedures of this study

Antibody (name referred as)	Target	Manufacturer (reference number)
Tau, clone Tau-5 (Tau5A)	Tau protein (210–241 aa)	Millipore (MAB361)
Tau, clone 46.1 (Tau 46.1)	Tau protein (315–352 aa)	Upstate, Millipore (05-838)
Tau, clone Tau-5 (Tau5P)	Tau protein	Biosource, Life Technologies (AHB0042)
Tau, paired235 (TauS235)	Tau protein (Ser 235)	Anaspec (55461)
JJ319	Rat CD 28	Given by Prof. T. Hünig
6E10 (6E10a)	Amyloid β 1–16 aa	Chemicon (MAB 1560; batch LV1375915)
6E10 (6E10b)	Amyloid β 1–16 aa	Covance (SIG-39320), batch unknown)
4G8 (4G8a)	Amyloid β 17–24 aa	Sigma (A1349; batch 116K1552)
4G8 (4G8b)	Amyloid β 17–24 aa	Covance (SIGNET-39220; batch 10BC00211)
12F4 (12F4a)	Amyloid β 42	Covance (SIG-39142; batch 08EC00916)
12F4 (12F4b)	Amyloid β 42	Covance (SIG-39142); batch 10CC00480)
Syndecan-2, clone T-17 (Syndecan-2)	Syndecan-2	Santa Cruz Biotechnology (sc-9494)
NeuN	NeuN protein	Millipore (MAB377)
MAP2	Microtubule associated protein –2	Millipore (AB5622)
Prp	Prion protein (Prp, 25–44 aa)	Abcam (ab703)
MMP2	Matrix metalloproteinase-2	Millipore (AB19167)
Calretinin	Calretinin	Schwann (PV25)
Calbindin	Calbindin	Schwann (CB-38a)
Parvalbumin	Parvalbumin	Schwann (7699/3H)
Novaclone A blood group IgM antibody	A1 and A2 human blood groups	Dominion Biologicals (0170-M100)
Novaclone B blood group IgM antibody	B human blood group	Dominion Biologicals (0175-M100)
A1, A2, A3 blood groups IgM, clone Z2-B1	A1, A2 and A3 human blood groups	Santa Cruz Biotechnology (sc-52367)
A blood group IgM, clone Z2A	A1 and A2 human blood groups	Santa Cruz Biotechnology (sc-69951)

concentration of Tau protein (Millipore). The solution obtained was incubated on the slides instead of the primary antibody in the immunohistochemical procedures.

Tau5A antibody preadsorption with carbohydrates

The Tau5A antibody containing IgM was preadsorbed with four different solutions of carbohydrates (D-galactose, L-fucose, GalNAc, D-mannose and D-glucose (Sigma-Aldrich)), dissolved in PBS at concentrations of 0.05, 0.1, 0.2 or 0.4 M. The Tau5A antibody was incubated with the solutions overnight at 4 °C with mild agitation. Each mixture was then used on different brain sections instead of the primary antibody in the immunohistochemistry procedures.

Haemagglutination test

The haemagglutination test was performed as described in Finstad et al. (1991). Briefly, Tau5A was incubated in

a microtitre plate alone or with goat anti-mouse IgG (Life Technologies) for 1 h at RT. Human erythrocytes (3 %) from blood types A Rh⁺, O Rh⁺, B Rh⁺ and B Rh[−] obtained from the *Banc de Sang i Teixits* (Barcelona) were washed in PBS and added to the microtitre plate. The haemagglutination reaction was evaluated by light microscopy after 1 h of incubation. Positive controls were performed with Novaclone blood grouping reagents for A and B blood groups (Dominion Biologicals, Canada), and negative controls consisted of replacing the primary antibody or both primary and secondary antibodies with PBS when incubating human erythrocytes.

Brain processing for transmission electron microscopy

The animals were anaesthetised i.p. with 80 mg/kg of sodium pentobarbital and perfused intracardially with 50 mL of saline solution followed by 50 mL of PBS with PF at 2 %. Coronal sections of 100 μ m thickness were obtained using a vibratome. Sections were post-

fixed with 2 % glutaraldehyde in PBS (Sigma-Aldrich). The samples were treated with osmium tetroxide (1 %) containing potassium ferricyanide for 1 h at 4 °C, dehydrated in acetone at 4 °C and finally embedded in Spurr resin. Semi-thin sections (1 µm thick) were obtained, and after methylene blue staining, hippocampal CA1 regions were localised. Ultra-thin sections (55 nm thick) were obtained using a Reichert-Jung Ultracut E ultramicrotome and a diamond knife (Diatome, Switzerland), and the sections were then placed on gold grids and post-stained with uranyl acetate and lead citrate.

Immunostaining for transmission electron microscopy

Ultra-thin sections were treated with hydrogen peroxide at 5 % in order to eliminate the osmium tetroxide and were then labelled at RT with nanogold particles as follows: incubation with 1 % BSA in 0.01 M PBS for 30 min, then incubation with Tau5A antibody at dilutions of 1/50 for 2 h, followed by gold conjugated (10-nm particles) rat anti-mouse IgG for 1 h. After washing in PBS, rinsing in distilled water and drying, the sections were stained with uranyl acetate and lead citrate.

Image acquisition

Images were taken with a fluorescence laser and optic microscope (BX41, Olympus, Germany) and stored in tiff format. All images were acquired using the same microscope, laser and software settings. Image treatment and analysis were performed by means of the ImageJ programme (National Institute of Health, USA).

Ultra-thin sections were examined using a Jeol 1010 transmission electron microscope operated at an accelerating voltage of 80 kV. The images were obtained using a Bioscan 792 camera (Gatan, CA).

Results

Failure of specific immunostaining of the SAMP8 hippocampal granules

Several commercial antibodies were used to stain brain slices from SAMP8 animals. Most of the antibodies obtained from mouse ascites or mouse and rabbit sera stained the hippocampal granules (Table 2). These antibodies were monoclonal or polyclonal and purified

or not purified. A representative image of these positive stainings is presented in Fig. 1a, in which the staining achieved using the Tau5A antibody shows the clusters of granules in the CA1 hippocampal region of a 9-month-old SAMP8 mouse. Although the commercial antibodies shown in Table 2 target antigens present in the nervous system, and some of them are monoclonal and purified antibodies, the large number of antibodies that stained the granules induced us to test for the presence of anti-mouse IgG Fc receptors in the granules. To test this hypothesis, normal mouse serum was incubated in brain sections of SAMP8 mice as a primary antibody, and AF488 anti-mouse IgG was used as a secondary antibody. In this case, no staining was observed, thus excluding the presence of the Fc receptor in the granules (data not shown). Moreover, the presence of endogenous IgG in the granules was also ruled out because incubation with only secondary antibodies directed against mouse IgG did not stain the granules (data not shown).

We then attempted to determine antibody specificity in granule staining. Two antibodies obtained from ascites fluid were used: (a) the Tau5A mouse IgG antibody directed against total tau protein and (b) the JJ319 mouse IgG directed against rat CD28 surface antigen in lymphocytes, with no reactivity in the brain. These antibodies were tested on consecutive brain slices using AF488 anti-mouse IgG as the secondary antibody. When the brain section was stained with Tau5A, we observed the presence of the granules (Fig. 1a) and the well-orientated dendrites of the stratum radiatum (inset in Fig. 1a). After preadsorption of Tau5A with tau protein, the staining of the hippocampal granules remained (Fig. 1b) but that of dendrites disappeared (inset in Fig. 1b), indicating that the first staining was not due to the anti-tau antibody. On the other hand, granules were also observed when the brain section was stained with the JJ319 antibody, although this antibody is not directed against brain structures (Fig. 1c). However, when this IgG antibody was purified by protein A, staining of the hippocampal granules disappeared (Fig. 1d). It should be noted that after protein A purification, JJ319 continued to stain positive control tissues such as T lymphocytes in peripheral blood (data not shown). Therefore, it can be inferred that the ascites fluid of JJ319 antibody without protein A purification contained a contaminant that was responsible for staining the granules. This contaminant should be susceptible to recognition by secondary anti-mouse IgG antibodies.

Table 2 List of antibodies which stained hippocampal granules from SAMP8 mice

Antibody	Host	Monoclonal/polyclonal	Source	Purification	Dilution	Granules staining	IgM
Tau5A	Mouse	Monoclonal	Ascites	Non-purified	1/100	+	+
Tau46.1	Mouse	Monoclonal	Ascites	Non-purified	1/100	+	+
Tau5P	Mouse	Monoclonal	Ascites	Purified ^{a, b}	1/100 ^e	+	+
JJ319	Mouse	Polyclonal	Ascites	Non-purified	1/50	+	+
JJ319	Mouse	Polyclonal	Ascites	Purified ^a	1/50	–	–
6E10a	Mouse	Monoclonal		Purified	1/100	+	?
6E10b	Mouse	Monoclonal		Purified ^b	1/100	–	–
4G8a	Mouse	Monoclonal		Purified ^b	1/100	+	?
4G8b	Mouse	Monoclonal		Purified ^b	1/100	–	–
12F4a	Mouse	Monoclonal		Purified ^b	1/100	+	+
12F4b	Mouse	Monoclonal		Purified ^b	1/100	–	–
Syndecan-2	Goat	Polyclonal		Purified ^c	1/100	+	+
NeuN	Mouse	Monoclonal	Serum	Non-purified	1/200	+	+
MAP2	Rabbit	Polyclonal		Purified ^d	1/100	+	+
Prp	Rabbit	Polyclonal	Serum	Non-purified	1/100	+	+
MMP2	Rabbit	Polyclonal		Purified ^a	1/200	+	–
Calretinin	Rabbit	Polyclonal	Serum	Non-purified	1/50 ^f	+	+
Calbindin	Rabbit	Polyclonal	Serum	Non-purified	1/50 ^f	+	+
Parvalbumin	Rabbit	Polyclonal	Serum	Non-purified	1/50 ^f	+	+

In the granule staining column, absence of staining (–), presence of staining (+) or not available for testing (?) is indicated. In the last column, the presence (+) or absence (–) of contaminant IgM or not available for testing (?) is shown. Immunostainings have been performed in acetone-fixed fresh frozen tissue except in IHC procedures on PFA and IHC procedures on PFA and previous antigen retrieval

^aProtein A chromatography, ^bProtein G chromatography, ^cAffinity purification method, ^dAmmonium sulphate precipitation, ^eIHC procedures on PFA, ^fIHC procedures on PFA and previous antigen retrieval

Tau5A stains membranous structures in the granules

A more precise characterisation of Tau5A staining of the granules was performed using transmission electron microscopy. As shown in Fig. 2a, the granules are dense-core deposits of haphazardly membranous structures surrounded by a peripheral translucent space containing degenerated mitochondria and other organelles. A discontinuous membrane was apparent around all these structures. The granules measured up to 3 µm in diameter, while the dense core measured between 0.5 and 2 µm. When staining with Tau5A with the respective nanogold-conjugated secondary antibody, gold particles appeared in the granule cores (Fig. 2b), mainly staining membranous-like components (Fig. 2c). The membranous staining on the granules is consistent with the MAG epitope, which is located in the cellular membranes of the Golgi compartment and in derivative membranous structures.

The large number of antibodies that stained granules, the non-disappearance of Tau5A staining on granules when the antibody was preadsorbed with tau protein, the capacity of Tau5A to stain membranous structures in the dense core of the granules and the presence of a contaminant in the JJ319 antibody ascites fluid prompted us to study the possible presence of an anti-MAG antibody as a possible ubiquitous contaminant responsible for such staining.

Presence of antibodies against MAG antigen in the Tau5A ascites fluid

In view of the reported reactivity of anti-MAG antibodies against erythrocytes from blood group A, a haemagglutination test was performed. Human A, B and O Rh⁺ and B Rh[–] blood group erythrocytes were incubated with Tau5A antibody with or without a secondary anti-mouse IgG antibody. Positive controls

Fig. 1 Immunohistochemical detection of SAMP8 CA1 hippocampal granules with antibodies obtained in ascites fluid: **a** anti-Tau5A antibody (in **a** inset: dendrites on stratum radiatum can be observed);

b anti-Tau5A antibody preadsorbed with tau protein (in **b** inset: dendrites on stratum radiatum are not stained); **c** JJ319A antibody; and **d** JJ319P antibody purified by protein A. Scale bar 100 μ m

Fig. 2 **a** Immunoelectron microscopy image of a SAMP8 hippocampal granule. **b** A granule stained with Tau5A antibody and 10 nm nanogold-labelled secondary antibody. The brightness has

been modified to enhance the visualisation of the nanogold particles. **c** Nanogold particles located in membranous-like components of the granules. Scale bar: **a** 1 μ m, **b** 0.2 μ m, **c** 0.1 μ m

were performed using haemagglutination antibody reagents against human blood groups A and B. The results for incubations with Tau5A carried out without the secondary antibody showed no agglutination of O or B group erythrocytes (Fig. 3a–c), whereas a positive haemagglutination of erythrocytes was observed for the A Rh⁺ group (Fig. 3d). Although this agglutination was not as strong as that obtained for the positive control (Fig. 3f), it was clearly evident when compared with the absence of agglutination found in B and O erythrocytes or in the negative control of type A blood group (Fig. 3e). When the process was repeated with the secondary antibody, the same results were obtained, with group A being the only one in which Tau5A produced agglutination (data not shown). The capacity of Tau5A to directly agglutinate blood group A erythrocytes supports the presence of an anti-MAG antibody in the ascites fluid and also suggests that this antibody may be of the IgM type.

Antibodies directed against MAG in Tau5A antibody are of IgM type

To ascertain whether Tau5A contained a contaminant antibody of IgM type, stainings with Tau5A were performed in SAMP8 hippocampal sections using a

secondary antibody directed specifically against the μ chain, which is only present in IgM antibodies, and labelled with AF555. The granules were positively stained, indicating the presence of an IgM antibody that recognised the granules (Fig. 4b). Moreover, the absence of the dendritic staining typical of tau indicated that this IgM antibody does not react against tau protein. The section shown in Fig. 4 was incubated with Tau5A as primary antibody and with both anti-IgM AF55 and anti-IgG AF488. The green channel (AF488) shows the presence of the granules and the dendrites (Fig. 4a). Dendritic staining revealed that, as expected, Tau5A contains an IgG directed against tau protein. However, the presence of granular staining when using the secondary anti-IgG antibody does not indicate the presence of tau in the granules because this anti-IgG recognises not only the γ chain of the IgG but also the light chains of both the IgG and the IgM. As indicated previously, preadsorption with tau protein blocked the dendritic but not the granular staining. Taken together, these results indicate that granular staining with Tau5A was due to the presence of anti-MAG IgM. The merged images obtained from anti-IgG and anti-IgM enabled visualisation of a high colocalisation of both stainings in the granules (Fig. 4c).

Fig. 3 Haemagglutination test of Tau5A antibody and different human blood group erythrocytes: **a** Group O Rh⁺ incubated with Tau5A, **b** group B Rh⁺ incubated with Tau5A, **c** group B Rh⁻ incubated with Tau5A, **d** group A Rh⁺ incubated with Tau5A, **e**

group A Rh⁺ incubated with PBS (negative control) and **f** group A Rh⁺ incubated with the Novaclone A blood group IgM antibody (positive control). Scale bar 100 μ m

Fig. 4 Immunohistochemical staining of the brain sections from SAMP8 mice with Tau5A antibody. **a** Secondary antibody against IgG. **b** Secondary antibody against IgM.

c Merging of **a** and **b**. Yellow corresponds to colocalisation of both stainings. Scale bar 100 μ m

Tau is not present in the granules of SAMP8 mice

Immunohistochemical stainings were also performed with TauS235 antibody directed against the surrounding Ser235 region of tau protein, equivalent to the epitope region recognised by the Tau5A antibody. TauS235, which does not contain IgM antibodies, stained the hippocampal dendrites with a similar pattern to Tau5A, but did not stain the hippocampal granules (data not shown). These results confirmed that the granules do not contain tau protein.

The epitope recognised by the IgM does not correspond exactly to type A blood group epitope, but carbohydrates are determinant in their antigenicity

As the IgM contaminant antibody produced a slight but persistent haemagglutination with human type A blood group antigen, several lectins and antibodies were tested in order to obtain more information about the MAG-like epitope present in the hippocampal granules. We focused on lectins that recognise carbohydrates containing GalNAc, which differentiates the A blood group from the others. None of the lectins tested (see ‘[Materials and methods](#)’ section) gave positive staining of the granules. Moreover, antibodies against A1, A2 and A3 blood group epitopes in the brain sections of SAMP8 mice did not stain the granules either (data not shown). Thus, the MAG-like epitope present in the granules does not exactly correspond to the epitope present in the A blood group antigen.

After incubating the Tau5A antibody together with the carbohydrate mixtures at different concentrations, we observed that the granular staining with anti-IgM labelled with AF555 showed a decrease that was inversely proportional to the carbohydrate concentrations (Fig. 5). This inhibition was specific for IgM staining, as the IgG labelling of hippocampal dendrites was not affected by the addition of carbohydrates. Moreover, this inhibition was due to the interaction of IgM with carbohydrates and not to that of carbohydrates with granules because the staining of granules with anti-IgM was not altered when the tissue was previously incubated with any of the carbohydrate mixtures. These results confirm that carbohydrates are at least part of the epitope recognised by IgM contaminant antibodies.

The JJ319 antibody also contains an IgM contaminant antibody that stains the granules

To evaluate the presence of IgM in the mouse ascites fluid of non-purified JJ319, staining with JJ319 was performed in brain sections of aged SAMP8 using the secondary antibody directed against mouse IgM. Observation of the stained hippocampal granules revealed that, as with Tau5A, an IgM antibody that stains granules was present in JJ319 (data not shown). We also observed that, as expected, IgM staining of the granules disappeared after purification of JJ319 with A protein (data not shown). This result also explained the previous findings, in which

Fig. 5 Immunohistochemical staining of the brain sections from SAMP8 mice with Tau5A after the incubation of the antibody with a mixture of carbohydrates (see text) at the following concentrations: 0 M (**a**, positive control); 0.05 M (**b**); 0.1 M

(**c**); 0.2 M (**d**); 0.4 M (**e**); 0 M and PBS instead of Tau5A (**f**, negative control). In all cases, secondary antibody is anti-IgM. Scale bar 50 μ m

JJ319 with anti-IgG as a secondary antibody only stained granules when not purified.

IgM antibody against MAG-like epitope is present in most of the antibodies that stain SAMP8 hippocampal granules

Immunohistochemical procedures performed on the brains of old SAMP8 mice using anti-IgM secondary antibodies enabled us to determine if IgM was responsible, at least in part, for these positive stainings. The results obtained with these antibodies are shown in Table 2. A large number of mouse and rabbit antibodies obtained from either sera or ascites contained IgM antibodies that stained the granules. Some of these antibodies were supplied following purification using A or G protein columns, but also contained a quantity of contaminant IgM that was sufficient to produce artefact stainings. As an example, 12F4a stained the granules with the anti-IgG as secondary antibody but also with the anti-IgM (Fig. 6(a1–a3)).

Among the tested antibodies, we observed two exceptions to the association between the presence of IgM and granule staining. First, the MMP2 antibody stained the granules when using an anti-IgG secondary

antibody but did not stain them when using an anti-IgM, thus indicating that it did not contain contaminating IgM (Fig. 6(b1–b3)). Second, the syndecan-2 antibody stained the granules more intensely when using an anti-IgG as a secondary antibody than when using an anti-IgM (Fig. 6(c1–c3)). In the first case, the possibility that granule staining was due to the presence of an IgM in the MMP2 antibody was therefore excluded, and in the second case, although IgM was present to some extent, it seems that some specific staining was also due to the syndecan-2 antibody.

β -Amyloid peptides are absent from SAMP8 hippocampal granules

As shown in Table 2, some batches of 6E10, 4G8 and 12F4 antibodies, directed against A β peptides, stained SAMP8 granules when using a secondary anti-IgG antibody, whereas other batches did not. We observed that all batches that did not stain the granules (i.e. 6E10b, 4G8b and 12F4b) had no IgM contamination. As indicated previously and shown in Fig. 6(a1–a3), 12F4a stained the granules with the anti-IgG as secondary antibody but also with the anti-IgM, indicating the presence of the IgM contaminant. The presence of IgM in the 6E10a

Fig. 6 Double immunohistochemical staining of SAMP8 hippocampal granules performed with a primary antibody and anti-IgG and anti-IgM as secondary antibodies. *A1* 12F4a with anti-IgG. *A2* 12F4a with anti-IgM. *A3* Merging of *A1* and *A2*. *B1*

MMP2 with anti-IgG. *B2* MMP2 with anti-IgM. *B3* Merging of *B1* and *B2*. *C1* Syndecan-2 with anti-IgG. *C2* Syndecan-2 with anti-IgM. *C3* Merging of *C1* and *C2*. Scale bar 50 μ m

and 4G8a batches could not be tested as there was no further stock in our laboratory. These results seem to indicate the absence of A β peptides from the hippocampal granules. Given the importance of these findings, further immunohistochemical stainings were performed on brain sections of APP/PS1 mice, which have amyloid plaques that can be used as a positive control for A β staining.

β -Amyloid peptides are absent from APP/PS1 hippocampal granules

When the hippocampus of 9-month-old APP/PS1 mice was stained with the 12F4a antibody and anti-IgG was used as a secondary antibody, both A β plaques and hippocampal granules were stained (Fig. 7(a1)). When staining with a secondary antibody against the IgM, only the staining of the hippocampal granules remained positive, whereas A β plaques were not stained (Fig. 7(a2)). Stainings with 12F4b, 6E10b and 4G8b,

which did not contain IgM, showed positive results for A β plaques but not for granules when an anti-IgG was used as the secondary antibody (Fig. 7(b, c)). Moreover, Hylite-AF488 and Thioflavin S did not stain granules, but they did stain the A β plaques present in the cortex and hippocampus of APP/PS1 mice (Fig. 7d, e). Given the findings described thus far, the results indicate that hippocampal granules from both SAMP8 and APP/PS1 do not contain A β peptides. It should also be borne in mind that 9-month-old APP/PS1 mice have fewer granules and clusters of granules than SAMP8 mice of the same age (Figs. 6(a1) and 7(a1)).

Discussion

In the present study, we describe the presence of a neopeptide of glycosidic nature in the pathological

Fig. 7 Stainings of brain sections from APP-PS1 mice. Staining with 12F4a antibody and both anti-IgG (*A1*) and anti-IgM (*A2*). *B* Staining with 4G8b antibody and anti-IgG. *C* Staining with 6E10b and anti-IgG. *D* Staining with Hylite-AF488. *E* Staining

with Thioflavin S. *White arrow* amyloid plaque. *Grey arrow* localisation of amyloid plaque that is not stained. *Yellow arrow-head* cluster of granules. Scale bar 50 μ m

hippocampal granules of aged mice. This epitope is present in the granular structures, but not in the other hippocampal or healthy brain regions. We also found that this epitope is recognised by a contaminant IgM present in several antibodies obtained from mouse ascites and both mouse and rabbit sera.

A range of findings indicates that the neo-epitope is constituted, at least in part, by carbohydrates. On one hand, the IgM that recognises the epitope provoked a certain level of agglutination of human type A erythrocytes, but not of the other human blood types, with the main difference between them being the presence of a GalNAc group. On the other

hand, preincubation of the antibodies with carbohydrates blocked the IgM staining.

Neo-epitopes related to carbohydrates have been described in several situations. For instance, in aging processes, there is an increase in the generation of advanced glycation end products (AGE) (Goldin et al. 2006), which are formed by modifications of proteins or lipids that become non-enzymatically glycosylated and oxidised (Schmidt et al. 1994; Singh et al. 2001). Early glycation and oxidation processes result in the formation of Schiff bases and Amadori products that finally lead to the generation of AGEs (Schmidt et al. 1994). On the other hand, neo-epitopes of carbohydrates have also been

described in malignant tumour cells (Vollmers and Brändelin 2006). In this case, the epitopes are post-transcriptional modifications of carbohydrate residues on cell surface glycolipids and glycoproteins (Cobb and Kasper 2005). These carbohydrate antigens are prominent targets of immune surveillance and natural IgM antibodies (Brändelin et al. 2003; Vollmers and Brändelin 2006). Natural IgM antibodies can be present from birth without external antigenic exposure and there is mounting evidence to suggest that they contribute to critical innate immune functions involved in the maintenance of tissue homeostasis (Grönwall et al. 2012). Natural IgM antibodies frequently present cross-reactivity, which is a required feature associated with their function in the first line of defence (Vollmers and Brändelin 2006). Hence, it is conceivable that the new carbohydrate epitope that appeared in the hippocampus granules of mice could be recognised by natural antibodies of IgM type present in ubiquitous form in the ascites or sera of mice or even other species.

The ubiquity and the cross-reactivity of these IgM antibodies may be an important source of misunderstanding in studies on granules. The presence of this contaminant IgM in many commercial and non-commercial antibodies, and even in some batches of purified antibodies, has produced false-positive results. In this study, we re-evaluated the presence of some components previously described in the hippocampal granules of SAMP8 mice, such as A β peptides and tau protein, and found that their reported presence is a misunderstanding generated by the presence of the contaminant IgM. It should be noted that as early as 1992, Jucker et al. stated that hippocampal granules in C57BL/6 aged mice do not contain A β and that the previously reported A β immunoreactivity of these granules was a staining artefact (Jucker et al. 1992).

Moreover, Jucker et al. (1994) later reported that based on distribution, morphology, staining properties and association with glial cells, it would appear that the deposits in C57BL/6 and SAMP8 are identical; this was confirmed by other studies that compared both strains (Kuo et al. 1996). Our results are consistent with all these observations and indicate that the granules present in SAMP8 and APP/PS1 mice do not contain A β peptides. Together, the results obtained to date indicate that the granules present in the hippocampus of different mouse strains do not contain A β peptides.

Due to IgM contamination, it may be necessary to test for the presence of the other reported granule components, especially in studies where the results are based

on immunohistochemical stainings. Among the 19 antibodies tested in the present study, the MMP2 antibody was the only one that did not contain IgM contaminant but stained the hippocampal granules. Moreover, the syndecan-2 antibody showed just faint IgM staining of the granules, suggesting that most of the staining was due to the genuine presence of syndecan-2.

Studies on the deposition of A β peptides in SAMP8 mouse brain have produced controversial results. The deposits described have included, among others, β /A4 protein-like immunoreactivity granular structures (β -LIGS) (Takemura et al. 1993), amyloid β -peptide-like immunoreactivity deposits (A β -LI) (Fukunari et al. 1994), reelin deposits that contained A β (Knuesel et al. 2009) and the hippocampal granules containing A β (1-40) and A β (1-42) (Del Valle et al. 2010; Currais et al. 2012; Porquet et al. 2013; Yamaguchi et al. 2012). Morley et al. (2000) found A β plaques that were not discernible before 16 months of age and became more prevalent at 22 months of age. However, Nomura et al. (1996) found no evidence of deposits or granular structures in SAMP8 brain although they reported an increase in APP-like immunoreactivity. From their description, the A β -LI and reelin deposits containing A β reported by Knuesel et al. (2009) would appear to be the pathological granules that we have investigated further in the present study. Given that we found that they do not stain with Thioflavin S or with Hylite-AF488, and that A β -staining is a mistake due to the presence of the contaminant IgM, we conclude that there is no deposition of A β in these granular structures. It should be noted that Doehner et al. (2010) reported that these granules stained with Thioflavin S, but we did not observe this positivity either in granules from SAMP8 mice or in those from APP/PS1 mice. In the latter, amyloid plaques were used as a positive control for Thioflavin S staining. Thus, the only structures that can be considered as A β deposits are the β -LIGS (Takemura et al. 1993) and the A β plaques that appear at advanced ages in SAMP8 animals, as described by the Morley group, although neither of these results has been confirmed by other authors or in other strains of mice. Given the above, the use of SAMP8 animals as a model of AD needs to be based not on amyloid deposition, but on other facts. In this regard, several authors have observed an increase with age of A β PP and its mRNA in the hippocampus of SAMP8 (Morley et al. 2000; Kumar et al. 2000). On the other hand, abnormal levels of phosphorylated tau protein

have also been described in 11-month-old SAMP8 mice (Wei et al. 1999) and there are higher levels of various forms of hyperphosphorylated tau in SAMP8 compared to SAMR1 (Canudas et al. 2005).

On the other hand, it can be deduced from this study that A β peptide levels do not influence the production of pathological granules in the hippocampus. In APP/PS1 animals, overexpression of A β PP and overproduction of A β peptides cause the formation of amyloid plaques at very early ages. However, at 9 months of age, the number of granules in APP/PS1 transgenic mice is lower than in SAMP8 animals. Thus, an increase in A β does not correlate with an increase in granule formation, and the accelerated formation of these granules in SAMP8 must be caused by other pathological mechanisms. Among these, we propose the increase in oxidative stress, as SAMP8 animals treated with resveratrol (Porquet et al. 2013) or ApoE-deficient mice fed with an antioxidant diet (Veurink et al. 2003) show a decrease in the number of hippocampal granules.

In conclusion, we have described the presence of a new neo-epitope of glycosidic nature in the pathological granules of the hippocampus of several strains of mice. This epitope is recognised by contaminant IgM antibodies present ubiquitously in antibodies obtained from mouse ascites and both mouse and rabbit sera and may be a new marker of the pathological process that takes place in the formation of these structures.

Acknowledgments This study was funded by grants BFU2010-22149, SAF2011-23631 and SAF2012-39852 from Spain's *Ministerio de Ciencia e Innovación* and *Centros de Investigación Biomédica en Red* from the *Instituto de Salud Carlos III*. We would like to thank the *Generalitat de Catalunya* for funding the research group (2009/SGR00853) and for awarding a predoctoral fellowship to G. Manich (FI-DGR 2011).

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