Periodic acid-Schiff granules in the hippocampus of aged mice: from amyloid aggregates to degenerative structures containing neo-epitopes

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ABSTRACT

Brain ageing in mice leads to the progressive appearance and expansion of degenerative granular structures in the hippocampus. Because the granules stain positively with periodic acid-Schiff (PAS), they have frequently been referred to as PAS granules. These structures have been erroneously identified as a wide range of brain deposits related to numerous neurodegenerative diseases, such as amyloid deposits, neurofibrillary tangles, Lafora bodies, corpora amylacea and polyglucosan bodies. All of these erroneous identifications, which have appeared and disappeared regularly over time, have generated controversy and particular theories about their significance. We recently reported that the principal cause of these misinterpretations is the high number of false immunostaining results produced by the presence of neo-epitopes in these structures and the presence of contaminant IgMs directed against them in numerous commercial antibodies produced in ascites and serum. These IgMs may in fact be natural antibodies, some of which contribute to the controlled elimination of cell remnants and apoptotic or altered cells. Now that this point has been clarified, this article reviews and reconsiders the nature and physiopathological significance of these degenerative granules. Moreover, we suggest that neo-epitopes could become a useful brain-ageing bio-marker and that autoimmunity could become a new focus in the study of age-related degenerative processes.

Keywords: Ageing, hippocampus, periodic acid-Schiff, neo-epitope, natural antibody, amyloid

HIGHLIGHTS

- PAS granules are degenerative structures formed in mice hippocampus with age
- Oxidative stress and genetic background play a significant role in granule formation
- Granules have been erroneously identified as amyloid deposits, among others
- Granules contain neo-epitopes that are a target of natural IgM auto-antibodies
- Autoimmunity could be a new focus in the study of age-related degenerative processes

CONTENTS

1. INTRODUCTION

Ageing has been broadly defined as a time-dependent functional decline affecting most living organisms and is mainly characterised by a progressive loss of physiological integrity that leads to impaired function and increased vulnerability to death (López-Otín et al., 2013). In the human brain, ageing involves alterations to learning and memory, as well as motor performance (Yeoman et al., 2012). Moreover, ageing is the main risk factor for several neurodegenerative diseases. Because the molecular mechanisms of human brain ageing are not fully understood, animal models of ageing and age-related diseases are useful for acquiring greater insight into these pathways.

Brain ageing in mice leads to morphological and functional changes that can be considered a result of abnormal or pathological processes. One of these is the appearance of pathological granular structures in the hippocampus and their progressive expansion with age. These round-to-ovoid structures are organised into clusters and one of their main features is that they stain positively with periodic acid-Schiff (PAS). They are found in a wide range of mouse strains, but a noticeable amount of PAS granules has been observed in the senescence-accelerated mouse prone 8 (SAMP8) strain, which exhibits an accelerated ageing process (Akiyama et al., 1986; Del Valle et al., 2010; Kuo et al., 1996). Although PAS granules have mainly been studied in mice, they have also been described in the brains of aged individuals of other animal species, such as other rodents and non-human primates (Knuesel et al., 2009; Kuo et al., 1996).

PAS granules were first described by Lamar et al. (1976) in old C57BL/6 mice and they were later reported in SAMP8 mice (Akiyama et al., 1986). These structures, alternatively named Wirak bodies, became relevant when they were accidentally identified as beta-amyloid (Aβ) aggregates developed in $A\beta PP_{(1-42)}$ transgenic mice (Wirak et al., 1991). The results reported in that article were shown to be actually caused by false-positive immunohistochemical stainings, due to a non-specific binding of the anti-Aβ antibodies to the PAS granules (Jucker et al., 1992). Since then, there has been a large disparity when describing PAS granules composition, cell origin and physiopathological role. However, there is a consensus on relating these structures with the aging phenomenon (Akiyama et al., 1986; Doehner et al., 2012; Jucker et al., 1994b; Kuo et al., 1996).

The recent identification of the reason behind the false-positive staining results (Manich et al., 2014b) has helped clarify current evidence relating to PAS granules and paved the way for a

new approach to studying neurodegenerative diseases. This review aims to address these questions.

2. MAIN FEATURES OF PAS GRANULES

2.1. Morphology and brain location of PAS granules

PAS granules are round-to-ovoid structures found in the hippocampus of several mice strains and first appear in the stratum radiatum of the CA1 region, from where they gradually extend to other hippocampal layers and regions. Granules measure up to 3 µm in diameter and tend to form clusters measuring about 80 µm in diameter, each containing approximately 40-50 granules (Akiyama et al., 1986; Jucker et al., 1994b; Kuo et al., 1996; Ye et al., 2004) and occasionally reaching 100 granules per cluster (Jucker et al., 1992; Robertson et al., 2000). Figure 1 shows a representative granule cluster in mouse hippocampus.

Clustered PAS granules have mainly been studied in the hippocampus, although they have also been found in other brain regions. A study on the topographical distribution of clustered granules in aged C57BL/6 mouse brains indicated the presence of these structures in the hippocampus, piriform and entorhinal cortex, olfactory bulb, cerebellum and trapezoid body (Jucker et al., 1994b). Clustered granules are present in a high amount in the *stratum radiatum* and *lacunosum moleculare* layers of the hippocampus, and are distributed throughout the CA1, CA2, CA3 and dentate gyrus regions (Nakamura et al., 1995). Slight differences have been encountered in the clusters of the cerebellum, since these have a smaller number of granules per cluster and a more diffused pattern compared to those of the hippocampus. Clustered granules have occasionally been reported in the diencephalon, striatum and amygdala of the oldest mice (Jucker et al., 1994b; Knuesel et al., 2009).

In general, the distribution and progression of granule clusters in the brain regions follow a similar pattern in all mice that develop these structures. The main differences relating to the onset, evolution and speed of expansion of granule clusters in mice are linked to age and strain. These determining factors will be explained in more detail in the following sections.

2.2. Strain specificity in granule cluster presence

Numerous studies have reported the presence of clustered granules in many mouse strains, but have not observed them in other strains (Table 1) (Jucker et al., 1994a, 1994b). These structures have been extensively studied in the brains of aged C57BL/6 mice (Jucker et al.,

1992; Lamar et al., 1976), SAMP8 and senescence-accelerated mouse resistant-1 (SAMR1) mice (Del Valle et al., 2010; Porquet et al., 2013), and AKR mice (Mitsuno et al., 1999). Their presence in BALB/c, DBA/2, C3H and CBA strains is unclear, or only small amounts have been described (Jucker et al., 1994a, 1994b; Krass et al., 2003). These structures also appear in transgenic mice with a genetic background of a strain that develops clustered granules. Some of these transgenic mice present variations in the speed of appearance and the quantity of hippocampal clustered granules compared to the parental strain (Robertson et al., 1998, 2000). The strain differences made it possible to study the inheritance of this trait and thus prove the importance of the genetic background of the strain in the onset of clustered granules (see Section 4.2).

2.3. Age and sex influences in the onset of granule cluster

Age is undoubtedly a key factor in the onset and growth of granule clusters in mice. Although the increase of these clusters in the hippocampus, entorhinal cortex and piriform cortex correlates with age (Madhusudan et al., 2009), the age at which granule clusters appear and grow varies substantially between mice strains. In SAMP8 mice, the granules appear as early as three months of age, which is earlier than in other strains, and the number of clusters and granules increases and spreads faster than in other strains (Del Valle et al., 2010; Jucker et al., 1994a, 1994b; Kuo et al., 1996). In a study that compared the number of hippocampal granule clusters between ICR-CD1, SAMR1 and SAMP8 mice from three to 15 months of age, the latter showed a greater increase in granule clusters at all ages analysed. At three months of age, the number of clusters in SAMP8 mice was higher, although not significantly, than the other strains. However, the differences were statistically significant at six, nine, 12 and 15 months of age. Nevertheless, the number of granule clusters increased slightly with age in both ICR-CD1 and SAMR1 strains, especially between 12 and 15 months of age. As shown in Figure 2, the granule clusters occupied almost the entire hippocampus in a 12-month-old SAMP8 mouse (Del Valle et al., 2010). Differences in the increase in granule clusters with age have been also observed in transgenic mice, with respect to controls, including reeler mice (Kocherhans et al., 2010), 3xTg-AD (Knuesel et al., 2009; Oddo et al., 2003), Ts65Dn (Kern et al., 2011; Rachubinski et al., 2012) and malin knockout mice (Valles-Ortega et al., 2011). However, transgenic ApoE-deficient mice have been reported to develop the earliest PAS granule structures, as early as four to six weeks of age (Robertson et al., 1998, 2000).

Generally, a higher variability is observed in the number of hippocampal granule clusters with advancing age. In the oldest mice, the accumulative trend of granule clusters stalls and may

even see a slight decrease (Jucker et al., 1994b). Moreover, there is greater granule cluster dispersion in these older animals, perhaps as a result of the increase in granule diameter or the blending of small granules into larger ones (Jucker et al., 1994b; Robertson et al., 2000), as previously observed at ultrastructural level (Doehner et al., 2012; Manich et al., 2014a).

While age clearly influences the onset of granule clusters, different results have been reported between male and female mice. Female C57BL/6 mice usually present more granule clusters than males (Jucker et al., 1992, 1994; Knuesel et al., 2009; Lamar et al., 1976). Therefore, age-related changes in steroidal hormones and the neuroendocrine axis have been hypothesised to influence hippocampal granule cluster formation. However, early studies in SAMP8 mice contradict these differences between sexes (Akiyama et al., 1986), so it is unclear whether there are real variations between male and female mice.

2.4. Granule ultrastructure

Analysing the granule ultrastructure makes it possible to differentiate a central core of electron-dense crystalline-like fibrillary deposits, generally surrounded by a halo or electron-lucent region (Figure 3). This halo is externally delimited by a slightly discontinuous plasma membrane, which suggests an intracellular location. The central core is round to ovoid in shape and contains membrane-like structures that are 5 nm to 8 nm thick, are haphazardly aggregated and do not resemble any cell organelles (Kuo et al., 1996; Manich et al., 2014a; Robertson et al., 2000). In fact, cytoplasmic organelles such as the endoplasmic reticulum, polyribosomes, intermediate filaments, multivesicular bodies and mitochondria have occasionally been described in the halo region, usually in a state of degeneration (Doehner et al., 2010; Kuo et al., 1996; Manich et al., 2014a; Mitsuno et al., 1999; Robertson et al., 1998). These organelles are accompanied by many blebs or large cisterns, some of which originate from the invagination of the surrounding plasma membrane, and which are located in the translucent area and show close contact with the core of the granule (Manich et al., 2014a). The membrane surrounding the granule has special contacts that are characteristic of astrocyte-astrocyte junctions (Kuo et al., 1996; Manich et al., 2014a). In general, the structures in the area immediately surrounding the mature granules do not present perceivable morphological alterations. However, some symptoms of deterioration such as degenerating dendrites have occasionally been detected (Manich et al., 2014a).

2.5. Granule composition

The first steps to identifying the PAS granule composition were performed using a wide range

of histochemical staining procedures in order to find out the general nature of the granule compounds (Table 2).

As the name of these granules suggests, the granule clusters characteristically stain intensely positive with PAS (Lamar et al., 1976). This staining method is used to detect glycogen and macromolecules that contain glycans, proteoglycans and glycolipids (Spicer and Schulte, 1992), although proteins containing serine and threonine may show slightly PAS-positive staining (Spicer, 1961). Furthermore, other general staining procedures related to glycoconjugates have been reported to positively stain the granules, e.g. iodine (Mitsuno et al., 1999), or modifications of the PAS staining, e.g. Gomori's methenamine silver stain (Akiyama et al., 1986; Jucker et al., 1994a, 1994b). Previous brain slice digestion with α-amylase or diastase only slightly reduced PAS granule staining, which rules out the possibility of high amounts of glycogen in these structures (Akiyama et al., 1986; Lamar et al., 1976; Mitsuno et al., 1999). In any case, although glycogen is not the main component, these staining results suggest that the granules contain high levels of polysaccharides.

However, some controversy has arisen from the results of other general staining methods. This was the case for thioflavin S stain, which has produced positive staining results in some cases (Doehner et al., 2010; Jucker et al., 1992; Kocherhans et al., 2010) and negative results in others (Jucker et al., 1994a; Mandybur et al., 1989; Manich et al., 2014b; Takemura et al., 1993), and the Congo red stain, another amyloid marker, which has also produced contradictory results (Jucker et al., 1994a; Robertson et al., 1998). Indeed, the ultrastructural observation of the hippocampal granules does not reveal the presence of amyloid in these structures, or at least not as a major constituent (Jucker et al., 1992; Manich et al, 2014a).

Tests performed using enzyme histochemistry reported monoamine oxidase (MAO)-B positive enzymatic activity in the hippocampal granules of aged SAMP8 mice (Nakamura et al., 1995). MAO-B is typically located in the external mitochondrial membrane of glial cells and in serotonergic and histaminergic neurons (Shih et al., 1999), and this enzyme has been also reported to be involved in Parkinson's disease and ageing (Jenner, 2012).

The granule composition has also been extensively addressed in immunohistochemistry studies. However, as we will explain later, many of these studies, perhaps all of them, may be revised.

2.6. Presence of a neo-epitope in PAS granules

In a recent work, we described the presence of a neo-epitope of carbohydrate nature in the PAS granules that is not present in other brain areas (Manich et al., 2014b). Ultrastructural studies indicated that the neo-epitopes are located in the core of the granules, specifically in the membranous fragments. However, some of them could also be observed in the plasma membrane of the astrocytic process and in the membranes of the structures nearest to the neuropil. The neo-epitope is similarly observed in these same areas in immature granules and in the core spread matrix (Manich et al., 2014a). These observations led to the conclusion that the neo-epitope is actually generated during the granule formation process. The neo-epitopes found in the PAS granules are a target of natural IgM auto-antibodies present in the serum of all mice strains tested and in other animal species (see Section 4.3). It is important to note that the profuse presence of these IgMs directed against the neo-epitope as a contaminant in commercial and non-commercial antibodies is responsible for numerous false-positive staining results when immunohistochemical procedures are used, as detailed below (Manich et al., 2014b).

2.7. Immunohistochemical studies of PAS granules

Because a wide range of antibodies produced in mouse and rabbit ascites and serum contained these IgMs, even some that were supplied as purified antibodies, they stained the granules thus providing false-positive stainings. These findings cast doubt on most of the information available on PAS granule composition from immunohistochemical studies.

Several immunostaining processes detected glycoproteins and proteoglycans (Akiyama et al., 1986; Kuo et al., 1996; Takemura et al., 1993), as well as polyglucosan components, which led to consider the PAS granules as equivalent to Lafora bodies (Valles-Ortega et al., 2011). The presence of several extracellular matrix proteins have also been described, including heparin sulphate proteoglycan and laminin (Jucker et al., 1992, 1994b; Kuo et al., 1996), syndecan-2 (Manich et al., 2011) and reelin (Knuesel et al., 2009; Doehner et al., 2012). Furthermore, the antibody KM279 generated against corpora amylacea intensely stained the granules in aged AKR mice (Mitsuno et al., 1999). Furthermore, some proteins involved in neurodegenerative diseases were also targeted, i.e. Aβ peptides (Doehner et al., 2012; Knuesel et al., 2009; Oddo et al., 2003; Robertson et al., 1998; Wirak et al., 1991; Del Valle 2010), ubiquitin (Robertson et al., 1998; Soontornniyomkij et al., 2012), α-synuclein (Krass et al., 2003) and tau protein (Kern et al., 2011; Manich et al., 2011; Rachubinski et al., 2012). These staining results suggested several comparisons with brain lesions observed in neuropathological conditions. Taking into

account the possible contaminant IgMs, the presence of all these components in granules reported by means of immunohistochemistry requires confirmation. The correspondence of the PAS granules with Lafora bodies and corpora amylacea based on immunohistochemical procedures seems to be inconsistent with ultrastructural and histochemical studies. However, the correspondence with senile plaques or neurofibrillary tangles, based on staining with antibodies directed against Aβ peptides and tau protein, has been rejected; the presence of Aβ peptides and tau protein has been revised and discarded (Jucker et al., 1992; Manich et al., 2014b), and the positive staining results explicitly attributed to the presence of IgMs directed against the neo-epitope (Manich et al., 2014b).

2.8. Granule formation

The granule formation process is still unclear, since the information available is minimal and confusing. However, ultrastructural studies have shed light on the fine granule structure and helped identify immature granules, which has generated relevant information about the formation of granules.

Although the dense membrane-like mesh that constitutes the core of the granule can be considered to be an accumulation of molecular waste, successive ultrastructural changes from the exterior to the interior of the granules have led some authors to suggest that the pathological process initiates outside the granules and that degenerative structures migrate inward (Kuo et al., 1996). However, Doehner et al. (2012) proposed that granules originate as a protective strategy of aged neurons that extrude damaged or misfolded proteins, which are subsequently engulfed by glia. This hypothesis is based on the presence of AβPP-derived proteolytic fragments on the granules (Doehner et al., 2010), which has since been demonstrated to be false. Nevertheless, the study of the granule formation in the early phases led us to hypothesise that the granules are the result of a degenerative process involving principally astrocytic processes (Figure 4). The generation of granules includes the appearance of abnormal membranous structures that form intracellular bubbles or blebs of variable sizes and irregular shapes and the instability of the plasmatic membrane. These structures and some organelles degenerate and produce some membranous fragments, and an assembly process of the resulting fragments generates the dense-core nucleus of the mature granule (Manich et al., 2014a). It is important to note that the plasma membrane of the immature granules, and some membranes of the adjacent structures, is often fragmented or unstable, and the boundaries between cells are therefore lost. This membrane disruption has also been observed in mature granules, but is particularly evident in granules being formed, and could

lead to part of the neuropil adjacent to the granule being incorporated into it (Manich et al., 2014a).

2.9. Cell origin of granules

The cell origin of the granules has been a controversial topic and conflicting opinions have been expressed about their neuronal or astrocytic origin.

A remarkably tight relationship between the granules and astrocytes has frequently been reported. Granules have been observed within protoplasmic astrocytic cell bodies and astrocytic processes that had expanded and whose cytoplasmic organelles were greatly diluted (Robertson et al., 1998). Furthermore, approximately 60% to 80% of the granules have been associated with glial fibrillary acidic protein (GFAP)-positive astrocyte processes (Akiyama et al., 1986; Jucker et al., 1994b; Madhusudan et al., 2009; Nakamura et al., 1995), and whole granule clusters have even been directly associated with GFAP-positive astrocytes (Jucker et al., 1994b; Manich et al., 2011, 2014a; Nakamura et al., 1995). Ultrastructural studies have identified mature granules in degenerated astrocytic processes containing glycogen accumulations, GFAP-immunostained fibrils and specific astrocyte-astrocyte junctions with adjacent membranes (Kuo et al., 1996; Manich et al., 2014a). Interestingly, immature granules have always been found to originate in astrocytes, and the formation of granules in the different processes of a specific astrocyte has been reported to cause the clustering pattern of the granules, each cluster being the set of granules formed in one astrocyte (Manich et al., 2014a). Granules have also been regularly associated with blood capillaries and detected in the astrocyte end-feet surrounding them (Doehner et al., 2010; Manich et al., 2014a; Robertson et al., 1998; Ye et al., 2004). Remarkably, MAO-B histochemical staining reinforces the suggestion that astrocytes participate in the formation of granules, since this enzyme is observed in both hippocampal astrocytes and granule clusters (Nakamura et al., 1995).

Neuronal origin has been suggested based on the fact that synaptic vesicles and terminals are in contact with the plasma membrane of the granules (Mitsuno et al., 1999), and on the ultrastructural observation of granules presumably located in abnormal synaptic terminals (Irino et al., 1994) or granules interconnected through dendrites (Doehner et al., 2012; Wirak et al., 1991). However, as mentioned above, locating the granules can be difficult due to the loss of boundaries between the astrocytes and the adjacent cells during their formation. In addition, neuronal origin has been postulated based on the neuronal components described in these structures, such as Aβ peptides (Del Valle et al., 2010; Knuesel et al., 2009; Robertson et al., 1998), neuronal nuclei protein and tau protein (Kern et al., 2011; Manich et al., 2011;

Rachubinski et al., 2012) and reelin protein or ApoE2 receptor (Knuesel et al., 2009). Moreover, some authors have suggested that these granules are dystrophic neurites due to the positive staining with anti-tau antibodies (Corsetti et al., 2008). Some of these components, such as Aβ and tau, have been demonstrated to be absent from granules, while other staining results are uncertain and require verification.

In any case, a general review of the findings reported up to now supports the astrocytic origin of the granules. The remaining cell types, including oligodendrocytes and microglia, do not appear to have a close relationship with granules, although they may be close to these structures (Manich et al., 2014a).

3. BRAIN PATHOLOGY ASSOCIATED WITH PAS GRANULES

3.1. Memory loss

Possibly age-related disturbances may be found in the immediate surrounding areas of PAS granules, as confirmed by the extent of the affected area and the ultrastructural observation of these structures. Because the hippocampus plays an essential role in memory, several tests to measure memory dysfunction have been used to relate it to the presence of PAS granules in this brain area. In aged C57BL/6 mice, a statistically significant relationship between granule clusters and memory loss was found in the Morris water maze test (Jucker et al., 1994b) and the radial arm maze task (Knuesel et al., 2009). Recent episodic memory was also evaluated in this strain by Soontornniyomkij et al. (2012) using the object recognition test. A signification correlation was observed with granule clusters stained with LC3 and p62, although this may have corresponded to IgM anti-neo-epitope false-positive staining.

3.2. Motor performance

Although granule clusters that are found in the hippocampus have been the main subject of researchers' interest, these structures have also been observed in other brain areas (Section 2.1). The cerebellum also exhibits a considerable presence of granule clusters, and age-related motor impairment could therefore be related to these structures. Nevertheless, a first attempt to study the relationship between motor performance and cerebellar granule clusters in SAMP8, SAMR1 and C57BL/6 did not reveal any significant correlation (Ingram et al., 1994).

4. FACTORS RELATED TO GRANULE ETIOPATHOGENY

4.1. Oxidative stress

One of the principal theories of ageing relates oxidative stress to the decline of physiological functions (Harman, 2006). Because granule clusters are related to ageing, it seems feasible to search for a relationship between oxidative stress and the onset and development of these structures. Indeed, the effect of oxidative stress and anti-oxidant compounds on these hippocampal structures has been assessed in several studies that have mainly focused on feeding mice on high-fat diets for long periods of time.

In an initial study performed using ApoE-deficient mice, the administration of a high-fat diet supplemented with antioxidants for a three-month period showed a significant reduction in the number of granule clusters compared to mice fed either the same high-fat diet without antioxidants or a normal diet (Veurink et al., 2003). In another study on the incidence of hippocampal granules in mice, a positive correlation was observed between plasma HDL levels and the number of granule clusters in mice fed an atherogenic diet (Krass et al., 2003). Moreover, a recent article reported a significantly low incidence of granule clusters in SAMP8 mice fed with resveratrol, a well-known antioxidant compound (Porquet et al., 2013). SAMP8 mice, one of the mouse strains with the highest numbers and earliest appearance of granule clusters, are considered a model of oxidative stress, since high levels of free radicals have been observed in their brains (Perluigi et al., 2014). Furthermore, the MAO-B enzyme, a mitochondrial enzyme that may be related to the formation of reactive oxygen species, has been detected in high amounts in the granules, and its expression is higher in the hippocampal astrocytes of aged animals (Nakamura et al., 1995).

On the whole, these findings reinforce the important role played by oxidative stress in the appearance of these structures and point to a definite but as yet unclear relationship between oxidative stress, ageing and the degenerative process that takes place in the mouse hippocampus and leads to granule formation.

4.2. Genetic background

According to previous studies, hippocampal granule clusters show a strain-specific expression with ageing, as mentioned in Section 2.2. In order to take advantage of this feature, intercrosses between susceptible and resistant mouse strains have been used to identify the

chromosomal regions involved in granule cluster development. Early studies on intercrosses between C57BL/6 and DBA/2, CH3 or BALB/c revealed a dominant heritability of this trait in the first generation progeny (Jucker et al., 1994a, 1994b). A subsequent study consisting of quantitative trait loci analysis using the first and second progenies of a C57BL/6 and DBA/J strain intercross provided evidence of possible major gene loci involved in the appearance of hippocampal granule clusters (Krass et al., 2003). Specifically, a major gene locus with a dominant effect was identified 26 cM from marker D7Mit91, and two other gene loci, D10Mit15 and D10Mit10, were found to influence this phenotypic trait. Krass et al. (2003) speculated about the possible genes located in these sequences based on the granule components detected by immunohistochemistry, and it was therefore difficult to identify an appropriate candidate. It has been reported that D7Mit91 is involved in fat maintenance under dietary restrictions and is related to the insulin growth factor-1 receptor, and that D10Mit15 is associated with life span and other aspects (www.informatics.jax.org). This data reveals a complex genetic basis related to the onset and development of the granules. Thus, different mutations or gene alleles affecting the metabolism or oxidative stress levels could determine and influence the different degrees of granule formation. For example, as indicated above, ApoE-deficient mice have higher numbers of granules than control animals (Miyata and Smith, 1996) and malin knock-out mice (Valles-Ortega et al., 2011). Therefore, the presence of granules is probably related to a variety of genes and not one specific gene, and the genetic background of each mouse strain could affect the number of granules they present. In any case, oxidative stress may underlie and influence granule formation.

4.3. Natural IgM antibodies directed against the neo-epitope

IgM antibodies directed against the neo-epitope have been found in the sera of mice. These antibodies have been detected in, among others, three-month-old ICR-CD1, SAMP8 and BALB/c animals, and in ICR-CD1 and BALB/c animals of the same age maintained in specific and opportunistic pathogen-free conditions (unpublished results). Thus, these IgM antibodies must be considered as natural antibodies that are present in the organism without previous exposure to external antigens. Actually, the presence of these natural IgM antibodies is the reason behind the false-positive staining results of the granules when using antibodies obtained from mouse or rabbit ascites or serum. Moreover, the IgM antibodies of each animal can recognise the neoepitopes present in the granules of their own brain, which would indicate that the anti-neoepitope IgMs are in fact natural auto-antibodies. Whether anti-neoepitope IgMs exert a functional role in the appearance or development of granule clusters is unclear. Because these IgMs are natural antibodies, they could be involved in the removal of

brain senescent structures, since one of their functions is the clearance of tissue debris following degradation (Avrameas and Selmi, 2013).

4.4. Other treatments and experimental manipulations

Since it has been alleged that granules have a variety of identities, various treatments with different experimental aims have been performed and have occasionally achieved an increase or a decrease in the quantity of granule clusters in mouse brains. These experimental treatments include bone marrow replacement in ApoE-deficient mice, which resulted in a reduction in the number of hippocampal granule clusters compared to untreated mice (Robertson et al., 2000), and chronically induced neuroinflammation, which provoked an increase in the size and number of granules with respect to the control group (Doehner et al., 2012; Knuesel et al., 2009). Finally, the implantation of neural stem cells in disomic and trisomic Ts65Dn mice showed a significant decrease in the number of granule clusters in the CA1 hippocampal area in both genotypes (Kern et al., 2011). However, further investigation is required in order to interpret and achieve a better understanding of these observations.

5. DISCUSSION AND CONCLUSIONS

Since the PAS granules were first described by Lamar et al. in 1976, these structures have been erroneously identified as different brain deposits related to numerous neurodegenerative diseases.

A wide range of studies have assimilated the granule clusters to the corpora amylacea in the human brain (Mitsuno et al., 1999; Sinadinos et al., 2014). This theory has been based on the PAS-positive staining of these deposits, their association with ageing and their formation in both astrocytes and neurons (Cavanagh, 1999). Nevertheless, some other important features of the granules are not consistent with these deposits, e.g. their characteristic and specific distribution in the hippocampus, the granule ultrastructure and the conflicting results in other histochemical stainings (Cavanagh, 1999; Robertson et al., 1998). Another glycosidic brain deposit with which the granules have been related are Lafora bodies. However, these deposits develop in neurons, their formation pattern does not present fragmentation or blebbing of the cytoplasm (Ganesh et al., 2002) and their identification has been based on immunohistochemical procedures using anti-advanced glycation end-product (AGE) and anti-ubiquitin antibodies (Valles-Ortega et al., 2011). The granules have been also identified as amyloid aggregates (Del Valle et al., 2010; Doehner et al., 2010; Robertson et al., 2000; Wirak

et al., 1991), although this interpretation should be ruled out due to the controversial results and false-positive staining results (Jucker et al., 1992; Kern et al., 2011; Krass et al., 2003; Manich et al., 2014b). Similarly, PAS granules have been interpreted as a sign of tauopathies (Kern et al., 2011; Krass et al., 2003; Manich et al., 2011) and even autophagosomes formed when the proteostasis has been altered (Soontornniyomkij et al., 2012). Again, these statements were based on immunohistochemical studies. Finally, a group of studies have hypothesised that the granules are reelin deposits extruded by aged neurons and phagocytised by astrocytes due to a failure in the proteasome/autophagosome system, a parallel process to reelin neuron loss (Doehner et al., 2010, 2012; Knuesel et al., 2009; Kocherhans et al., 2010; Madhusudan et al., 2009). The false-positive staining results could explain the detection of reelin as a granule component.

Each of these identifications has generated a particular theory about their significance and a different set of experiments and proposals. These misinterpretations, which have appeared and disappeared regularly over time, have generated disagreements and controversy. The principal cause of these misinterpretations is the high number of false immunostaining results produced by the presence of some neo-epitopes on these structures and the existence of natural IgMs directed against them. Now that this point has been clarified, and according to the items exposed previously, the nature and significance of these granules should now be reconsidered.

Throughout this review, PAS granules have been shown to be structures of a degenerative nature that have been described and studied primarily in the hippocampus of mice, although they may also exist in other brain structures and other mammals. The granules mainly form in astrocyte processes and tend to appear in clusters, where each cluster corresponds to the set of granules of a determined astrocyte. During formation of the granules, the degeneration of cytoplasmic organelles, especially mitochondria, takes place, irregular vacuolar structures emerge and the cytoplasmic membrane occasionally fragments and alters. The cytoplasmic membranes surrounding the neuropil structures may also suffer alterations, and in some cases the cellular boundaries are lost. Once constituted, the granules contain a dense central part formed by the accumulation of membranous fragments and a peripheral zone with a very low density of organelles.

The relationship between hippocampal PAS granules and ageing has been repeatedly and consistently shown. A comprehensive observation of granule formation leads to the identification of global ageing-related features that may be connected to the functional

impairment of the ageing brain. In fact, several hallmarks of ageing described by López-Otín et al. (2013) may be present in the granule formation process, including cellular senescence, mitochondrial dysfunction and deregulated nutrient sensing, and some others may be involved in the process, e.g. altered intercellular communication and loss of proteostasis. Therefore, considering the multifactorial characteristics of ageing, it is not surprising that the factors related to the appearance of granules could be numerous and diverse. One seemingly important factor is the level of oxidative stress, since in different experimental designs in which oxidative stress levels are modified (alterations in energy metabolism, use of antioxidants, studies using high-fat diets, etc.), the onset and progression of the granules is altered. Mitochondrial degeneration and the increased enzyme activity of MAO-B in the granules may also be associated with increased oxidative stress. Another factor that seems important is the genetic background of the animal, since different strains of mice have shown different trends in the presentation of these granules. This genetic background might be related to the presence of different alleles or gene mutations affecting the metabolism or the levels of oxidative stress.

Nevertheless, the appearance of neo-epitopes in hippocampal granules may also be related to ageing and increased oxidative stress. In ageing processes, there is an increase in the generation of AGEs (Goldin et al., 2006), which are formed by the modification of proteins or lipids that become non-enzymatically glycated 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, which eventually lead to the generation of AGEs (Schmidt et al., 1994). Although it is unclear whether or not the glycosidic neo-epitopes found in the granules are AGEs, it is important to note that glycosidic neo-epitopes can be targets of immune surveillance and natural IgM antibodies (Brändlein et al., 2003; Vollmers and Brändelin, 2006). Therefore, it is not surprising that the new carbohydrate epitope that appears in the hippocampus granules of mice would be recognised by natural IgM antibodies present in ubiquitous form in the ascites or serum of mice or other species.

From our point of view, the neo-epitopes present in the PAS granules could become a useful brain-ageing bio-marker in degenerative brain processes. Moreover, these neo-epitopes and the natural IgMs directed against them suggest that autoimmunity could become a new focus in the study of age-related degenerative processes. Natural antibodies could become a useful new tool for the treatment of age-related neurodegenerative diseases.

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FIGURE LEGENDS

Figure 1. Clusters of granules in the CA1 region of the hippocampus of a 12-month-old SAMP8 mice stained with PAS. As it can be observed in B and C magnifications, clusters constituted by 40-50 granules are intensively stained. Or: Oriens layer, Py: pyramidal layer, Rad: *stratum radiatum.* Scale bars: A) 200 µm, B and C) 50 µm.

Figure 2. Coronal hippocampal sections presenting granule clusters of SAMP8 mice aged 3, 6 and 12 months (A, B and C, respectively). Granules were immunostained with 4G8 antibody directed against β-amyloid peptides but containing anti-neo-epitope IgM antibodies. Granule clusters start to appear in the CA1 hippocampal region and they spread throughout the hippocampus with increasing age. Or: *oriens* layer, Py: pyramidal layer, Rad: *stratum radiatum*, LMol: *lacunosum moleculare* layer, Mol: molecular layer of the dentate gyrus. (adapted from Del Valle et al., 2010).

Figure 3. Electron microscopy images of hippocampal mature granules from **a** 14-month-old SAMP8 mice. Mature granules measured up to 3 µm. When tissue was embedded in Spurr and treated with OsO_4, granules showed an electron-dense core formed by a dense mesh of fibrillar membranous-like structures, generally encircled by a translucent halo (A, C and D). When tissue was embedded in Lowicryl, the core appeared smoothly stained (B). Blebs or big cisterns (*), as well as degenerating mitochondria (full arrowhead) were located in the translucent area. A characteristic junction with a membrane of an adjacent cell could be observed (empty arrowhead). In the surroundings of the mature granule, a degenerating dendrite (full arrow) and a mitophagy process (empty arrow) could be visualized. Scale bar: 1 µm. (Adapted from Manich et al., 2014a).

Figure 4. Electron microscopy images of hippocampal immature granules from a 14-month-old SAMP8 mice. A) Immature granules showed some sparse membranous-like structures instead of the electron-dense core. B and C) Granules in a more advanced stage of development. Big blebs or bubbles (*) and degenerating mitochondria (full arrowheads) appeared in the peripheral zone. The plasma membrane and membranes of adjacent structures showed instability and fragmentation (full arrows), causing a loose of the boundaries of these structures. This process of rupture can affect dendritic spines (empty arrowheads), as clearly shown in C). D) Inset from A in which the instability and the fragmentation of the membranes could be better observed. Scale bar: A and B): 1 µm; C, D and E) 200 nm. (Adapted from Manich et al., 2014a).

Figure 5. Confocal microscopy image of a double immunohistochemical staining of the CA1 hippocampus area from a 9-month-old SAMP8 mice. Granules were detected with a mouse IgM antibody against the neo-epitope (red), and reactive astrocytes were detected with an anti-GFAP antibody (green). Nuclei were stained by Hoechst (blue). In this image the close association of some granule clusters and astrocytes is shown. (Adapted from Manich et al., 2014a)