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Decreased myeloperoxidase expressing cells in the aged rat brain after excitotoxic damage

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ABSTRACT

Brain aging is associated to several morphological and functional alterations that influence the evolution and outcome of CNS damage. Acute brain injury such as an excitotoxic insult induces initial tissue damage followed by associated inflammation and oxidative stress, partly attributed to neutrophil recruitment and the expression of oxidative enzymes such as myeloperoxidase (MPO), among others. However, to date, very few studies have focused on how age can influence neutrophil infiltration after acute brain damage. Therefore, to evaluate the age-dependent pattern of neutrophil cell infiltration following an excitotoxic injury, intrastriatal injection of N-methyl-D-aspartate was performed in young and aged male Wistar rats. Animals were sacrificed at different times between 12 h post-lesion (hpl) to 14 days post-lesion (dpl). Cryostat sections were processed for myeloperoxidase (MPO) immunohistochemistry, and double labeling for either neuronal cells (NeuN), astrocytes (GFAP), perivascular macrophages (ED-2), or microglia/macrophages (tomato lectin histochemistry). Our observations showed that MPO + cells were observed in the injured striatum from 12 hpl (when maximum values were found) until 7 dpl, when cell density was strongly diminished. However, at all survival times analyzed, the overall density of MPO + cells was lower in the aged versus the adult injured striatum. MPO + cells were mainly identified as neutrophils (especially at 12 hpl and 1 dpl), but it should be noted that MPO + neurons and microglia/macrophages were also found. MPO + neurons were most commonly observed at 12 hpl and reduced in the aged. MPO + microglia/macrophages were the main population expressing MPO from 3 dpl, when density was also reduced in aged subjects.
These results point to neutrophil infiltration as another important factor contributing to the different responses of the adult and aged brain to damage, highlighting the need of using aged animals for the study of acute age-related brain insults.

1. Introduction

Acute injury to the central nervous system (CNS), such as traumatic brain injury, brain ischemia or excitotoxicity induces primary tissue damage which elicits an inflammatory response (Jin et al., 2010). This response is associated with secondary damage to surrounding tissue, contributing to delayed cell death and determining the final extent of tissue damage and lesion outcome (Mancuso et al., 2007). Besides the endogenous glial cell population, it is known that endothelial cells and leukocytes recruited from the blood are also significant cellular components of the neuroinflammatory process (McColl et al., 2007; Justicia et al., 2003; Wang et al., 2007). Among leukocytes, neutrophils enter into the sites of CNS injury during the first hours after the insult, forming the first line of defense (Man et al., 2007; Pardridge, 2007), being followed by monocytes at longer survival times (Chen et al., 2005). Neutrophils are the most abundant type of circulating white blood cells and belong to the polymorphonuclear cell family (PMNs) due to their multilobulated nuclei (Dale et al., 2008). Massive neutrophil infiltration and accumulation in sites of CNS injury induces the release of toxic substances such as reactive oxygen species (ROS) (Wang et al., 2007), which contribute to tissue damage (Weston et al., 2007), BBB dysfunction (Pun et al., 2009; Abbott et al., 2010) and delayed neuronal death. Myeloperoxidase (MPO) is a heme-containing peroxidase that constitutes the most abundant component in azurophilic granules in neutrophils, has often been used as an histopathological marker for neutrophils (Rausch et al., 1978; Klebanoff, 2005), and has recently been used as an in vivo marker of brain inflammation (Breckwoldt et al., 2008).

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It is well known that aging is associated with an enhanced susceptibility to suffer CNS chronic and acute diseases combined with poor post-injury recovery, which becomes an important health challenge considering the increasing life expectancy (Popa-Wagner et al., 2007; Rutten et al., 2007; Hayflick, 2000). At the cellular level, non-pathological age-related brain changes include metabolic disarrangements, excessive production of ROS and other morphological and functional alterations in neuronal and glial cells, inducing basal glial reactivity and decreased BBB permeability (Peinado et al., 2000; Campbell et al., 2007; Popescu et al., 2009; Farrall and Wardlaw, 2009). However, although it is assumed that these characteristics influence the response to brain injury, currently most studies still use young adult animals for the analysis of post-injury inflammatory processes.

In this regard, we have previously reported that neuronal, glial and inflammatory responses to acute striatal excitotoxic injury show significant age-differences in rats (Castillo-Ruiz et al., 2007; Campuzano et al., 2008). Aged (22–24 months old) animals display a delay in the neurodegenerative process, tissue damage and edema, concomitant with an earlier astroglial and microglial/macrophagic responses (Castillo-Ruiz et al., 2007; Campuzano et al., 2008). In addition, aged animals display increased basal levels of cytokines, but attenuated post-injury induced cytokine response (Campuzano et al., 2008; Campuzano et al., 2009), in parallel with reduced expression of the inflammatory enzyme inducible nitric oxide synthase (iNOS) and increased expression of cyclooxygenase-2 (COX2) (Campuzano et al., 2008; Campuzano et al., 2009). As neutrophils are important contributors to the production of these inflammatory mediators which show age-related differences, and MPO is an enzyme contributing to oxidative stress, the aim of the present study was to identify and assess putative changes in the presence of MPO expressing cells after acute striatal damage in the aged versus the adult rat brain.

2. Materials and methods

2.1. Animals

For this study we have used a total of 94 male Wistar rats: 47 adult (3–4 months of age) and 47 aged (22–24 months) rats. Animals of both ages were distributed into three groups: intact controls (n = 5 in each age group), saline-injected controls (n = 12 in each age group) and N-methyl-D-aspartate (NMDA)-lesioned animals (n = 30 in each age group). Animals were housed individually in cages with free access to food and water, controlled temperature (22 °C), humidity (55%) and light/dark cycle (12 h, 8 pm–8 am). All experimental work was conducted according to established European Union bioethical directives and was approved by the ethical commission of the Universitat Autònoma de Barcelona. During all experimental procedures, unnecessary animal suffering was avoided.

2.2. Excitotoxic lesions

Rats were placed in a stereotaxic frame under isofluorane anesthesia, the skull was opened using a surgical drill, and 1 μl of saline solution (0.9% NaCl, pH 7.4) containing 120 nmol of N-methyl-D-aspartate (NMDA) (Sigma, M-3262) was injected into the right striatum (caudate–putamen, bregma coordinates A = +0.12, L = −0.3; V = −0.45 cm) (Paxinos and Watson, 1986) using a 1 μl Hamilton microsyringe coupled to an automatic microinjector (Stoelting, Illinois, USA) at a speed of 0.2 μl/min. After the injection, the needle was kept for 10 min in order to facilitate diffusion into the striatum and minimize reflux. Saline-injected control animals received an injection of 1 μl of the vehicle saline solution. After suture, rats were placed in a thermal pad and maintained at normothermia until they recovered from anesthesia.

2.3. Sacrifice and tissue processing

For histological and immunohistochemical analyses, after 12 h (hpl), 1 day, 3 days, 5 days, 7 days and 14 days after injection (dpl), a minimum of 5 NMDA-lesioned animals and 2 saline-injected controls for each age group and survival time were anesthetized and intracardially perfused for 10 min with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, brains were quickly removed, postfixed for 4 h at 4 °C in the same fixative, washed twice in buffer and immersed for cryoprotection in a 30% sucrose solution in 0.1 M phosphate buffer (pH 7.4) until they sunk and were quickly frozen with dry CO. Intact control animals were processed equally. Thirty-micron-thick parallel frozen coronal sections were obtained with a Leica cryostat and were either stored floating free in antifreeze solution at −20 °C or mounted on gelatin-coated slides.

2.4. Toluidine blue staining

Sections mounted on slides were rinsed, stained with toluidine blue (0.1% toluidine blue solution in Walpole buffer −pH 4.5 −for 15 min), rinsed, dehydrated, and differentiated in n-butyl alcohol. After rinsing, slides were air dried, cleared in xylene, and coverslipped in DPX. These sections were used for the histological control of the intracerebral injection and to determinate the sites of cell counting (Fig. 1).

2.5. Single immunohistochemistry

Free-floating parallel sections were rinsed thoroughly in Tris buffered saline (TBS) (0.05 M Trizma base, 150 mM NaCl, pH 7.4) and endogenous peroxidase was blocked with 2% H_2O_2 in 70% methanol. After rinsing, sections were treated with blocking buffer (BB) (10% fetal calf serum in Tris buffered saline (TBS) + 1% triton X-100) for 30 min, and incubated overnight at 4 °C and then for 1 h at room temperature (RT) with primary antibody diluted in BB (Table 1). Sections were rinsed and incubated for 1 h at room temperature with the corresponding secondary antibody diluted in BB (Table 1). After incubation, sections were rinsed with TBS + 1% triton (TBS-T) and incubated in Avidin–Peroxidase (P-0364, Dakopatts) (1:400 in BB) for 1 h at RT. Finally, the peroxidase reaction product was visualized in 100 ml of TB (0.05 M Trizma base, pH 7.4) with 50 mg of 3, 3′-diaminobenzidine (DAB) and 33 μl of hydrogen peroxide. Negative controls were incubated without primary antibodies (data not shown). After washing, slides were mounted on gelatin-coated slides, air-dried, dehydrated in alcohol, cleared in xylene, and cover slipped with DPX.

2.6. Double immunolabeling

For double immunohistochemical labeling, free-floating parallel sections were rinsed in TBS, treated with BB for 30 min, and incubated overnight at 4 °C and then for 1 h at RT with the first primary antibody diluted in BB (Table 1). Sections were then rinsed and further incubated for 1 h with the corresponding secondary antibody, linked to a cyanine fluorochrome. For the second immunostaining, sections were rinsed in TBS-T and incubated overnight at 4 °C and then for 1 h at RT in a second primary antibody obtained in different species. After rinsing, sections were incubated for 1 h at RT with the corresponding secondary antibody linked to a fluorochrome or conjugated with biotin (Table 1). In the case of biotin-conjugated secondary antibodies, sections were then further incubated for 1 h at RT in Streptavidin-Cy2 or Avidin-Cy3 (Table 1).

For the demonstration of microglia/macrophages, tomato lectin (TL) histochemistry was used. Briefly, sections were incubated in biotin-labeled tomato lectin (6 μg/ml, Sigma, L0651) diluted in TBS-T overnight at 4 °C and 1 h RT. After rinsing with TBS-T, sections were incubated for 1 h RT with Streptavidin-Cy3 (Table 1). After the last

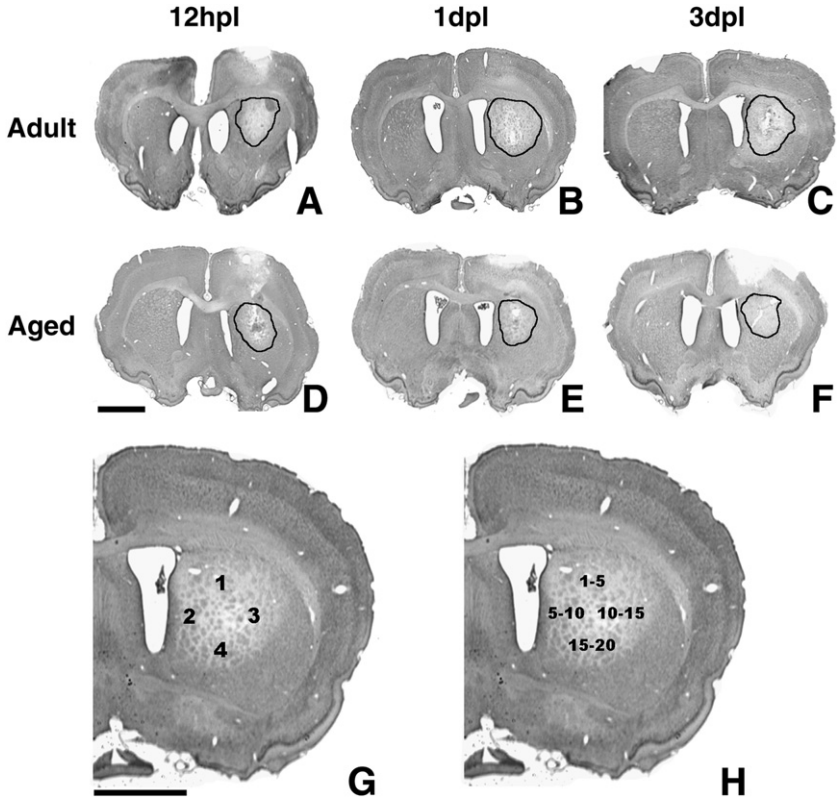

Fig. 1. Toluidine blue stained coronal sections of excitotoxically damaged adult and aged brains. Representative micrographs showing the striatal injured area (surrounded by a line) in the adult (A–C) and the aged (D–F) at different post-lesion survival times. Representative micrographs showing the area and the number of photographs performed at 10× for single MPO + cell counts (G), and at 40× for double-stained cells (H). Scale bars = 2 mm.

incubation, sections were rinsed in TBS and TB, mounted on gelatin-coated slides, air-dried, counterstained with DAPI (Sigma, D9542) and coverslipped with fluorescent mounting media (Dakopatts). Sections processed for double immunofluorescence were first analyzed using a Nikon Eclipse E-600 and next, using a LEICA-TCS-SP2 confocal microscope in order to perform an exhaustive double labeling study.

2.7. Cell density quantification

MPO labeled sections from NMDA-injected animals were analyzed using a Nikon Eclipse E 600 microscope interfaced to a DXM 1200 camera (Nikon) and a PC. Pictures of representative areas were taken at different magnifications using the software ACT-1 2.20 (Nikon Corporation). A minimum of 4 NMDA-injected animals for each survival time and age group were analyzed. For each animal, 4 selected sections ranging from 1 mm to −0.3 mm from bregma and containing the lesion core were analyzed (Fig. 1). Anatomical landmarks (size and position of the anterior commissures, corpus callosum, septum, lateral ventricles, striatum, and nucleus accumbens) were used to ensure that parameters were analyzed at similar levels within and between groups. For MPO + cell density quantification, the striatum was delimited in each section by the lateral ventricle medially, the corpus callosum dorsally and laterally, and by a line drawn between the two anterior commissures ventrally. In each section, 4 pictures of the striatum were taken at 10× (covering an area of 0.6 mm^2 each) and only MPO positive profiles clearly identified as specifically labeled cell somas were counted. The obtained number of profiles per section was corrected using the Abercrombie method to adjust for potential biases in counting methodology (Abercrombie, 1946; Guillery, 2002). Results are expressed as MPO + cells/mm^2. For the quantification of double immunolabeled cells, 15–20 pictures covering the whole striatal area were taken at 40× (area of 0.037 mm^2) in each section. Only profiles clearly identified as specifically double labeled cell somas were counted.

Table 1
Primary and secondary antibodies used for immunohistochemical labeling.

Primary antibodies	Species	Dilution	Source	Code
Anti-ED2	Mouse	1:500	Serotec	MCA342R
Anti-glial fibrillary acidic protein (GFAP)	Rabbit	1:1000	DAKO	Z0334
Anti-myeloperoxidase (MPO)	Rabbit	1:400	DAKO	A0398
Anti-neuronal nuclear antigen (NeuN)	Mouse	1:500	Chemicon	MAB377

Secondary antibodies and reagents	Dilution	Source	Code
Cy2-linked goat anti-rabbit	1:750	Amersham	PA42004
Cy3-linked goat anti-rabbit	1:1000	Amersham	PA43004
Biotinylated donkey anti-rabbit	1:200	Amersham	RPN1004
Cy3-linked goat anti-mouse	1:1000	Amersham	PA43002
Biotinylated sheep anti-mouse	1:200	Amersham	RPN1001
Cy2-linked Avidin	1:750	Amersham	PA42000
Streptavidin-peroxidase	1:400	Vector	SA5004
Cy3-linked Streptavidin	1:1000	Amersham	PA43000

2.8. Statistical analysis

Statistical analysis was performed using StatView 4.5. One-way ANOVA and Fisher post-hoc comparisons were used. All results are presented as mean values ± S.E.M. and $p \leq 0.05$ was considered significant.

3. Results

In both age groups injection of saline solution induced slight tissue disruption in the striatum, restricted to the needle track up to 3 days

post-injection. However, intrastriatal injection of NMDA produced tissue degeneration involving neuronal loss mainly in the striatum (caudate–putamen) but also in the frontal cortex and the corpus callosum (Fig. 1), as previously described in detail (Castillo-Ruiz et al., 2007).

3.1. The aged injured striatum showed decreased density of MPO + cells

In intact control animals of both ages, no MPO expression was observed in the cortex or striatum. Similarly, no MPO + cells were detected in the contralateral non-lesioned hemispheres of lesioned animals of both ages. Saline-injected control aged and adult animals showed scattered MPO + cells in the mechanically injured cortex and in the striatal tissue surrounding the needle track (data not shown).

Following intrastriatal NMDA injection, animals of both ages showed slight MPO immunoreactivity in the cortex (similar to that observed in saline-injected animals, data not shown), although most MPO + cells were seen in the damaged striatum, mainly in the lesion core but also in its periphery at both ages (Fig. 2B–G). From 1 dpl, MPO + cells were often observed around blood vessels of the injured striatal zone. Quantitative analysis by cell counting showed that in both age groups, peak values of MPO + cell density were observed at 12 hpl (Fig. 2A). However, the density of MPO + cells at this survival time was 24.32% lower in the aged versus the adult injured striatum; at 1 dpl and 3 dpl, the aged injured striatum showed a further 36.76% and 50.14% reduction, respectively. By 5 dpl a 25.40% reduction was still seen in the aged versus the adult injured striatum, whereas at 7 dpl the number of MPO + cells had decreased and there were no significant differences between ages. No MPO labeling was observed at 14 dpl at any age (data not shown).

3.2. MPO is expressed in neutrophils but is also present in neurons and microglia/macrophages

Analysis of MPO immunostaining showed that, whereas many MPO + cells displayed typical polymorphonuclear morphology and MPO + vesicular cytoplasmic structures (Fig. 4 and the bottom of this section), significant numbers of MPO + cells lacking neutrophil morphology were also observed.

Double immunohistochemical analysis of MPO and several glial and neuronal specific markers demonstrated that, in lesioned animals of both ages, MPO was also found in some NeuN + neurons and TL + microglia/macrophages showing amoeboid morphology in the injured striatum (Fig. 3), however, MPO expression was never found in

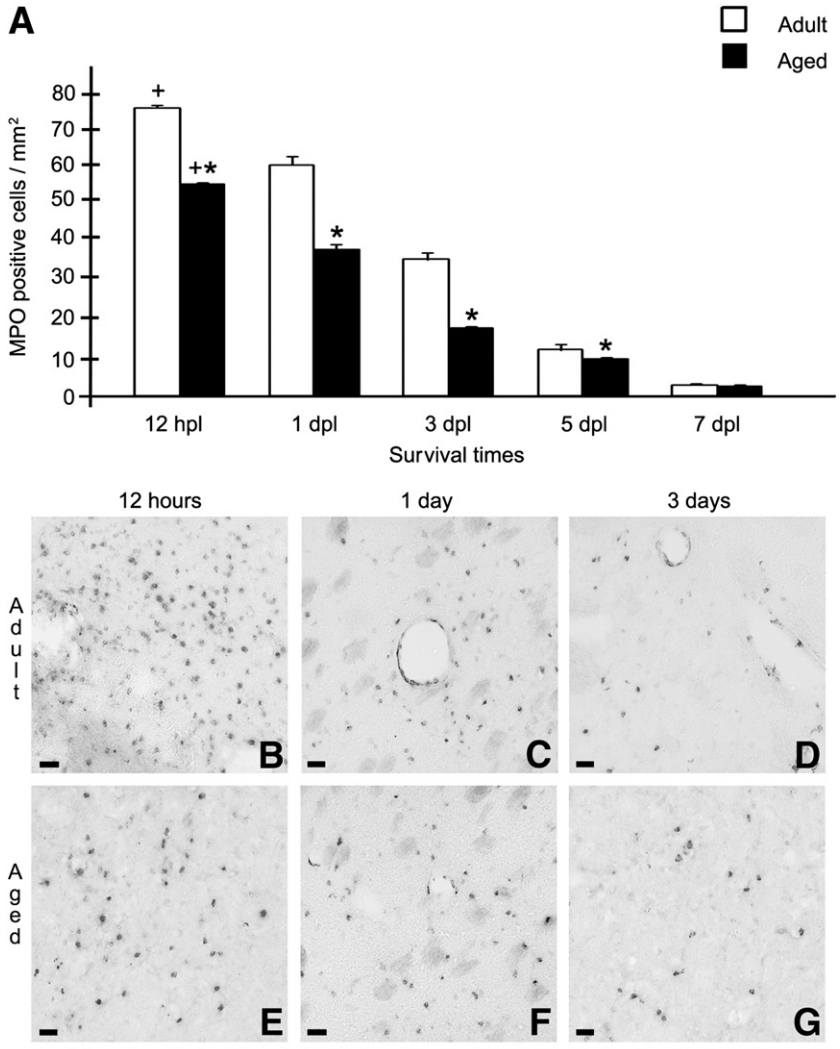

Fig. 2. Temporal pattern of MPO + cell expression (A) both in the adult (white columns) and aged (black columns) animals. The analysis of MPO + cell density shows peak values at 12 hpl at both ages but significantly lower values in aged animals at all survival times except at 7 dpl. Data are presented as mean ± s.e.m. Asterisks (*) show statistically significant differences between ages at the specific time-point ($p<0.05$) while pluses (+) shows statistically significant maximum values within each age group ($p<0.05$). Representative micrographs (B–G) show MPO + cells both in the adult (B–D) and the aged (E–G) at different survival times. At 12 hpl cells were scattered all over the injured striatum, while they were closer related to vessels later on. Scale bars = 30 μm.

(GFAP)+ astrocytes or ED2+ perivascular macrophages (data not shown). Quantitative analysis showed that MPO expression in NeuN+ neurons followed a similar temporal pattern compared to that of overall MPO labeling, peaking at 12 hpl at both ages (Fig. 3A). Similarly, the overall density of neuronal cells expressing MPO was decreased in the aged injured striatum when compared to the adult. However, as total MPO+ cells were decreased in the aged, at 12 hpl, when peak density of MPO+ cells occurred, MPO+/NeuN+ represented a 25.60% of total MPO cells in the adult, while they were 26.19% of all MPO+ cells respectively injured striatum. At 1 and 3 dpl, the percentage of MPO+/NeuN+ decreased, representing, in the aged, a 14.16% and 7.75% respectively, and 18.18% and 18.50% of total MPO+ cells in the adult. At 5 dpl, MPO+/NeuN+ cells represented the 22.22% of MPO cells in the adult, whereas no labeling of MPO/NeuN+ cells was detected in the aged injured striatum. At 7 dpl neither adult nor aged injured striatum showed double MPO+/NeuN+ cells (Fig. 3).

MPO expression in TL+ microglia/macrophages, showed peak values at 12 hpl in the aged injured striatum, and then progressively decreased until 7 dpl, when MPO+/TL+ microglia/macrophages were still observed at both ages (Fig. 3B). In contrast, in the adult, maximum density of MPO+ microglia/macrophages was not observed until 3 dpl, the only time point when the density of TL+/MPO+ cells was significantly higher than in the aged. Notably, the proportion of MPO+ cells identified as microglia/macrophages showed important age-related differences and was generally increased in the aged. At 12 hpl and 1 dpl, whereas in the adult injured striatum 21.94% and 25%, respectively, of MPO+ cells were identified as microglia/macrophages, this population accounted for the 30.95% and the 35.71% of total MPO+ cells in the aged. Notably, from 3 dpl, microglia/macrophages were the main population expressing MPO, representing 55.51% and 55.55% of total MPO+ cells in the adult injured striatum at days 3 and 5 respectively, and the 61.53% and the 85.74% in the same survival times in the aged (Fig. 3).

Quantification of MPO+ cells with polymorphonuclear morphology and cytoplasmatic MPO labeling lacking NeuN and TL labeling (Fig. 4) showed that neutrophil cell density followed a similar temporal pattern than total MPO+ cells, showing maximum neutrophil presence at 12 hpl at both ages (Fig. 3A) and decreasing thereafter. In comparison to the adult brain, the aged striatum showed a significant neutrophil reduction of 38.16% at 12 hpl, 44.21% at 1 dpl, a 41.05% seen at 3 dpl and 52.11% at 5 dpl. Data analysis showed that at 12 hpl, when peak density of total MPO+ cells and MPO+ neutrophils occurred, MPO+ neutrophils represented a 52.44% of total MPO cells in the adult, while they were 42.85% of all MPO+ cells in the aged injured striatum. This percentage increased at 1 dpl for both ages, when MPO+ neutrophils represented a 56.81% of total MPO cells in the adult, and 50.11% in the aged injured striatum, decreasing at later time points (25.97% vs 30.70% of total MPO+ cells at 3 dpl and 22.22% vs 14.28% at 5 dpl) (Fig. 4).

4. Discussion

In this study we describe important age-differences in the rapid MPO+ cell accumulation that takes place throughout the striatum after excitotoxicity. Notably, MPO+ cell density was decreased in the aged

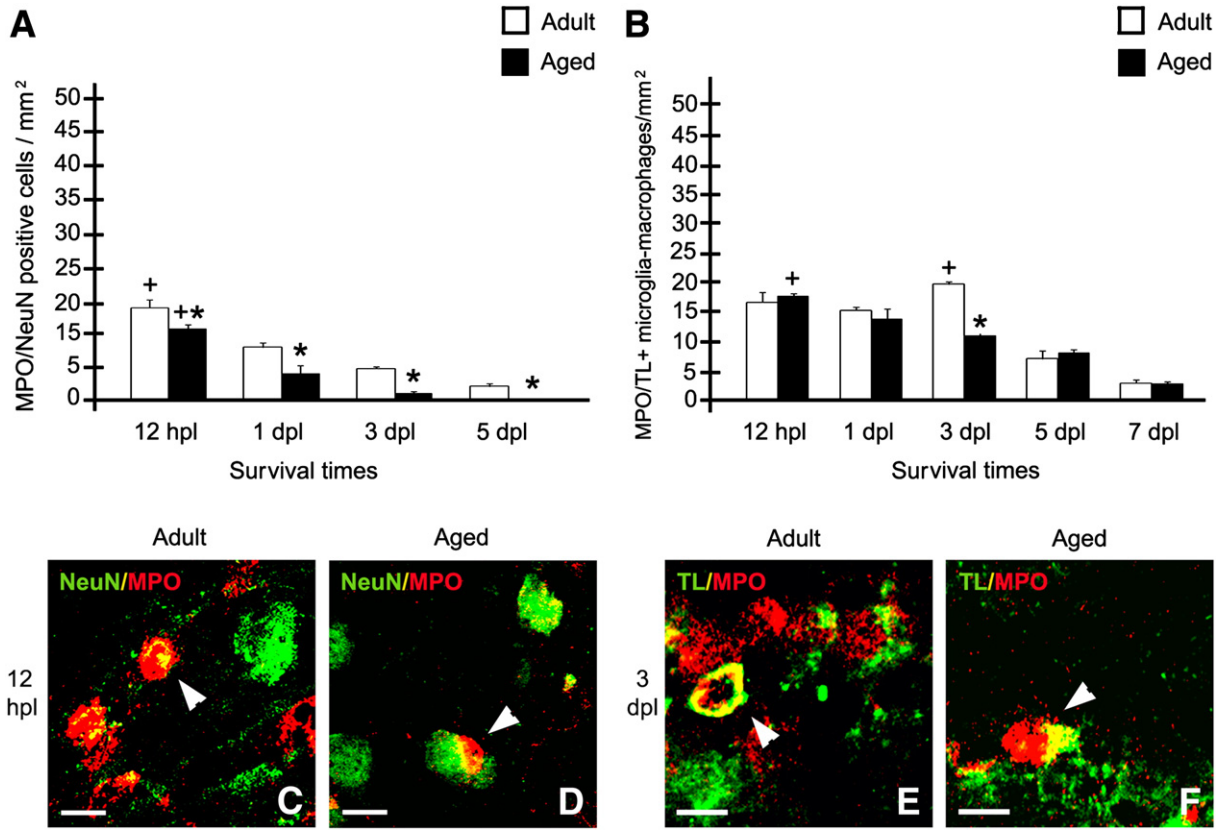

Fig. 3. Temporal pattern of MPO/NeuN (A) and MPO/TL (B) cell expression both in the adult (white columns) and aged (black columns) injured striatum. In A, the analysis of MPO/NeuN+ cell density shows significantly lower values in the aged striatum at all survival times and a significant peak in MPO/NeuN+ cell density at 12 hpl at both ages. In B, the analysis of MPO/TL+ cell density shows significantly lower values in the aged striatum only at 3 dpl and a significant peak in MPO/TL+ cell density at 12 hpl in the aged and at 3 dpl in the adult. Data are presented as means ± s.e.m. Asterisks (*) show statistically significant differences between ages at the specific timepoint ($p<0.05$), while pluses (+) show statistically significant maximum values within each age group ($p<0.05$). (C–F) Representative confocal images of both ages showing double labeling for MPO+ cells and NeuN (C,D), and TL (E,F) at different survival times. Arrowheads show cytoplasmatic co-localization of MPO with NeuN and TL. Scale bars = 10 μm.

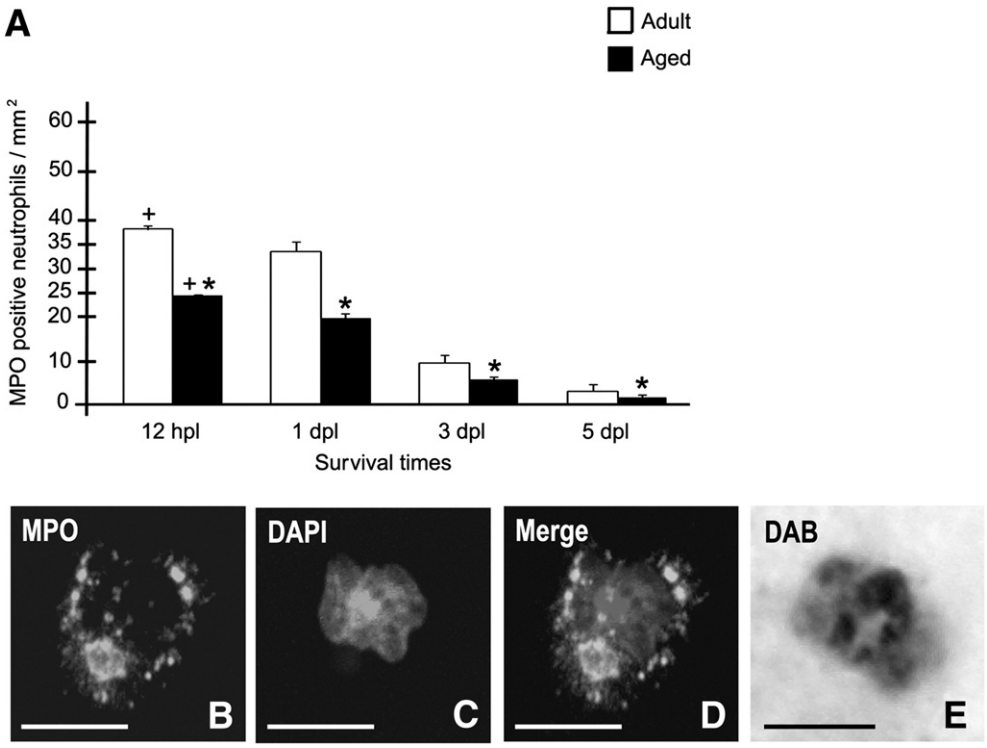

Fig. 4. Temporal pattern of MPO expression in neutrophils (A) both in the adult (white columns) and aged (black columns) animals. The analysis of MPO + neutrophil density shows labeling from 12 hpl to 5 dpl at both ages but significantly lower values in the aged striatum at all survival times. The peak of MPO + neutrophil density occurs at 12 hpl at both ages. Data are presented as means ± s.e.m. Asterisks (*) show statistically significant differences between ages at the specific time-point ($p<0.05$), while pluses (+) show statistically significant maximum values within each age group ($p<0.05$). (B–D) Confocal images showing MPO + neutrophils (B), nuclear morphology labeled with DAPI (C) and the merged image (D). (E) Representative image of typical neutrophil morphology labeled with DAB. Scale bars = 10 μm.

injured striatum when compared to the adult. Although most MPO + cells showed typical morphology of neutrophils (especially at 12 hpl and 1 dpl), we also identified MPO + cells showing neuronal or reactive microglia/macrophage markers. At both ages, neuronal MPO expression in injured striatum showed peak values at 12 hpl. In contrast, the maximal density of MPO + reactive microglia/macrophages was found at earlier times in the aged striatum.

4.1. Aged-induced changes in neutrophil density

The tightly regulated and restrictive permeability to cells and molecules is the most important feature of the BBB (Abbott et al., 2010; Farrall and Wardlaw, 2009), including control of leukocyte trafficking to the healthy CNS (Hickey, 1999). In the normal aged brain, BBB morphology is modified and permeability is altered as compared to the adult brain (Mooradian, 1988a; 1988b; Shah and Mooradian, 1997). Aging-associated BBB modifications are due, at least in part, to a significant age-dependent reduction in the number of laminin-positive (Campbell et al., 2007) and collagen-type-IV-containing vessels (Uspenskaia et al., 2004), and reduced capillary density (Farkas and Luiten, 2001). In addition, in several pathological conditions of the CNS, associated inflammation alters BBB morphology and function, inducing adherence of leukocytes to the vasculature, and penetration into the CNS (Pun et al., 2009; Turowski et al., 2005; Joice et al., 2009).

In the present study we show that following excitotoxic damage to the adult and aged rat striatum, peak values of infiltrated neutrophils are seen at 12 hpl in both ages. However, MPO + neutrophils are significantly reduced in the aged injured striatum at all survival times, in agreement with a previous study reporting diminished neutrophil infiltration in aged rats after intracerebral hemorrhage (Gong et al., 2008). Reduced presence of MPO + neutrophils at sites of neuroinflammation in the aged may be attributed to several factors: A first explanation could be an age decrease in circulating neutrophils (Schroder and Rink, 2003); however, it has been shown that the number of bone marrow precursor cells remain unaltered in human aging, and the number of circulating polymorphonuclear cells has been claimed to be either significantly increased in the elderly (Cakman et al., 1997), or remain unchanged with age (Chatta et al., 1993; Born et al., 1995). Secondly, changes may be attributed to an impairment of neutrophil recruitment in the elderly, but literature is again contradictory: whereas Biasi et al. (1996) reported no differences in migration between elderly and young neutrophils in vivo, and Esparza et al. (1996) found that the migratory response of PMN is also not affected by age; other groups have reported reduced chemotaxis with increasing age (Gong et al., 2008; Niwa et al., 1989; Wenisch et al., 2000). Thirdly, age-induced reduction in CNS neutrophil presence could also be attributed to a compromised function by an accelerated entry into apoptosis at the site of injury (Koedel et al., 2009) as it is known that neutrophils have a short lifespan and die by apoptosis few hours after activation (Savill et al., 1989; Savill, 1997; Arruda and Barja-Fidalgo, 2009). In this sense, although there are few studies analyzing neutrophil apoptosis in the elderly, they have shown that the susceptibility of unstimulated PMNs to apoptosis is slightly increased in aging, which has been related to the fact that aged neutrophils show a diminished capacity to be rescued by anti-apoptotic mediators (Fulop et al., 1997; Tortorella et al., 1998). Finally, age-related changes in lymphocyte function might also further compromise efficiency of neutrophils, reducing their phagocytic capacity, superoxide generation and/or degranulation (Lord et al., 2001).

Besides the age-related reduction in the number of infiltrated neutrophils, which is reported here, important factors determining the extent of tissue injury include neutrophil capacity for the production of inflammatory mediators, which may also change in aging. In addition to MPO, infiltrated neutrophils express other proinflammatory

enzymes like cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS) which have been implicated in delayed neuronal death by increasing oxidative stress (Gong et al., 2000; Lerouet et al., 2002; Beck et al., 2007). Interestingly, we have previously reported, after striatal excitotoxicity, maximum number of MPO + neutrophils expressing COX2 at 1 dpl at both ages; although adult animals showed double the density of MPO/COX-2+ neutrophils than aged rats. In addition, we also described a maximum number of MPO + neutrophils expressing iNOS at 1 dpl in adult injured striatum while in aged rats the maximum value was seen at 12 hpl (Campuzano et al., 2008). In this sense, It has been suggested that early endogenous NO production exerts a neuroprotective effect reducing neutrophil infiltration after brain damage (Batteur-Parmentier et al., 2000). As such, the reduced number of neutrophils and their earlier expression of iNOS in the injured aged striatum could be implicated, among other factors, in the delayed expansion of tissue injury and the later onset of neurodegeneration previously described in the excitotoxically damaged aged striatum (Castillo-Ruiz et al., 2007).

Another factor determining the extension of neuronal damage after acute injury in the aged brain is BBB disruption and edema formation that potentiate lesion extension (DiNapoli et al., 2008). In this regard, we have previously shown that edema formation is delayed after striatal excitotoxicity in the aged rat brain (Castillo-Ruiz et al., 2007), which may possibly be related to a reduction in BBB permeability (Kawai et al., 2001), and is certainly related to the reduced neutrophil infiltration reported in this study. Neutrophils have been implicated in the generation of vasogenic cerebral edema and may play a role in BBB breakdown (Plateel et al., 1997). Therefore, in addition to being influenced by edema, the reduced neutrophil infiltration in the aged injured brain could in turn, contribute to the reduced edema observed in the aged brains at 1 dpl (Castillo-Ruiz et al., 2007).

4.2. MPO expression in microglia/macrophages and neurons

MPO expression has been described in circulating neutrophils and monocytes (Dale et al., 2008; Nauseef, 1988) as well as in tissue infiltrated neutrophils and monocytes at early times post-lesion, and in macrophages at later times (Breckwoldt et al., 2008; Daugherty et al., 1994; Nagra et al., 1997; Sugiyama et al., 2001), as we also report here. This delayed presence of MPO in macrophages may be in part attributed to the engulfment of other MPO expressing cells by reactive microglia/macrophages. Notably, the higher density of MPO microglia/macrophages we report in the adult injured striatum at 3 dpl coincides with an earlier neuronal cell death and a higher phagocytic capacity of microglia/macrophages after adult lesions, observations suggested by our previous research (Castillo-Ruiz et al., 2007; Campuzano et al., 2008).

In addition, MPO expression was also seen in injured neurons at early time points, as previously reported in neuronal cultures and in studies of patients with Alzheimer disease (Reynolds et al., 2000), where MPO expression was found both at the mRNA and protein levels, and was associated with aging-associated increase in neuronal oxidative stress. This data together with our results suggest that neuronal MPO expression may contribute to increased oxidative stress in progressive neurodegenerative diseases as well as after acute injuries (Green et al., 2004; Reynolds et al., 1999).

In conclusion, this work points to reduced neutrophil infiltration in the aged striatum as another factor involved in the age-differences in the response to striatal excitotoxic injury. Notably, the presence of MPO in neurons and microglia/macrophages should not be underestimated and further research to elucidate its role in injured aged brain is promising. All these results highlight the importance of using aged animals for experimental models of brain lesions associated to the elderly.

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