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Increased Levels of Proinflammatory Cytokines in the Aged Rat Brain Attenuate Injury-induced Cytokine Response After Excitotoxic Damage

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In order to evaluate proinflammatory cytokine levels and their producing cell types in the control aged rat brain and after acute excitotoxic damage, both adult and aged male Wistar rats were injected with N-methyl-D-aspartate in the striatum. At different survival times between 6 hr and 7 days after lesioning, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were analyzed by enzyme-linked immunosorbent assay and by double immunofluorescence of cryostat sections by using cell-specific markers. Basal cytokine expression was attributed to astrocytes and was increased in the normal aged brain showing region specificity: TNF-α and IL-6 displayed age-dependent higher levels in the aged cortex, and IL-1β and IL-6 in the aged striatum. After excitotoxic striatal damage, notable age-dependent differences in cytokine induction in the aged vs. the adult were seen. The adult injured striatum exhibited a rapid induction of all cytokines analyzed, but the aged injured striatum showed a weak induction of cytokine expression: IL-1β showed no injury-induced changes at any time, TNF-α presented a late induction at 5 days after lesioning, and IL-6 was only induced at 6 hr after lesioning. At both ages, in the lesion core, all cytokines were early expressed by neurons and astrocytes, and by microglia/macrophages later on. However, in the adjacent lesion border, cytokines were found in reactive astrocytes. This study highlights the particular inflammatory response of the aged brain and suggests an important role of increased basal levels of proinflammatory cytokines in the reduced ability to induce their expression after damage.

Key words: aging; microglia; astrocyte; interleukin-1β; TNF-α; interleukin-6; striatum; cortex

Normal brain aging is characterized by region specific changes in the vasculature, the extracellular compartment, and cellular and biochemical alterations in glia and neurons (Scahill et al., 2003; Shah and Mooradian, 1997; Sykova et al., 1998). The aging-induced increase in oxidative stress and the important decline in neurotransmission induce a progressive loss of neuronal function (Finkel and Holbrook, 2000; Monti et al., 2004; Segovia et al., 2001). Glial cells show generally activated phenotypes with changes in cell morphology and metabolism, including astroglial hypertrophy associated with a significant increase in the expression of the intermediate filament component glial fibrillary acidic protein (GFAP); and expression of antigen-presenting molecules primarily in white matter microglial cells (Amenta et al., 1998; Finch, 2002; Finch et al., 2002; Perry et al., 1993; Yu et al., 2002). These activated phenotypes are accompanied with changes in inflammatory gene expression: microarray technology studies have identified that about 35% of total mRNA increases in the aged mouse cortex and cerebellum are inflammatory-related proteins (Lee et al., 2000). Accordingly, several reports have described increased levels of the proinflammatory cytokines interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) mainly in the aged hippocampus but also in cortical regions (Katafuchi et al., 2003; Maher et al., 2004; Murray et al., 1997; Nolan et al., 2005; Sierra et al., 2007; Terao et al., 2002; Xie et al., 2003; Ye and Johnson, 1999). Augmented basal levels of these inflammatory-related molecules, mainly IL-1β, are thought to disrupt normal physiology and contribute to age-dependent deficits in behavioral function (Gemma et al., 2005; Lynch and Lynch, 2002; Yirmiya et al., 2002).

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Undoubtedly, these characteristics influence the susceptibility and response of the aging brain to damage and are often used to explain why aging is the major risk factor for higher incidence of brain injuries and worse outcome after an insult (Brown et al., 2003; Bruns and Hauser, 2003; Petcu et al., 2008; Woo et al., 1992). However, it should be noted that although there is an extensive literature reporting cytokine gene and protein changes after adult brain damage, including traumatic injury (Giulian and Lachman, 1985; Holmin et al., 1997; Woodroofe et al., 1991), ischemia (Gregersen et al., 2000; Lambertsen et al., 2005; Orzylowska et al., 1999), and excitotoxicity (de Bock et al., 1996; Pearson et al., 1999; Yabuuchi et al., 1993), few studies are available on the expression of cytokines after acute damage to the aged brain. These studies have reported increases in IL-1β, TNF-α and/or IL-6 after traumatic brain injury (Kyrkanides et al., 2001; Sandhir et al., 2004; Shah et al., 2006), but no studies have focused on the cytokine induction after excitotoxicity to the aged brain.

Previous work in our laboratory found that aged rats respond to excitotoxic damage by showing slower progression of neurodegeneration and an exacerbation of the astroglial response when compared with adult rats (Castillo-Ruiz et al., 2007). In addition, these changes are accompanied by an earlier onset of microglial changes and an altered microglial expression of the proinflammatory enzymes cyclooxygenase-2 and inducible nitric oxide synthase (Campuzano et al., 2008). Because cytokines are known to determine the final outcome of brain damage by modulating cell activation, proliferation, and migration, as well as producing other injury-induced molecules and acting as important intercellular messengers involved in neuronal-glial interrelationships and the cross talk between astrocytes and microglial cells (Gosselin and Rivest, 2007; Mrak and Griffin, 2005; Wang and Campbell, 2002), we hypothesized that excitotoxic acute damage to the aged brain may induce a different pattern of cytokine expression than in the adult brain. Therefore, the aim of the present study was to determine the expression levels of IL-1β, IL-6, and TNF-α and their spatiotemporal and cellular expression pattern in the aged vs. the adult striatum in intact control brains and after an excitotoxic lesion.

MATERIALS AND METHODS

Experimental Animals

Experimental animal work was conducted with male Wistar rats 3–4 months old (adult animals) and 22–24 months old (aged animals). Animals were distributed into three groups: intact controls, saline-injected controls, and N-methyl-D-aspartate (NMDA)-lesioned animals. Animals were housed individually in cages with free food and water supply, in an environment with controlled temperature (22°C), humidity (55%), and light/dark cycle (12 hr). All experimental work was conducted according to established European Union bioethics directives and was approved by the ethical commission of the Universitat Autonoma Barcelona. During the entire process, unnecessary animal suffering was avoided.

Excitotoxic Lesions

Rats were placed in a Kopf stereotaxic frame under isoflurane anesthesia. The skull was opened with a surgical drill, and 1 μl of sterile saline solution (0.9% NaCl, pH 7.4) containing 120 nmol of NMDA (Sigma, M-3262) was injected into the right striatum (caudate–putamen, A = +0.12, L = −0.3; V = −0.45 cm from bregma) (Paxinos, 1986) with a 1-μl Hamilton microsyringe coupled to an automatic microinjector (Stoelting, Wood Dale, IL) at a speed of 0.2 μl/min. After the injection, before retraction, the needle was kept at the same depth for 10 min 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 suturing, rats were placed in a thermal pad and maintained at normothermia until recovery from anesthesia, when they were caged individually until sacrifice.

Sacrifice and Tissue Processing

For histological and immunohistochemical analysis, after survival times of 6 hr, 12 hr, 1 day, 3 days, and 5 days after injection, a minimum of five NMDA-lesioned animals and two saline-injected controls for each age group and survival time were anesthetized and intracardially perfused for 10 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, brains were quickly removed, postfixed for 4 hr at 4°C in the same fixative, and immersed for cryoprotection in a 30% saccharose solution in 0.1 M phosphate buffer (pH 7.4) until freezing. Intact control animals ($n = 4$ adult; $n = 8$ aged) that did not receive any injection were processed the same way. Thirty-micron-thick parallel frozen coronal sections were obtained with a Leitz cryostat and were either stored floating free in antifreeze solution at −20°C or mounted on gelatin-coated slides.

For enzyme-linked immunosorbent assay (ELISA) analysis, 50 NMDA-lesioned animals (5 for each survival time and age group) were killed by decapitation at 6 and 12 hr, and 1, 3, and 5 days after injection. In addition, five intact adult controls and five intact aged controls were also processed. Brains were quickly removed and the ipsilateral cortex and striatum were dissected out, frozen separately on dry ice, and kept at −80°C. Samples were weighed and homogenized in ice-cold Tris/EDTA/HCl buffer containing a cocktail of protease inhibitors (Roche, 11697498001) and phosphatase inhibition cocktail (Sigma, P2850) with a Potter homogenizer. Total protein content in each sample was measured by the bicinchoninic acid method (Sigma, B9643), as previously described (Smith et al., 1985).

Toluidine Blue and Fluoro-Jade B Staining

Parallel cryostat sections mounted on slides were either stained with toluidine blue or processed for Fluoro-Jade B staining, a fluorescent marker for the localization of degenerating neurons (Castillo-Ruiz et al., 2007; Schmued and Hopkins, 2000). For toluidine blue staining, sections were stained with 0.1% toluidine blue solution in Walpole buffer (pH 4.5)

for 15 min, rinsed, dehydrated, and differentiated in *n*-butyl alcohol. For Fluoro-Jade B staining, slides were immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min, rehydrated in graded ethanol, rinsed in water, and oxidized with 0,06% potassium permanganate (MnO_4K) for 15 min. Next, sections were rinsed with distilled water and immersed in 0,0004% Fluoro-Jade B (1FjB, Histo-Chem Inc., Jefferson, NC) plus 1% glacial acetic acid for 20 min. After rinsing, slides were air dried, cleared in xylene, and coverslipped in DPX. Toluidine blue– and Fluoro-Jade B–stained sections were used for the histologic control of the intracerebral injection and injury size (Fig. 4).

Double Immunofluorescence for Cytokines and Specific Cellular Markers

We used double-staining techniques for the simultaneous visualization of each cytokine and microglial cells by tomato lectin (TL) histochemistry (Acarin et al., 1994), astroglial cells by GFAP immunocytochemistry (Bignami et al., 1972), or neuronal cells by neuronal nuclear antigen (NeuN) immunocytochemistry (Mullen et al., 1992). After rinsing in Tris-buffered saline (TBS), free-floating parallel sections were treated with buffer blocking (BB) (10% fetal calf serum in TBS with 1% Triton X-100) for 30 min and incubated overnight at 4°C with one of the following antibodies diluted in BB: goat polyclonal anti-rat IL-1β (AF-501-NA, R&D Systems, Abingdon, UK) (1:1,000), goat polyclonal anti-rat IL-6 (AF-506-NA, R&D Systems) (1:1,000), or goat polyclonal anti-rat TNF-α (AF-510-NA, R&D Systems) (1:50). Afterward, sections were rinsed and incubated for 1 hr at room temperature with Cy3-conjugated anti-goat secondary antibody (Jackson Immunoresearch, West Grove, PA) (1:400). After rinsing, sections were then incubated in either rabbit anti-GFAP primary antibody (Dakopatts, Z-0334, Denmark) (1:1800) or mouse anti-NeuN monoclonal antibody (MAB 377; Chemicon International, Temecula, CA) (1:1,000), and further incubated with Cy2-conjugated anti-rabbit secondary antibody (Amersham, PA-42004) (1:1,000) or Cy2-conjugated anti-mouse secondary antibody (Amersham, PA-42002) (1:1,000), respectively. Sections for double staining with TL

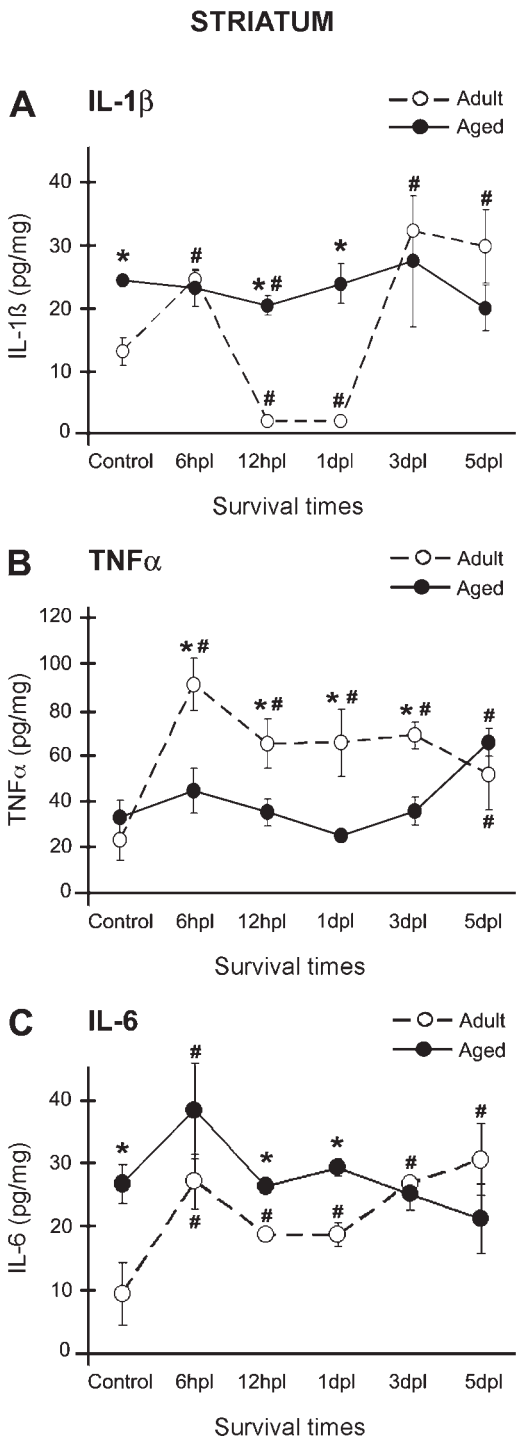
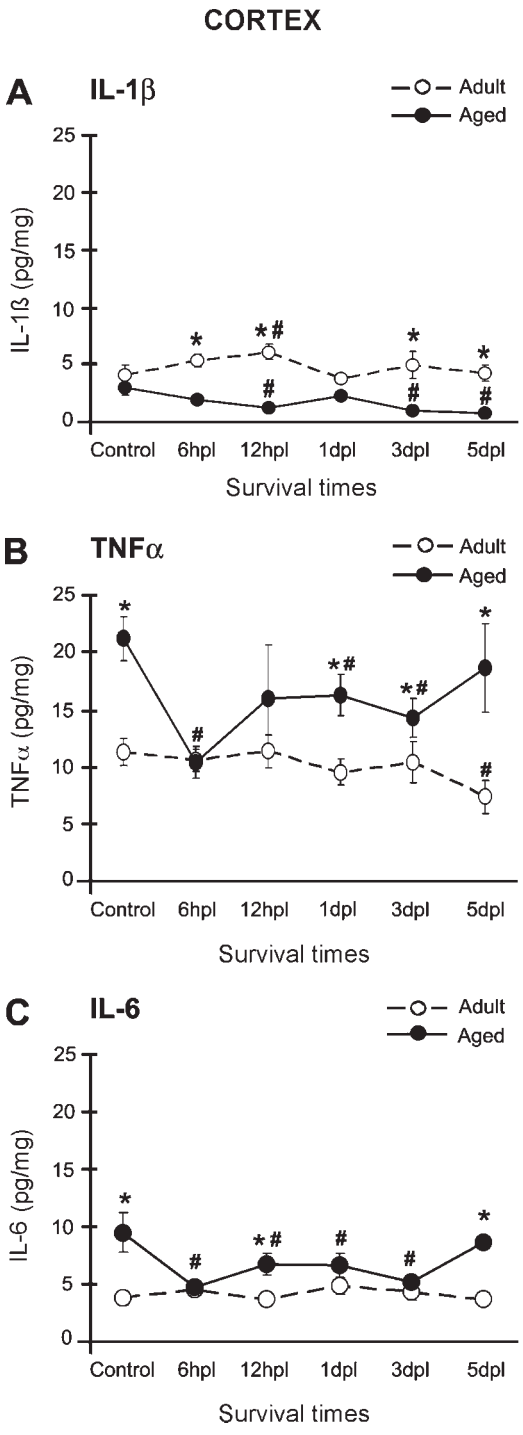


Fig. 1. Age-dependent differences in striatal levels of IL-1β (**A**), TNF-α (**B**), and IL-6 (**C**), comparing adult (white dots) vs. aged (black dots) rats. Data are shown as mean cytokine content (pg) per mg of total protein ± SEM. Significant differences in relation to their age-matched controls ($P < 0.05$) are indicated by #, and asterisks show significant differences between ages at a specific time point ($P < 0.05$). **A:** IL-1β quantification shows significant differences between ages in intact control animals, with higher values in the aged. After lesioning, striatal IL-1β in adult animals shows a biphasic induction with values over controls at 6 hpl and at 3 and 5 dpl, but a decrease at 12 hpl and 1 dpl, when significant age differences are seen. In the aged injured striatum, no IL-1β induction is observed, and a mild reduction occurs at 12 hpl. **B:** TNF-α ELISA assay shows no significant differences in TNF-α levels between ages in controls, but age differences in postinjury values at 6 hpl, 12 hpl, 1 dpl, and 3 dpl are seen. The adult injured striatum shows induction of TNF-α, with significantly increased TNF-α values at all survival times until 3 dpl, but in the aged injured striatum, induction of TNF-α is not observed until 5 dpl, when levels differ from the age-matched control. **C:** IL-6 ELISA assay shows significantly higher values of IL-6 in aged control striatum, and at 12 hpl and 1 dpl. In the adult striatum, there is a significant injury-induced increase in IL-6 at all survival times studied, but in the aged striatum, significant induction is only seen at 6 hpl, and values decrease to control values later on.

histochemistry were incubated with the biotinylated lectin obtained from *Lycopersicon esculentum* (tomato) (Sigma, L-9389, Germany) diluted to 6 μg/ml, and with Cy2-conjugated streptavidin (Amersham, PA-42001) (1:1,000). Double-stained sections were analyzed with a Nikon Eclipse E600 and a Leica TCS SP2 confocal microscope. Qualitative analysis of the estimated amount of double-labeled cells was performed. Intact controls, saline-injected controls, and a minimum of four



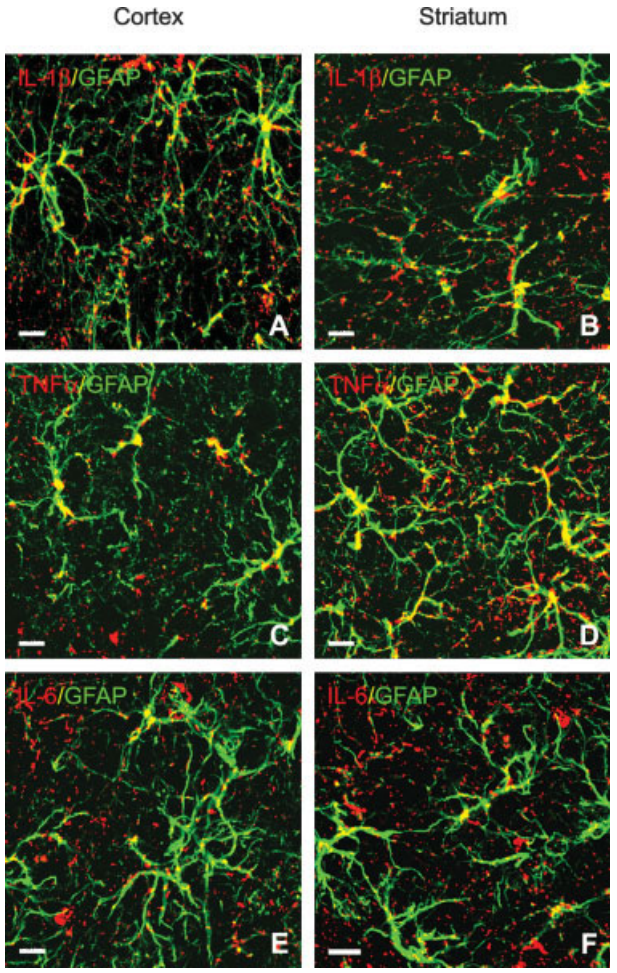


Fig. 3. Confocal immunofluorescence images showing double labeling for IL-1β (red in **A,B**), TNF-α (red in **C,D**) and IL-6 (red in **E,F**), with GFAP (green) as an astroglial marker in the aged cortex (A,C,E) and striatum (B,D,F) in intact controls. IL-1β-positive (A,B), TNF-α-positive (C,D) and IL-6-positive (E,F) cells are mainly identified as astrocytes in both regions. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. Age-dependent differences in neocortical levels of IL-1β (**A**), TNF-α (**B**), and IL-6 (**C**), comparing adult (white dots) vs. aged (black dots) rats. Data are shown as mean cytokine content (pg) per mg of total protein ± SEM. Significant differences in relation to their age-matched controls ($P < 0.05$) are indicated by #, and asterisks show significant differences between ages at a specific time point ($P < 0.05$). **A:** IL-1β ELISA assay shows that cortical IL-1β changes after striatal lesioning were wild, with a significant increase in relation to control values at 12 hpl in the adult cortex, but decreases at 12 hpl, 3 and 5 dpl in the aged. Significant differences between ages were seen at 6 and 12 hpl, and at 3 and 5 dpl. **B:** Quantification of TNF-α ELISA assay shows significant differences in TNF-α levels between ages in control animals and at 1 dpl, 3 dpl, and 5 dpl. After striatal lesioning, adult cortex shows no induction of TNF-α, and aged cortex shows higher variability with significantly decreased levels at several survival times in relation to its age-matched control but no induction. **C:** IL-6 ELISA assay shows significant differences between ages in control animals and at 12 hpl and 5 dpl. Again, in the adult injured cortex no induction of IL-6 is seen, but in the aged, significantly reduced values in relation to its control occur at 6 hpl, 12 hpl, 1 dpl, and 3 dpl.

TABLE I. Qualitative Analysis of Striatal IL-1β Expression in Neuronal Cells (NeuN+), Astrocytes (GFAP+), and Microglia/macrophages (TL+) After Excitotoxic Injury in the Adult and Aged Striatum★

	Lesion core			Adjacent lesion border		
Animal	IL-1β/NeuN neurons	IL-1β/GFAP astrocytes	IL-1β/TL microglia/Mø	IL-1β/NeuN neurons	IL-1β/GFAP astrocytes	IL-1β/TL microglia/Mø
Adult						
Control	−	++++[a]	−	−	++++[a]	−
6 hpl	++[a]	++[a]	−	+	+++	−
12 hpl	++[a]	++[a]	−	+	+++[a]	−
1 dpl	+	++[a]	+	+	+++[a]	−
3 dpl	−	+	+++[a]	+	+++[a]	−
Aged	−	++++[a]	−	−	++++[a]	−
Control	++[a]	+	+	+	+++[a]	−
6 hpl	++[a]	+	+	+	+++[a]	−
12 hpl	+	++[a]	+	+	+++[a]	−
1 dpl	±	±	+++[a]	+	+++[a]	−
3 dpl	−	++++[a]	−	−	++++[a]	−

★hpl, hours after lesioning; dpl, days after lesioning; IL-1β, interleukin-1 beta; NeuN, neuronal nuclei antigen; GFAP, glial fibrillary acidic protein; TL, tomato lectin; Mø, macrophages; −, no double-labeled cells; ±, few double-labeled cells; +, approximately 25% of IL-1β-labeled cells; ++, approximately 50% of IL-1β-labeled cells; +++, approximately 75% of IL-1β-labeled cells; ++++, almost all IL-1β-labeled cells.
[a]Predominant cell type at each time point and region.

NMDA-injected animals for each survival time, double labeling, and age group were analyzed. For each animal, four selected sections comprising 1 mm/−0.3 mm from bregma, containing the striatum, were evaluated with a 40× objective. Only positive profiles clearly identified as specifically double-labeled cell somas were considered. Data are shown as approximate percentages of double-labeled cells for each cytokine and cell type in each condition.

Cytokine ELISA Assays

Cytokine assays were performed with the following commercially available kits with a sensitivity of <10 pg/ml: IL-1β rat enzyme-linked immunoabsorbent assay (ELISA) system (Amersham, Biotrak RPN 2743), TNF-α rat ELISA system (Amersham, Biotrak RPN 2744), and IL-6 rat ELISA system (Amersham, Biotrak RPN 2742) according to the manufacturer's instructions. Briefly, 50 μl of supernatant or recombinant protein standards were added to each well coated with primary antibodies against the specific cytokine, rinsed with buffer, incubated with the corresponding biotinylated secondary antibodies, and rinsed again before adding the streptavidin–horseradish peroxidase solution and developing the reaction with the tetramethylbenzidine (TMB) substrate solution. Absorbance of resulting reagent was determined at 450 nm within 30 min after developing the reaction.

Statistical Analysis

Statistical analysis was performed by StatView 4.5. Data of cytokine levels were analyzed by means of one-way ANOVA followed by Fisher post hoc comparisons. Results are presented as mean values ± SEM, and $P \leq 0.05$ was used as a limit for statistical significance.

RESULTS

In both aged and adult rats, injection of saline solution caused no neuronal degeneration and only a very focal and transient glial response up to 3 days after injection restricted to the needle track. However, the intrastriatal injection of NMDA induced tissue degeneration involving neuronal loss and glial response mainly in the striatum (caudate–putamen), but also in the cortex and the corpus callosum, as previously described in detail (data not shown) (Castillo-Ruiz et al., 2007). The cortical lesion affected mainly the primary and secondary motor cortex, and in the most injured animals, it expanded caudally.

Cytokine expression was mainly found in the damaged ipsilateral striatum but also in the adjacent injured cortex and in intact and saline-injected control brains at lower amounts. In general, cytokine levels in both controls and in NMDA-injured striatum were higher than in the control and injured cortex, respectively (Figs. 1 and 2). In addition, in the injured striatum, the cytokine cellular pattern varied depending on the specific cytokine, age, survival time, and location in the lesion core (LC) containing few remaining neurons and strong Fluoro-Jade B staining; and the adjacent lesion border (LB), which included the tissue surrounding the LC and contained few scattered Fluoro-Jade B–positive neurons and a higher proportion of Nissl-stained cells and NeuN-positive neurons (Fig. 4A–K).

IL-1β

In intact control animals, quantification by ELISA analysis showed significantly augmented IL-1β expression in the intact control aged striatum when compared

with the adult (Fig. 1A). No changes were induced by saline injection (data not shown). In both control groups, IL-1β-positive cells mainly corresponded to GFAP-positive astrocytes (Fig. 3B; Table I).

In the aged injured striatum, IL-1β levels were comparable to values in intact controls at all survival times except at 12 hr after lesioning (hpl), when IL-1β was slightly decreased (Fig. 1A). However, the adult injured striatum showed IL-1β levels different from intact controls at all survival times (Fig. 1A); IL-1β was increased at 6 hpl, reduced at 12–24 hpl (when values differed from the corresponding survival times in the aged) and increased again at the longer survival times of 3 and 5 days after lesioning (dpl) (Fig. 1A). In addition, changes in the cellular pattern of IL-1β expression in the LC, in comparison to the adjacent LB, as well as mild differences between ages, were observed in the injured striatum (Fig. 4F,G). In the striatal LC at both ages, IL-1β was expressed in neurons (Fig. 5A,B) and astrocytes (Fig. 5A,B) at 6–12 hpl; however, IL-1β was also observed in microglia/macrophages in the aged but not in adult animals (Table I, Fig. 5E,F). From 24 hpl and until 5 dpl (the last time point examined), neuronal expression decreased, and microglia/macrophages became the main cell type expressing IL-1β in the LC at both ages (Table I, Fig. 5G,H). No age differences were found in the adjacent LB, where minimal IL-1β neuronal expression occurred, and most IL-1β-expressing cells were identified as reactive astrocytes (Table I, Figs. 4F,G, 5I,J).

In regard to cortical IL-1β expression, no age differences were observed in the control cortex (Fig. 2A), when IL-1β-positive cells were mainly astrocytes identified by GFAP double labeling (Fig. 3A). However, the intrastriatal injection of NMDA induced a slight decrease in IL-1β expression in the aged injured cortex at different survival times (Fig. 2A) and induced a slight transient increase in cortical IL-1β at 12 hpl in the adult cortex (Fig. 2A). Analysis of double immunohistochemical labeling showed IL-1β expression mainly in astrocytes at all survival times and at both ages, although it was also observed in a reduced number of cortical neurons during the first 24 hpl, and in scattered microglia/macrophages later on (data not shown).

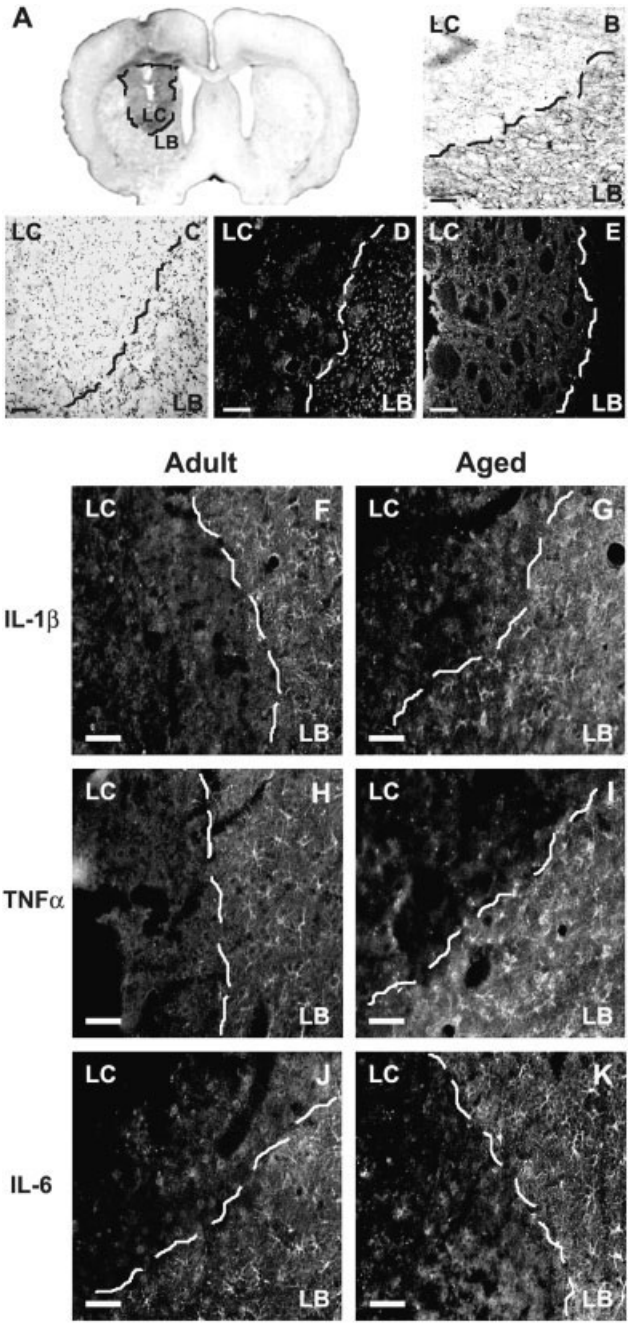


Fig. 4. Coronal sections showing different stainings and immunolabeling differentiating the striatal LC from the striatal LB (A–E) and changes in cytokine expression in the two areas (F–K). **A:** Low-magnification section processed for GFAP immunolabeling. **B:** Higher magnification of GFAP staining at 1 day after lesioning (dpl). **C:** After striatal injury, decreased toluidine blue staining in the LC at 1 dpl in comparison with LB shows massive neuronal loss. **D:** Decreased NeuN immunostaining showing neuron depletion in the LC at 1 dpl in comparison with LB. **E:** Fluoro-Jade B staining showing labeled degenerating neurons within the LC at 1 dpl. Immunolabeling for IL-1β (**F,G**), TNF-α (**H,I**), and IL-6 (**J,K**) both in the adult (F,H,J) and the aged (G,I,H) injured striatum, at 3 dpl, showing the decreased and heterogeneous cytokine labeling within the LC and the immunolabeling of star-shaped cells resembling astrocytes in the LB at both ages. Scale bars = 50 μm.

TNF-α

In intact control animals, striatal TNF-α values were similar at both ages (Fig. 1B), and no changes were induced by saline injection (data not shown). In both control groups, TNF-α-expressing cells mainly corresponded to GFAP-positive astrocytes (Fig. 3D; Table II). After the lesion, whereas the aged striatum showed TNF-α levels comparable to values in intact controls at all survival times except at 5 dpl, when TNF-α was increased (Fig. 1B), the adult injured striatum showed lesion-induced increase in TNF-α levels at all survival times (Fig. 1B). As a result, TNF-α levels in the aged injured striatum were significantly lower than in the

adult at all survival times except at 5 dpl (Fig. 1B). Analysis of double immunohistochemical techniques showed that, in the LC at both ages, TNF-α was expressed both in neurons and astrocytes from 6 hpl until 1 dpl, when neuronal expression decreased (Table II, Fig. 6A–D). At 3–5 dpl, microglia/macrophages was the main cell type expressing TNF-α in the LC at both ages (Fig. 6E,F). However, in the adjacent LB, most TNF-α-expressing cells were reactive astrocytes, although scattered TNF-α-positive neuronal cell were also seen (Table II, Figs. 4H,I, 6G,H).

Cortical TNF-α expression in the aged control cortex was significantly higher than in the adult, although TNF-α was found in astroglial cells at both ages (Figs. 2B, 3C). Generally, and in contrast to IL-1β, in the cortex of the NMDA-injected hemisphere, TNF-α content was slightly higher in the aged, vs. the adult, although values were variable and corresponded to a decrease in TNF-α levels when compared with aged controls (Fig. 2B). Double labeling for TNF-α and specific markers, showed expression mainly in astrocytes at all survival times, although some labeled neurons were seen at early times and a few microglia/macrophages later on (data not shown).

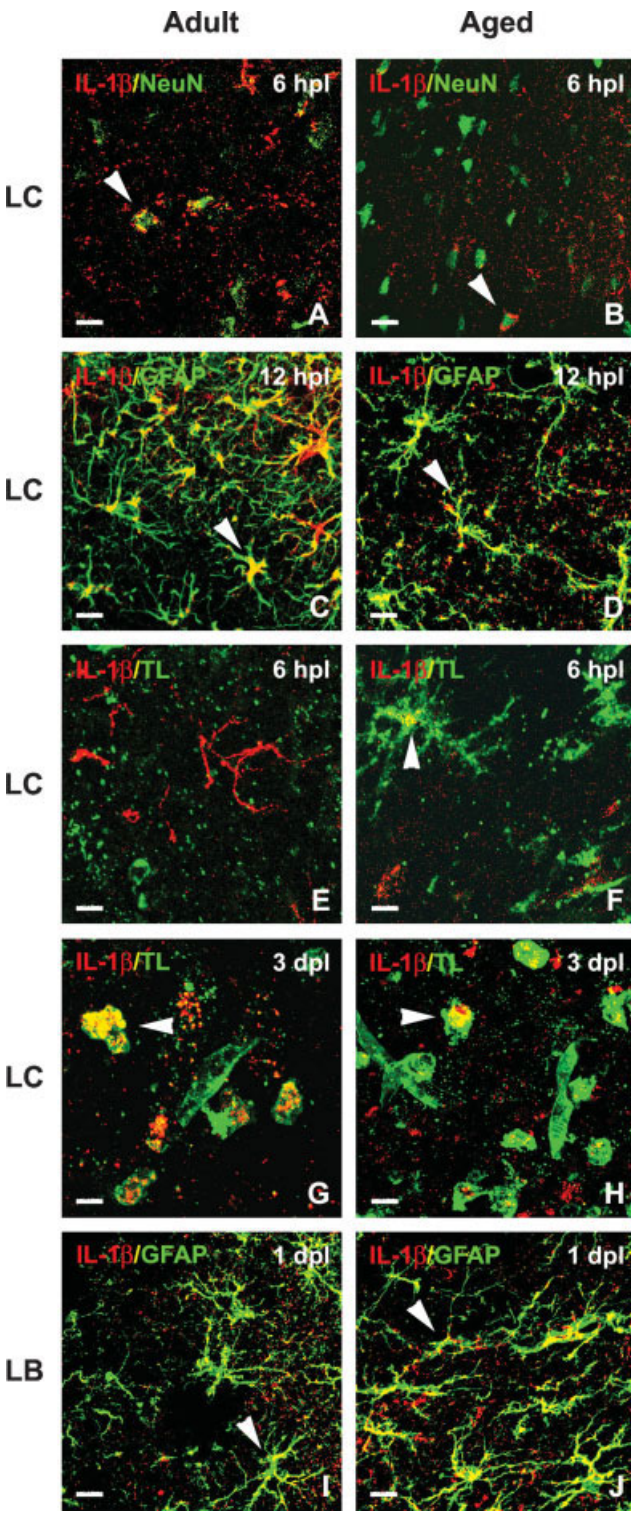


Fig. 5. Confocal immunofluorescence images showing IL-1β (red), and double labeling with the cell-specific markers NeuN (green in A,B), GFAP (green in C,D,I,J), and TL (green in E–H) in the adult and the aged LC (A–H), and in the adult and the aged adjacent LB (I,J). Yellow-orange labeling shows colocalization in all micrographs. In the LC, at 6 or 12 hpl, IL-1β-positive cells are identified at both ages as NeuN-positive neuronal cells (A,B) or GFAP-positive astrocytes (**C,D**). At 6 hpl, in the aged LC, but not in the adult (**E**), some IL-1β-positive cells are also identified as TL-positive microglial cells (**F**). At 3 dpl, IL-1β-positive microglia/macrophages showing a round/ameboid morphology are seen in the LC (**G,H**). In the LB, IL-1β-positive cells are mainly identified as astrocytes at all survival times and at both ages (I,J show 1 dpl). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IL-6

Intact control animals showed increased IL-6 expression in the aged striatum (Fig. 1C), and no changes were observed after saline injection (data not shown). In both control groups, IL-6-positive cells were also primarily identified as GFAP-expressing astrocytes (Fig. 3F; Table III). After NMDA injection, whereas the aged injured striatum showed a transient increase in IL-6 levels at 6 hpl, which decreased to control levels later on (Fig. 1C), the adult injured striatum displayed increased levels of IL-6 at all survival times (Fig. 1C). Consequently, comparison between ages showed that IL-6 levels in the aged injured striatum were significantly higher than in the adult at 12 hpl and 1 dpl, and close to significance at 6 hpl (Fig. 1C). Similar to the other cytokines analyzed, double immunofluorescence analysis showed that in the LC of both the adult and aged striatum, IL-6 was expressed in similar proportion in neurons and astrocytes at 6 and 12 hpl, prevailed in astrocytes at 1 dpl, and was clearly predominant in microglia/macrophages at 3–5 dpl, especially in the adult (Table III, Fig. 7A–F). In the adjacent LB, no age differences were found, and in addition to a minimal expression of IL-6 in neurons,

TABLE II. Qualitative Analysis of Striatal TNFα Expression in Neuronal Cells (NeuN+), Astrocytes (GFAP+), and Microglia/macrophages (TL+) After Excitotoxic Injury in the Adult and Aged Striatum*

	Lesion core			Adjacent lesion border		
Animal	TNFα/NeuN neurons	TNFα/GFAP astrocytes	TNFα/TL microglia/Mø	TNFα/NeuN neurons	TNFα/GFAP astrocytes	TNFα/TL microglia/Mø
Adult						
Control	±	++++[a]	−	±	++++[a]	−
6 hpl	++[a]	++[a]	−	+	+++[a]	−
12 hpl	++[a]	++[a]	−	+	+++[a]	−
1 dpl	+	++[a]	+	+	+++[a]	−
3 dpl	±	±	+++[a]	±	+++[a]	±
Aged						
Control	+	+++[a]	−	+	+++[a]	−
6 hpl	++[a]	++[a]	±	+	+++[a]	−
12 hpl	++[a]	++[a]	±	+	+++[a]	−
1 dpl	+	++[a]	+	+	+++[a]	±
3 dpl	±	±	+++[a]	+	++[a]	+

*hpl, hours after lesioning; dpl, days after lesioning; TNFα, tumor necrosis factor alpha; NeuN, neuronal nuclei antigen; GFAP, glial fibrillary acidic protein; TL, tomato lectin; Mø, macrophages; −, no double-labeled cells; ±, few double-labeled cells; +, approximately 25% of TNFα-labeled cells; ++, approximately 50% of TNFα-labeled cells; +++, approximately 75% of TNFα-labeled cells; ++++, almost all TNFα-labeled cells.
[a]Predominant cell type at each time point and region.

most IL-6 expression occurred in reactive astrocytes (Table III, Figs. 4J,K, 7G,H).

In the cortex of intact controls, higher IL-6 expression levels was also observed (Fig. 2C), mainly found in astrocytes (Fig. 3E). The intrastriatal NMDA injection induced no IL-6 changes in the adult cortex at any survival time, but a slight decrease in IL-6 values in the aged injured cortex at all survival times (Fig. 2C). Double labeling for IL-6 in the injured cortex showed, again, expression in astrocytes at all survival times after lesioning. However, it was also observed in a reduced number of neurons during the first 24 hpl and in a few microglia/macrophages at longer survival times (data not shown).

DISCUSSION

Proinflammatory Cytokine Expression Is Enhanced in the Aged Control Brain

This study demonstrates that overall basal levels of the main proinflammatory cytokines are increased in the normal aged rat striatum and cortex showing region and cytokine specificity: IL-1β and IL-6 display age-dependent higher levels in the aged striatum, and TNF-α and IL-6 in the aged cortex. Age-dependent increases in the expression of several cytokines have already been described, mainly in the aged hippocampus but also in cortex and thalamus (Bodles and Barger, 2004; Maher et al., 2004; Murray et al., 1997; Sandhir et al., 2004; Terao et al., 2002; Ye and Johnson, 1999), although no studies have previously studied cytokine levels in the aged striatum in vivo. Interestingly, besides age differences, notable region-specific changes were also observed: both in the adult and aged brain, cytokine levels were much higher in the striatum than in the cortex (cf. Figs. 1 and 2). In this regard, a previous study analyzing the heterogeneity in the astroglial production of proinflammatory molecules reported a higher production of cytokines by striatal astrocytes in vitro (Morga et al., 1999), and Xie and coworkers (2003) have described that both adult and aged cultured striatal glia are able to produce elevated levels of cytokines when compared with glia obtained from the cortex or hippocampus. Because cytokine expression in both areas and ages was mainly observed in astroglial cells, these overall findings suggest an important region-specific regulation of astroglial cytokine expression. Notably, because the cellular expression profile is similar at both ages, whether changes in total protein levels found are attributed to an increase in the number of cytokine-expressing cells or increased cytokine expression in a similar number of cells is unknown. However, in previous studies, we did not find evident differences in GFAP immunoreactivity in the control aged striatum (Castillo-Ruiz et al., 2007), pointing to an apparent lack of age-associated astroglial changes in normal striatum in this strain at this age, that could explain the significant increase in cytokine-producing astrocytes in basal conditions.

One of the cytokines showing increased levels in the aged striatum is IL-1β. Age-related increases in hippocampal IL-1β levels have been extensively studied, and IL-1β overexpression has been correlated with increased signaling through the IL-1β receptor I (IL-1RI) and enhanced microglial activation (Griffin et al., 2006; Moore et al., 2007), with concomitant decreased values of the anti-inflammatory cytokine IL-4 and down-regulation of intracellular survival signals in neuronal cells (Maher et al., 2005; Nolan et al., 2005). In agreement, increased hippocampal IL-1β in aging has been directly implicated in the decline of synaptic plasticity and impaired performance of cognitive tasks in aged rats because IL-1β inhibition induces an ameliora-

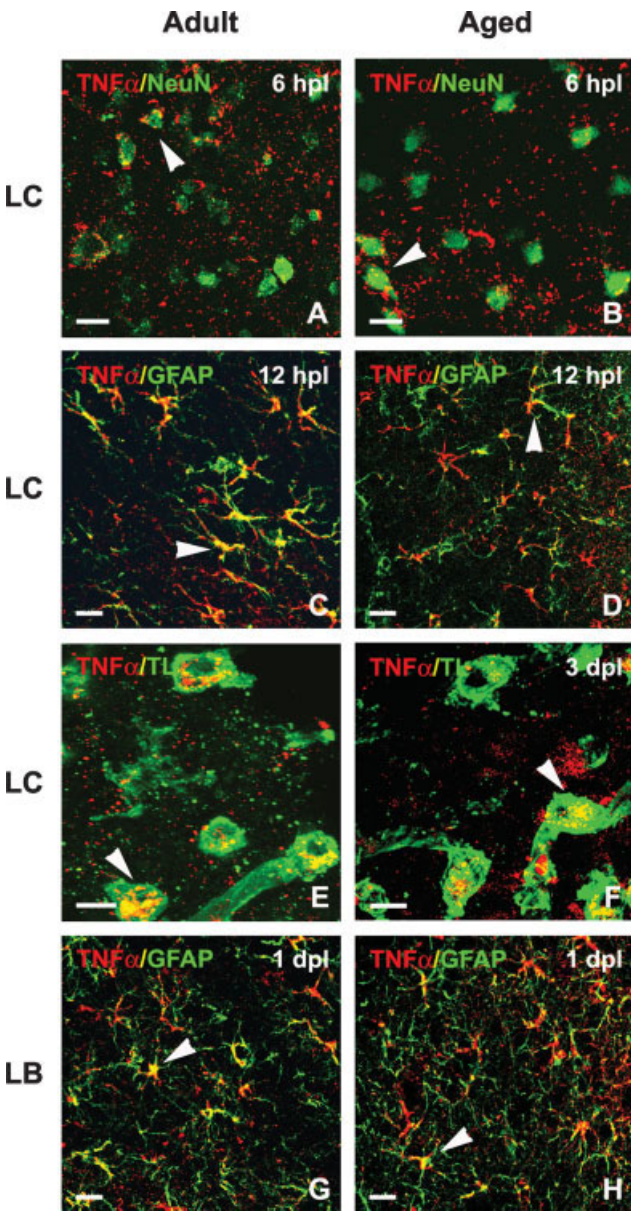


Fig. 6. Confocal immunofluorescence images showing TNF-α (red), and double labeling with the cell-specific markers NeuN (green in A,B), GFAP (green in C,D,G,H), and TL (green in E,F) in the adult and the aged LC (A–F), and in the adult and the aged adjacent LB (G,H). Yellow-orange labeling shows colocalization in all micrographs. In the LC, at 6 or 12 hpl, TNF-α-positive cells are NeuN-positive neurons (**A,B**) or GFAP-positive astrocytes (**C,D**) at both ages. At longer survival times, such as 3 dpl, microglia/macrophages are the main cell type, showing TNF-α in the LC (**E,F**). In the LB, TNF-α is primarily seen in GFAP-positive astrocytes at all survival times and at both ages (**I,J** show 1 dpl). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion and improved memory (Gemma et al., 2005; Maher et al., 2005). In regard to other brain regions, few and contradictory studies are available on IL-1β changes in the aged cortex. Whereas increased mRNA and protein levels are described in some studies (Katafuchi et al., 2003; Maher et al., 2004), other reports have shown increased IL-1β mRNA but no changes in protein expression (Gee et al., 2006), in agreement with our results.

Surprisingly, opposite results to those of IL-1β were found when analyzing TNF-α: no age-related changes in basal levels were found in the striatum, but a greatly enhanced TNF-α expression was seen in the aged cortex, in agreement with studies that used senescence-accelerated mice (Tha et al., 2000) and paralleling previously shown enhanced TNF-α values in the hippocampus and thalamus of aged mice (Casolini et al., 2002; Sandhir et al., 2004; Terao et al., 2002). In regard to IL-6, our results demonstrate higher levels both in the cortex and striatum of aged rats. Actually, the age-dependent increase in IL-6 levels is considered one of the most consistent findings in aging studies in rodents (Godbout and Johnson, 2004), as has been demonstrated in whole brain extracts (Ye and Johnson, 2001a, 2001b) and in several brain regions such as the hippocampus, cerebral cortex, cerebellum and thalamus (Prechel et al., 1996; Sandhir et al., 2004; Ye and Johnson, 1999). However, to our knowledge, this is the first study reporting increased IL-6 levels in the aged striatum, further supporting the age-related up-regulation of IL-6, which has been attributed to an increase in binding of the transcription factor NFκB to its promoter (Ye and Johnson, 2001b), and to reduced levels of the anti-inflammatory cytokine IL-10 (Ye and Johnson, 2001a), observed in the aged brain.

In view of these findings, our results support an increasing body of evidence suggesting that normal brain aging is characterized by a basal inflammatory activity caused by an imbalance between pro- and anti-inflammatory molecules, whose functions are not yet fully understood but that may certainly influence the aged brain's neurodegenerative process and inflammatory response after damage.

Injury-induced Cytokine Response Is Attenuated in the Aged Brain

Most available studies on stimuli-induced cytokine expression changes in aging come from reports on peripheral immune system challenge such as the injection of bacterial lipopolysaccharide (LPS), generally describing an exaggerated inflammatory response shown as increased production of cytokines in glial cells of the hippocampus, cortex and cerebellum of aged mice and rats (Buchanan et al., 2008; Chen et al., 2008; Godbout et al., 2005; Huang et al., 2007; Kalehua et al., 2000; Sierra et al., 2007; Siren et al., 1993; Yu and Li, 2000), associated with a delayed recovery from the behavioral symptoms of sickness (Huang et al., 2007). Here we describe that after an excitotoxic striatal lesion that induces massive neurodegeneration in the striatum (Castillo-Ruiz et al., 2007), we have observed a notable increase in cytokine levels in the adult, but very moderate induc-

TABLE III. Qualitative Analysis of Striatal IL-6 Expression in Neuronal Cells (NeuN+), Astrocytes (GFAP+), and Microglia/macrophages (TL+) After Excitotoxic Injury in the Adult and Aged Striatum*

	Lesion core			Adjacent lesion border		
Animal	IL-6/NeuN neurons	IL-6/GFAP astrocytes	IL-6/TL microglia/Mø	IL-6/NeuN neurons	IL-6/GFAP astrocytes	IL-6/TL microglia/Mø
Adult						
Control	−	++++[a]	−	−	++++[a]	−
6 hpl	++[a]	++[a]	−	+	+++[a]	−
12 hpl	++[a]	++[a]	±	+	+++[a]	−
1 dpl	+	++[a]	+	+	+++[a]	−
3 dpl	±	±	+++[a]	±	+++[a]	±
Aged						
Control	±	++++[a]	−	±	++++[a]	−
6 hpl	++[a]	++[a]	−	+	+++[a]	−
12 hpl	++[a]	++[a]	±	+	+++[a]	−
1 dpl	+	++[a]	+	±	+++[a]	±
3 dpl	+	+	++[a]	±	+++[a]	±

hpl, hours after lesioning; dpl, days after lesioning; IL-6, interleukin-6; NeuN, neuronal nuclei antigen; GFAP, glial fibrillary acidic protein; TL, tomato lectin; Mø, macrophages; −, no double-labeled cells; ±, few double-labeled cells; +, approximately 25% of IL-6-labeled cells; ++, approximately 50% of IL-6-labeled cells; +++, approximately 75% of IL-6-labeled cells; ++++, almost all IL-6-labeled cells.
[a]Predominant cell type at each time point and region.

tion of cytokine expression in the aged injured striatum. In addition, very low cytokine induction was found in the cortex, probably as a result of the mild cortical damage observed in this region at both ages and not to the region itself because cortical traumatic injury is known to induce cytokine changes, in the adult brain (Wang and Campbell, 2002), and in the 36 month old rat cortex after cortical stab wound, where injury-induced increases in IL-1β mRNA at several time points and TNF-α and IL-6 transiently at early times have been shown (Kyrkanides et al., 2001). In addition, cortical ablation of the visual cortex also increased cytokine levels in aged mice, where higher TNF-α and IL-1β expression, but no age-related changes in IL-6 could be observed (Sandhir et al., 2004).

One of the main findings of this study is the notable age-dependent difference in cytokine induction in the aged vs. the adult excitotoxically damaged striatum. The adult injured striatum exhibits increased levels of TNF-α, IL-6 and IL-1β when compared with age-matched controls at several survival times, correlating with earlier reports that used excitotoxic damage paradigms in adult animals in several brain regions (de Bock et al., 1996; Pearson et al., 1999; Schiefer et al., 1998; Vezzani et al., 1999). However, the aged injured striatum shows a very weak injury-induced up-regulation of cytokine expression that is only significantly elevated from control levels at very specific times, supporting the previously postulated hypothesis that constant basal expression of cytokines in the aged brain may create in glial cells a level of tolerance such that greater levels of stimulation would be needed to induce an increase in cytokine expression (Yu et al., 2002). Notably, despite the weak response in total cytokine levels, injury-induced changes in cell expression occur at both ages. Whereas within the LC expression of the three cytokines analyzed is attributed to neurons and astrocytes during the first 24 hr after lesioning, microglia/macrophages are the main cytokine-producing cells from day 3 after lesioning. However, cytokine expression in the adjacent LB is always found in reactive astrocytes.

After striatal damage in the aged, it was surprising to observe that IL-1β expression did not show a further induction and high basal levels of this cytokine were maintained at all survival times. IL-1β is a multifunctional proinflammatory cytokine that potentiates the inflammatory reaction by raising the production of other cytokines (Baldwin, 1996; Basu et al., 2004; Gosselin and Rivest, 2007; Wang and Campbell, 2002) and adhesion molecules that enhance neutrophil infiltration (Basu et al., 2004; Garcia et al., 1995; Jander et al., 1995; Yang et al., 1999). Moreover, IL-1β has been specifically shown to participate in the exacerbation of excitotoxic striatal damage as infusion of the IL-1β receptor antagonist, IL-1ra, markedly inhibited striatal neuronal damage caused by NMDA receptor activation (Lawrence et al., 1998; Stone and Behan, 2007; Touzani et al., 1999). However, neurotoxic properties can be compensated by the induction of neurotrophic actions, triggering growth factor expression (Gadient et al., 1990; Jankowsky and Patterson, 2001). In this sense, the beneficial or detrimental action of IL-1β depends on a wide range of interacting factors including the timing, duration as well as the level and location of IL-1β expression (Bernardino et al., 2005; Gosselin and Rivest, 2007; Grundy et al., 2002; Rothwell and Luheshi, 2000). In this sense, further studies are needed to elucidate the role of IL-1β in the neurodegenerative process in the aged, and whether higher levels but attenuated injury-induced changes are beneficial or detrimental for lesion outcome.

TNF-α, although not showing age-dependent increases in basal levels, is not induced after damage to

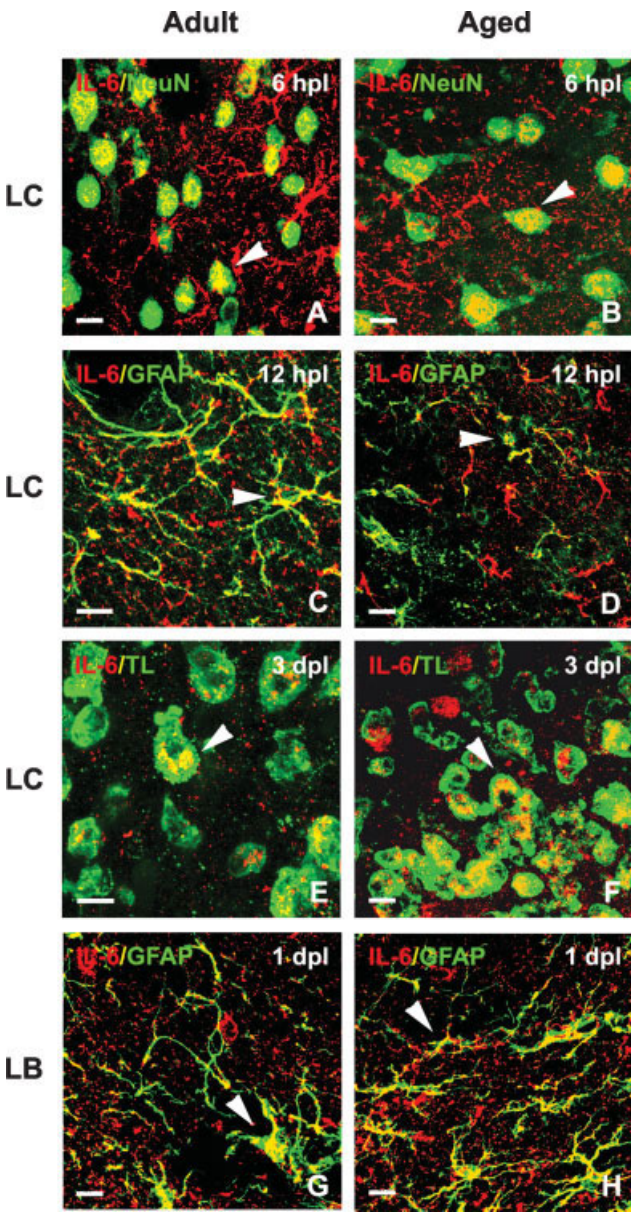


Fig. 7. Confocal immunofluorescence images showing IL-6 (red), and double labeling with the cell-specific markers NeuN (green in **A,B**), GFAP (green in **C,D,G,H**), and TL (green in **E,F**) in the adult and the aged LC (A–F), and in the adult and the aged adjacent LB (G,H). Yellow-orange labeling shows colocalization in all micrographs. In the LC, at both ages, NeuN-positive cells show IL-6 expression at 6 hpl, but astrocytes are also IL-6 positive (C,D). Note that at this survival time, GFAP immunostaining is very mild. Later, at 3 dpl, microglia/macrophages are the main cell type showing IL-6 in the LC (E,F). In the LB, GFAP-positive astrocytes are the main cell type showing IL-6 expression at all times analyzed (I,J, 1 dpl). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the aged striatum until 5 dpl, therefore showing overall lower levels than the adult at all survival times, and contrasting with the rapid and sustained increase in TNF-α induced by excitotoxicity in the adult, as shown here and in other studies (Allan et al., 2001; Botchkina et al., 1997; de Bock et al., 1996; Gong et al., 1998; Lambertsen et al., 2007; Uno et al., 1997). The importance of this lack of TNF-α induction in the progression of the neurodegenerative and inflammatory process in the aged striatum is not known. In this regard, several findings have specifically demonstrated a protective role of neuronal TNF-α against excitotoxicity induced by glutamate receptor agonists (Allan, 2002) or nitric oxide (Turrin and Rivest, 2006), although we have previously shown that aged rats show a slower progression of neurodegeneration to NMDA-induced excitotoxicity than adult rats (Castillo-Ruiz et al., 2007), occurring concomitantly to the expression of TNF-α in neuronal cells of the LC, when overall levels are lower than in the adult.

In regard to IL-6, adult striatal values are increased from 6 hr to 5 dpl in neurons, astrocytes and microglial cells, as previously reported (Ali et al., 2000; Hariri et al., 1994; Orzylowska et al., 1999; Schiefer et al., 1998; Suzuki et al., 1999a, 1999b). In contrast, in the aged striatum we report higher expression levels at all survival times, and an injury-induced early up-regulation at 6 hr after lesioning, partly occurring in neuronal cells. Interestingly, it has been demonstrated that IL-6 protects neurons against toxicity induced by glutamate agonists in a dose dependent response, suggesting that IL-6 may be an endogenous neuroprotective mechanism against excitotoxic injury (Ali et al., 2000; Toulmond et al., 1992; Yamada and Hatanaka, 1994), specifically in striatal neurons where the neuroprotective effect of both IL-6 and IL6/IL6R chimera has been demonstrated (Bensadoun et al., 2001). In this view, the elevated IL-6 expression in aged injured striatum at early survival times may partly explain the slower progression of neurodegeneration observed in this model (Castillo-Ruiz et al., 2007) and after intracerebral hemorrhage (Wasserman et al., 2008). In addition, IL-6 could also contribute to the exacerbated astroglial response observed in the aged brain after several types of injury (Castillo-Ruiz et al., 2007; Sandhir et al., 2008; Wasserman et al., 2008) because its action on astroglial cell activation is also well established (Gruol and Nelson, 1997), and IL-6 is a well-known inducer of GFAP transcription (Takizawa et al., 2001). However, it should be noted that GFAP transcription is repressed by IL-1β (Krohn et al., 1999), pointing that modulation of GFAP expression in the aged may not only be explained by single cytokine levels but by the balance between activating and repressing signals.

In conclusion, this study clearly demonstrates age- and region-specific higher basal levels of proinflammatory cytokines in the aged vs. the adult rat brain. In addition, after excitotoxic damage to the striatum, whereas the adult brain shows increased injury-induced levels of IL-1β, TNF-α, and IL-6 both in neurons and glial cells, the aged brain presents an attenuated injury-induced cytokine response. Because cytokines are important players in the regenerative attempts of neurons, initiation of the glial response, and the development of the inflammatory reaction, the age-specific cytokine pattern

demonstrated here may modulate a different injury evolution. Further studies that use acute models of injury in the aged brain are needed to elucidate the consequences of this increased basal cytokine expression and to indicate how the aged cells respond to it after damage.

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