Published in final edited form as:

Neurobiol Aging. 2012 June ; 33(6): 1123.e1–1123.16. doi:10.1016/j.neurobiolaging.2011.11.007.

Age-related severity of focal ischemia in female rats is associated with impaired astrocyte function

Danielle K. Lewis, Kristen T. Thomas, Amutha Selvamani, and Farida Sohrabji*

Women's Health in Neuroscience Program, Department of Neuroscience and Experimental Therapeutics, Texas A & M Health Science Center, College Station, TX, USA

Abstract

In middle-aged female rats, focal ischemia leads to a larger cortical infarction as compared with younger females. To determine if stroke-induced cytotoxicity in middle-aged females was associated with impaired astrocyte function, astrocytes were harvested and cultured from the ischemic cortex of young and middle-aged female rats. Middle-aged astrocytes cleared significantly less glutamate from media as compared with young female astrocytes. Furthermore, astrocyte-conditioned media from middle-aged female astrocytes induced greater migration of peripheral blood monocyte cells (PBMCs) and expressed higher levels of the chemoattractant macrophage inflammatory protein-1 (MIP-1). Middle-aged astrocytes also induced greater migration of neural progenitor cells (NPCs), however, their ability to promote neuronal differentiation of neural progenitor cells was similar to young astrocytes. In males, where cortical infarct volume is similar in young and middle-aged animals, no age-related impairment was observed in astrocyte function. These studies show that the aging astrocyte may directly contribute to infarct severity by inefficient glutamate clearance and enhanced cytokine production and suggest a cellular target for improved stroke therapy among older females.

Keywords

Reproductive senescence; MIP-1; Sex differences; Neuronal differentiation; GLT-1; PBMC

1. Introduction

Stroke is a major cause of mortality and long-term disability and results from a blood vessel occlusion that prevents blood flow, reducing both the amount of oxygen and glucose that can reach the brain. As neural tissue has a high demand for both, this initial event causes a disruption in metabolic processes and leads to a cascade of excitotoxic and inflammatory pathways (for review see Bramlett and Dietrich, 2004; Dirnagl et al., 1999; Hazell, 2007) affecting glutamate regulation (Benveniste et al., 1984; Globus et al., 1988) and increased

*Corresponding author at: TAM Health Science Center, Department of Neuroscience and Experimental Therapeutics, 228 Reynolds Medical Building, College Station, TX 77843-1114, USA. Tel.: +1 979 845 4072; fax: +1 979 845 0790. sohrabji@medicine.tamhsc.edu (S. Sohrabji).

Disclosure statement

The authors disclose no conflicts of interest.

All animal work was approved by the Institutional IACUC.

expression of cytokines and/or chemokines such as nuclear factor- κ B (NF- κ B), tumor necrosis factor (TNF)- α , hypoxia-inducible factor (HIF)-1, interleukins (Dirnagl et al., 1999), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 (Dirnagl et al., 1999; Kim et al., 1995).

Astrocytes play a key role in modulating these early events. Following cerebral ischemia reactive astrocytes are observed at the injury site (Ordry et al., 1993; Petito et al., 1990; Yamamoto et al., 1987) and can provide either trophic support or exacerbate the tissue damage. During pathological glucose shortages, astrocyte-derived glycogen can be degraded to form lactate as an alternative energy source (Brown et al., 2004; Ransom and Fern, 1997; Suh et al., 2007). Astrocytes are also involved in glutamate uptake (Schousboe et al., 1977) through the glial glutamate transporters (glutamate-aspartate transporter; GLAST) which is also known as the excitatory amino acid transporter (EAAT1) (Storck et al., 1992) and the glial glutamate transporter (GLT)-1 also known as the excitatory amino acid transporter-2 (EAAT2) (Pines et al., 1992; for review also see Amara and Fontana, 2002; Tzingounis and Wadiche, 2007). However, with injury this uptake mechanism can be inhibited (Volterra et al., 1994; Yu et al., 1989). Further, astrocytes are important sources for both inflammatory cytokines/chemokines such as interleukin (IL)-1 and IL-6, TNF- α , interferon (IFN)- γ (Lau and Yu, 2001; Ridet et al., 1997), MCP-1 (Ransohoff et al., 1993), and MIP-1 (Kim et al., 1995) as well as neurotrophins (Schwartz and Nishiyama, 1994; Wu et al., 2004) and growth factors (Garcia-Estrada et al., 1992; Ridet et al., 1997) after injury.

The extent of neural damage may be related to how well astrocytes modulate these injury-induced events, and age-related astrocyte impairment could exacerbate neurodegeneration. In aging rats, where functional recovery is impaired, glial reactivity is accelerated after ischemia (Badan et al., 2003; Popa-Wagner et al., 2007), but this rapid increase drops dramatically 7 days postischemia (Popa-Wagner et al., 2007). Aging astrocytes may also be more sensitive to parenchymal acidosis after ischemia. In aged murine astrocyte cultures, exposure to kainate in combination with Zn^{2+} was only detrimental to astrocyte survival when the pH was lowered (Sensi et al., 2006). Furthermore, the duration of ischemia-induced activation of signal transducers and activators of transcription 3, a factor regulating astrocyte reactivity, was significantly reduced in aged rats as compared with young rats (Dinapoli et al., 2010). Thus, astrocyte activity after injury may be perturbed in aging animals.

Reproductive senescence in rats, which is a midlife event physiologically similar to menopause, may play a strong role in the brain's ability to cope with a neural insult. Our previous studies show reduced neurotrophin expression (Jeziński and Sohrabji, 2001), increased blood brain permeability (Bake and Sohrabji, 2004), and an elevated brain inflammatory response to an excitotoxic injury (Nordell et al., 2003) in this middle-aged group. Furthermore, ischemic injury caused by middle cerebral artery occlusion results in a significantly larger infarct volume in this reproductively senescent, middle-aged group of females as compared with younger females (Selvamani and Sohrabji, 2010a; Selvamani and Sohrabji, 2010b). Astrocytes play a key role in providing trophic support for neurons (Liesi and Silver, 1988; Parish et al., 2002; Ullian et al., 2001; Wujek and Akeson, 1987), are a component of the blood-brain barrier (Janzer and Raff, 1987; Kim et al., 2006; recent

review), and regulate the neuroinflammatory response (Fontana et al., 1982; Robbins et al., 1987). Hence we hypothesized that the greater infarct severity seen in middle-aged females is associated with impaired astrocyte function. Additionally, because our studies indicate that cortical infarct volume is not increased in middle-aged males as compared with young males (Supplementary Fig. 1), we hypothesized that the function of ischemia-activated cortical astrocytes would not differ between these 2 ages of male rats. Here we examined the possible role of ischemia-activated cortical astrocytes in reproductively competent female and male rats (young, 6–8 months), reproductively senescent female rats (middle-aged, 10–12 months), and age-matched (reproductively competent) male rats (middle-aged, 10–12 months).

2. Methods

2.1. Animals

Young (6–8 month, average wt: 273.67 ± 17.09 g) and reproductively senescent, middle-aged (10–12 months; average wt: 325.75 ± 11.7 g) Sprague-Dawley female rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). For some studies, age-matched males (young: 6–8 months, average wt: 443.56 ± 45.5 g; middle-aged: 10–12 months; average wt: 498.56 ± 37.52 g) were also used. In females, the estrus cycle was determined by vaginal smears taken each morning for a period of 14–20 days. Vaginal smears were obtained with a cotton swab, placed on a slide and the cytology was examined (Olympus, BX2 microscope, Leeds Precision Instruments, Minneapolis, MN, USA) for estrus cycle classification. In the young cohort, only those females that had a normal estrus cycle were used. Reproductively senescent middle-aged females that persisted in 1 stage for 7 days were considered acyclic and were only used when vaginal smears confirmed that they were in constant diestrus (persistent low estrogen state). All procedures were in accordance with National Institutes of Health (NIH) and institutional guidelines governing animal welfare.

2.2. Generation of adult cortical astrocyte cultures

2.2.1. Intracerebral transient middle cerebral artery occlusion—Cortical astrocytes were collected from deeply anesthetized (ketamine: 87 mg/kg; xylazine: 13 mg/kg) animals that had been subjected to an intracerebral transient middle-cerebral artery occlusion (MCAo) 2 days poststroke. Transient MCAo was performed according to published studies (Biernaskie et al., 2001; Luke et al., 2004) and established laboratory procedures (Selvamani and Sohrabji, 2010a, 2010b). Briefly, animals were deeply anesthetized and placed into a Kopf stereotaxic apparatus. A midline incision was made in the scalp, the skull was exposed and a small hole was drilled on the left side of the skull. Endothelin-1 (ET-1; 3 μ L of 0.5 μ g/ μ L, American Peptide Company, Inc., Sunnyvale, CA, USA) was injected adjacent to the middle-cerebral artery (MCA; coordinates: Anteroposterior: +0.9, Mediolateral: +3.4, Dorsoventral: –8.5 relative to bregma) at a rate of 1.0 μ L per 30 seconds using a 5 μ L Hamilton syringe with a 26s-gauge/2-inch needle. The syringe was slowly removed 3 minutes after the injection to minimize backflow. Rats were maintained at 37 °C throughout the surgical procedures and respiratory rate and oxygen saturation were constantly monitored using the MouseOximeter (STARR Life Sciences

Corp., Oakmont, PA, USA) (see Supplementary Table 1). The ET-1 model results in a prolonged ischemia and gradual reperfusion, which lasts approximately 7 hours in the striatum and 16 hours in the cortex (Biernaskie et al., 2001). Optical imaging studies using a small fluorescent probe (Cy5.5; 1-kDa molecular weight) indicates that ET-1-induced vasoconstriction is clearly observed 5 hours after ET-1, whereas reperfusion is apparent at 10 hours in the midline (striatum) and is fully reestablished when imaged at 21 hours after ET-1 (Selvamani and Sohrabji, 2010b). The ET-1 dose (3 μL of 0.5 $\mu\text{g}/\mu\text{L}$) was based on published reports (Biernaskie et al., 2001; Luke et al., 2004) and our previous studies (Selvamani and Sohrabji, 2010a, 2010b) and results in consistent infarct volumes. Because ET-1 injections are stereotactically guided to the MCA, the dose of ET-1 is not influenced by the animal's body weight. Furthermore, this is also supported by our previous data where the same dose of ET-1 results in larger infarcts in middle-aged females (that are heavier) than young females (that weigh less) (Selvamani and Sohrabji, 2010a). Hence, if the dose of ET-1 was influenced by body weight, we would expect to find smaller lesions in the heavier, middle-aged females as compared with young females.

2.2.2. Astrocyte preparations—For each type of assay involving ischemic-activated cortical astrocytes (glutamate uptake, leukocyte recruitment, neural progenitor cell [NPC] migration, cytokine expression), young and middle-aged female or age-matched male rats were prepared for cerebral ischemia as described above. After MCA occlusion, 2 animals of each age and sex were euthanized and their brains pooled to harvest astrocytes using our previously published procedures (Lewis et al., 2008a, 2008b). This procedure was repeated 3–4 times each time using a new set of animals of each sex and age. Thus for each assay, 6–8 animals of each sex and age were used. Two days poststroke, deeply anesthetized rats were decapitated and a large slice (3 mm to 11 mm rostral to caudal) of the infarcted cortex was removed in a semisterile hood. The meninges were removed from the infarcted cortex and the cortical tissue was washed 5 times with Dulbecco phosphate-buffered saline (dPBS) containing a 1% streptomycin/penicillin antibiotic mixture. Cortical tissue was placed in a sterile hood and washed with Opti-MEM and N2 supplement (99:1, Invitrogen, Carlsbad, CA, USA). The tissue was mechanically dissociated with a surgical blade and then chemically dissociated with $1\times$ trypsin/ethylenediaminetetraacetic acid (Invitrogen) for 20 minutes at room temperature. The cell suspension was centrifuged at 700 rpm for 5 minutes to pellet the cells. The supernatant was removed and the cells were resuspended in an astrocyte-specific growth media (AGM: Dulbecco's modified eagle medium [DMEM]/F12 [1:1], 10% fetal bovine serum [FBS], 4.6 g/L glucose) and plated onto poly-d-lysine hydrobromide (0.05 mg/mL; Sigma, St. Louis, MO, USA) coated dishes. Cells were maintained at 37 °C with 5% CO₂ for 10 days. The cells were cultured with a 1% streptomycin/penicillin antibiotic mixture for the first 4 days. At Day 10, cells were shaken at 600 rpm on a plate shaker for 15 minutes at room temperature to remove microglia and oligodendrocytes. Microglia and oligodendrocytes were removed from the plate by washing the dishes 3 times with DMEM. Following the 3 washes in DMEM the cells were fed astrocyte-specific growth media and treated with L-leucine methyl ester (400 μM ; Sigma) and incubated for 1 hour at 37 °C to remove any macrophage contamination (Thiele et al., 1983). After the 1-hour incubation, the cortical astrocytes were washed 3 times with DMEM, trypsinized for 2–3 minutes and replated at a density of 150,000 cells for 35 mm

culture dishes (immunohistochemistry, immunoblotting) or at 50,000 cells for 24-well plates (glutamate experiments). Because ex vivo cultured cells will likely lose the characteristics they maintained in vivo over time, astrocytes were used directly after they were replated following removal of any macrophage and/or oligodendrocyte contamination to maintain the “ischemic” cortical astrocyte phenotype. Astrocytes used for immunohistochemistry were plated onto poly-d-lysine coated glass coverslips. The purity of the astrocyte population was examined by determining the ratio of the total number of glial fibrillary acidic protein (GFAP) (astrocytic marker GFAP; Sigma) and microglial marker (Mac-1) (microglial marker, CD11b, Serotec, Raleigh, NC, USA) immunolabeled cells relative to the total number of stained nuclei using the nuclear stain Hoechst 33258. These cultures contain 2%–3% CD11b positive cells and no significant differences were observed between young and middle-aged astrocytes (Young: 2.63 ± 0.58 ; Middle-aged: 3.30 ± 1.59 Supplementary Fig. 2).

2.2.3. Astrocyte treatments—All experiments were performed using an insulin-free medium (IFM: Neurobasal medium, glutamine, 0.5 mM, Invitrogen; human apotransferrin 100 $\mu\text{g}/\text{mL}$, sodium selenite 30 nM, progesterone, 20 nM, Sigma). Earlier studies showed that secreted insulin-like growth factor (IGF)-1 was significantly decreased by 25% in middle-aged astrocyte media as compared with young astrocyte media ($F(1,6) = 11.2$, $p < 0.05$; Supplementary Fig. 3), thus for all experiments insulin was removed from the media to maximize any age-dependent differences. At confluence, the cortical astrocytes were washed 3 times with Neurobasal medium and then treated with IFM for 24 hours. In studies where glutamate was added, the same treatment paradigm was followed except in some wells, glutamate was added at a concentration of 250 μM (glutamic acid assay) and incubated with the cells for 1 hour.

2.3. Immunohistochemistry

The purity of the astrocyte population was determined using the astrocytic marker, GFAP and the microglial marker, CD11b. The presence of GLT-1/EAAT2 (Millipore, Temecula, CA, USA) was also examined. In other studies where NPCs were cultured directly with astrocytes an early neuronal marker, microtubule-associated protein-2 (MAP-2; Millipore) was used. Cultures were washed briefly in $1 \times \text{dPBS}$ and fixed for 1 hour in 2% paraformaldehyde. Astrocytes were washed and incubated in block solution for 1 hour followed by incubation with the primary overnight at 4 °C (GFAP, 1:80; CD11b, 1:100, GLT-1/EAAT2, 1:1000; MAP-2, 1:500). The primary was diluted in dPBS, 1% normal goat serum and 0.3% Triton. Controls were incubated in diluent only. After washes, astrocytes were labeled with a fluorescent secondary Alexa Fluor 594 (CD11b, GLT-1/EAAT2, MAP-2, 1:2000, Molecular Probes, Carlsbad, CA, USA) or Oregon Green 488 (GFAP, 1:500, Molecular Probes) to visualize the labeling for 1 hour at room temperature. Astrocytes were counterstained with Hoechst 33258 (Polysciences, Inc., Warrington, PA, USA) for 1 hour at room temperature and cover-slipped with ProLong Antifade kit (Molecular Probes). Slides were visualized by epifluorescence and photographed using an Olympus BX2 microscope and QCapture imaging software (v2.7.3, QImaging Corporation, Ontario, Canada).

2.4. ELISA for protein expression of IGF-1

IGF-1 expression was determined using the Mouse/Rat IGF-1 Quantikine enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, MN, USA) using manufacturer's instructions and laboratory protocol (Selvamani and Sohrabji, 2010b). Briefly, 50 μ L of standard, control, and astrocyte media were incubated on an IGF-1 coated 96-well plate for 2 hours at room temperature. After the 2-hour incubation, the plate was washed 5 times with wash buffer and 100 μ L of conjugate was added and incubated for an additional 2 hours. The plate was again washed 5 times and the color reaction was initiated using 100 μ L of substrate solution, which was incubated on the plate for 30 minutes. The reaction was stopped using an equal volume of stop solution and the plate read in a plate reader (Infinite 200, Tecan, CA, USA) at 450 nm.

2.5. Glutamic acid assay

Glutamic acid uptake was examined using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Invitrogen, A12221). Manufacturer's instructions were followed and are described here briefly. Samples (50 μ L) were diluted (1:2000) in 1 $\times$ reaction buffer and added to a 96-well plate along with standards and controls. To each well, 50 μ L of the Amplex Red solution was added (100 μ M Amplex Red reagent; 0.25 units/mL horseradish peroxidase, 0.08 U/mL L-glutamate oxidase, 0.5 U/mL L-glutamate-pyruvate transaminase, and 200 μ M L-alanine). The plate was read in a plate reader (excitation: 540–560 nm; emission: 590 nm) at time 0 and then incubated at 37 °C for 15 minutes and read again. The time 0 reading was subtracted from the 15-minute reading to obtain the value for glutamic acid present in the astrocyte media.

2.6. Protein collection

Proteins were isolated by homogenizing the tissue in previously described lysis buffer (Sohrabji et al., 2000). Protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. The plates were read at 505 nm in an ELISA plate reader and concentrations of the samples were interpolated from the standards using Magellan software (Tecan, Männedorf, Switzerland). Total protein content was determined as a product of protein concentration (μ g/ μ L) $\times$ total fluid content of sample.

2.7. Immunoblotting

Expression of the GLT-1/EAAT2 was analyzed by immunoblotting. Total protein from astrocyte cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% Bis-Tris gel, Invitrogen) and transferred to a nylon membrane using the iBlot gel transfer system (Invitrogen). Standard laboratory procedures were followed for Western blot analysis (Jezierski and Sohrabji, 2000, 2001). Protein expression of GLT-1 (1:1000, Millipore) was normalized to α -tubulin (1:3000, Sigma). A horseradish peroxidase-conjugated secondary (GLT-1: anti-guinea pig, 1:5000, Millipore; α -tubulin: anti-mouse, 1:2000, BD Biosciences, San Jose, CA, USA) was used to capture the primary signal. Western Lightening chemiluminescence Reagent (PerkinElmer, Waltham, MA, USA) was utilized for immunodetection and X-ray film was used to capture the

intensity of the signal and the densitometry program Quantity One (Bio-Rad, Hercules, CA, USA) for quantifying the signal.

2.8. Migration of neural progenitor cells

The effect of astrocyte-secreted factors on migration of NPCs was examined using the QCM Chemotaxis 96-well Cell Migration Assay (8 μm pore size, ECM510; Millipore). Gestational Day 12.5 (E12.5)-derived fetal murine cortical neuroepithelial cells, maintained as neurosphere cultures (kind gift from Dr. R.C. Miranda) (Camarillo et al., 2007; Prock and Miranda, 2007; Santillano et al., 2005) were plated in the top insert at a density of 50,000 cells in 100 μL . These neurosphere aggregates have been previously shown to contain a mixed population of cells expressing several stem and progenitor cell markers (Santillano et al., 2005), but have the capacity to rapidly differentiate into neurons (Camarillo et al., 2007), astrocytes, or oligodendrocytes in response to culture conditions. In the bottom chamber, 150 μL of the appropriate cortical astrocyte-conditioned media (ACM) diluted 1:1 with IFM was added, and the 96-well plate was incubated at 37 °C for 24 hours. After 24 hours the insert was removed and any media in the top insert was removed. The insert was then incubated in 150 μL of detachment buffer to collect any cells that had migrated across the membrane but were still attached to the membrane for 30 minutes at 37 °C. At the end of the 30-minute incubation, 50 μL of a nuclear dye: lysis buffer mixture (1:75) was added to the 150 μL of ACM/cell mixture from the bottom chamber and incubated for 15 minutes at room temperature. A total of 150 μL of this mixture was placed into a flat-black 96-well plate and read in a fluorescent reader at 480 excitation/520 emission.

2.9. Migration of peripheral blood monocyte cells

2.9.1. PBMC collection—In the female studies, peripheral blood monocyte cells (PBMCs) were collected from young (6–8 months) female rats and used for the migration assays. In the male studies we used the same age of rat (6–8 months) however the migration assay was performed using PBMCs from both male and female animals. Young rats were deeply anesthetized and transcardially perfused with cold phosphate-buffered saline (pH 7.4) after the blood was collected from the left ventricle of the heart using a heparin (500 U/mL) coated syringe (16-gauge needle). Blood was added to a 15 mL conical containing 10% heparin (500 U/mL) and centrifuged at 1600 rpm for 7 minutes. The supernatant was removed and an equal volume of Neurobasal medium was added to the blood. Ficoll was added at 1/3 the volume of the blood/neurobasal medium mixture to a new conical and the blood/neurobasal mixture was overlaid on top of the Ficoll to sediment the PBMCs. The blood/Neurobasal/Ficoll mixture was centrifuged for 25 minutes at 1800 rpm to collect the PBMC layer. After centrifugation, the PBMC layer was carefully removed and placed in a 1.5 mL microcentrifuge tube. After a 7-minute spin at 1800 rpm, the PBMCs were washed with dPBS once, resuspended in IFM treatment medium, and counted.

2.9.2. PBMC migration—For PBMC analysis, the Chemotaxis Migration Assay (3 μm pore, ECM515, Millipore) was used. Manufacturer's instructions were followed and are described briefly. The PBMCs were plated on to the top insert of a 96-well plate at a concentration of 15,000 per well, 100- μL total was added to the upper chamber. In the bottom chamber, 150 μL of the appropriate cortical ACM diluted 1:1 with IFM was added

and the 96-well plate was incubated at 37 °C for 4 hours. After 4 hours the insert was removed and any media in the top insert was removed. The insert was then incubated in 150 μ L of detachment buffer to collect any cells that had migrated across the membrane but were still attached to the membrane for 30 minutes at 37 °C. At the end of the 30-minute incubation, 75 μ L of the cells/detachment buffer mixture was added to a flat black plate. To collect any cells that had migrated across the membrane and had entered the bottom chamber, 75 μ L of the ACM/cell mixture from the bottom chamber was also added to the same well. A nuclear dye:lysis buffer mixture was then added (1:75) to each well and incubated at room temperature for 15 minutes. The plate was read in a fluorescent reader at 480 excitation/520 emission.

2.10. Protein expression of cytokines/chemokines and inflammatory markers

Cytokine/chemokine protein expression in female cortical astrocyte media was performed using the rat Milliplex MAP kit (Millipore). The Bio-Plex Suspension System was calibrated using CAL2 (High PMT, Bio-Plex Calibration Kit, Millipore) as previously described (Lewis et al., 2008b, 2010). In male cortical astrocyte media only IL-6 and MIP-1 were examined as those were the only 2 analytes that were significantly regulated in females. For the Milliplex MAP (Millipore) rat cytokine/chemokine panel, 50 μ L of sample/standard/controls were added to each well followed by addition of the beads. The plate containing the samples/standards/controls plus the premixed beads were incubated overnight at 4 °C with shaking. The plate was washed twice, the washes were removed by vacuum filtration, the detection antibody (25 μ L) was added and the plate incubated for 2 hours at room temperature. Following a 2-hour incubation, streptavidin-phycoerythrin (25 μ L) was added and the plate incubated at room temperature for 30 minutes. The plate was washed twice and then the beads were resuspended in 150 μ L assay buffer and read. Cytokines and chemokine levels were normalized to total protein values.

2.11. Neural progenitor cells: astrocyte cocultures

Gestational day 12.5 (E12.5)-derived fetal murine cortical neuroepithelial cells, maintained as neurosphere cultures (described above) (Camarillo et al., 2007; Prock and Miranda, 2007; Santillano et al., 2005) were plated onto a confluent astrocyte monolayer derived from young and middle-aged females at a density of 50,000 cells per 35 mm dish. NPCs were cocultured with astrocytes for 4 days in IFM. To examine the impact of cortical astrocytes on neuronal differentiation, cells were immunolabeled for the early neuronal marker MAP-2 at termination of the culturing period. Immunohistochemical analysis of young and middle-aged astrocyte:NPC cocultures were performed simultaneously. The number of MAP-2 positive cells and the number of processes on each MAP-2 positive cell was determined. This measure was performed in 5 field of views and at least 100 cells were counted per slide using a 40 $\times$ objective lens. A total of 3 replicates were used for statistical analysis.

2.12. BrdU-doublecortin quantitation following transient MCAo

Animals were subjected to an intracerebral transient middle-cerebral artery occlusion (MCAo) by ET-1 injection or a sham surgery (saline injection). Animals received intraperitoneal injections of BrdU (50 mg/kg, Sigma) every 12 hours for 7 days following stroke. BrdU was used as marker for NPC proliferation (Nowakowski et al., 1989). At 14

days animals were deeply anesthetized and transcardially perfused with PBS pH 7.4 followed by perfusion with 4% paraformaldehyde. They were then decapitated and their brains rapidly dissected. The brains were postfixed in 4% paraformaldehyde for 2 hours at room temperature and sucrose (15%) loaded at 4 °C. The brains were placed in blocks containing mounting media and flash frozen.

2.13. Immunohistochemistry of tissue sections

Immunohistochemistry was performed to quantify the expression of BrdU and doublecortin (DCX) following MCAo. BrdU was used as a marker of cell proliferation (Nowakowski et al., 1989) and DCX as a marker for neuroblasts (Gleeson et al., 1999). Tissue sections were briefly washed in dPBS and fixed for 15 minutes in 4% paraformaldehyde. Sections were denatured in 1M hydrochloric acid for 30 minutes at 65 °C for 30 minutes and neutralized in 0.1 M sodium borate for 10 minutes. Following 3 washes with 1× dPBS the sections were incubated in block solution for 1 hour followed by incubation with the primary overnight at 4 °C (BrdU, 1:500, Chemicon, Temecula, CA; DCX, 1:500, Santa Cruz, Santa Cruz, CA, USA). The primary was diluted in dPBS, 1% normal horse serum and 0.3% Triton. Controls were incubated in diluent only. Following washes, sections were labeled with a fluorescent secondary Alexa Fluor 594 (DCX; 1:2000, Molecular Probes) or Oregon Green 488 (BrdU, 1:500, Molecular Probes) to visualize the labeling for 1 hour at room temperature. Sections were counterstained with Hoechst 33258 (Polysciences, Inc.) for 30 minutes at room temperature and cover-slipped with ProLong Antifade Kit (Molecular Probes). Slides were visualized by epifluorescence and photographed using an Olympus BX2 microscope and QCapture imaging software (v2.7.3, QImaging Corporation). BrdU, DCX, and double-labeled cells were counted in the striatum and cortex of 5 sections per animal and a total of 3–5 animals were used for quantification in each group. Cell counts were totaled and totals from stroke-injured animals was normalized to age-matched saline-treated animals.

2.14. Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS, V.13.0 Inc., IL, USA), and group differences were considered significant at $p < 0.05$. Data were analyzed using a 1-way analysis of variance (ANOVA) for young and middle-aged animals. All data are expressed in terms of $\pm$ the standard error of the mean (SEM) for each study. All experimental groups had 3–4 replicates, which is indicated in each figure legend.

3. Results

3.1. Astrocyte cultures

Astrocytes were harvested from the ischemic cortex of young and middle-aged males and females. Cerebral ischemia was induced by MCAo caused by stereotaxic injections of endothelin-1 directed to the MCA. As reported before, this method results in a typical cortical-striatal infarct common to MCAo models (Selvamani and Sohrabji, 2010a, 2010b). Astrocytes were harvested based on our previously published procedures (Lewis et al., 2008a, 2008b) and cultures were virtually pure for astrocytes (97%) as determined by GFAP immunohistochemistry, with low microglial contamination as determined by CD11b immunohistochemistry (Supplementary Fig. 2).

3.2. Glutamic acid assay

To assess age-dependent differences in the ability of cortical astrocytes to clear glutamate, astrocytes derived from young females, middle-aged females, and age-matched males were cultured in 250 μ M glutamic acid for 1 hour. The astrocyte media was then assayed for glutamate. While both young- and middle age-derived astrocytes clear glutamate from the media, we detected a 15% decrease in the amount of glutamate clearance per hour by ischemia-activated astrocytes from middle-aged females as compared with astrocytes derived from young females (Fig. 1; $F(1,4) = 153.0$; $p = 0.05$). Further, no significant differences in glutamate clearance were observed in astrocytes from age-matched males (Fig. 1).

3.3. GLT-1/EAAT2 expression in cortical cell lysates

To determine if there were differences in expression of GLT-1/EAAT2, young cortical astrocytes and middle-aged astrocytes derived from female rats were incubated in 250 μ M glutamic acid for 1 hour and cells were collected for immunoblotting. After glutamate treatment, GLT-1/EAAT2 expression increased in young and middle-aged ischemia-activated astrocytes as shown by immunolabeling (Fig. 2a), however no significant differences in GLT-1/EAAT2 protein expression were observed by immunoblotting (Fig. 2b).

3.4. Effect of astrocyte-conditioned medium on neural progenitor cells

Given that stroke increases the migration of NPCs from the subventricular zone (Arvidsson et al., 2002; Parent et al., 2002; Zhang et al., 2001), we next examined the effect of ischemia-activated cortical astrocytes on NPC migration (gift of R.C. Miranda) in vitro. When ischemia-activated cortical ACM from middle-aged females was used for the cell migration assay, increased cell migration of NPCs was observed (Fig. 3a) as compared with when young ACM was used. Specifically, a 29% increase in NPC migration was observed when the ACM was derived from female-derived middle-aged astrocytes as compared with ACM from young astrocytes ($F(1,8) = 15.8$; $p = 0.05$; Fig. 3a). To determine if the effects on cell migration were sex-dependent, ischemia-activated cortical astrocytes derived from age-matched males were also examined. When ACM from age-matched male astrocytes was cultured with NPCs, no differences were observed in NPC migration (Fig. 3).

In view of the greater recruitment of NPCs by ACM from older female astrocytes, we next determined the ability of young and middle-aged female astrocytes to promote neuronal differentiation in these NPCs. Previous studies have shown that the NPCs used in these studies can differentiate into neurons, astrocytes, and oligodendrocytes (Camarillo et al., 2007; Santillano et al., 2005). NPCs were cocultured with young or middle-aged astrocytes derived from female rats and immunolabeled for MAP-2, an early marker of neuronal differentiation. There were no age-dependent effects of ACM on neuronal differentiation as determined by the proportion of MAP-2 positive cells (Fig. 4a and b) and by the number of processes on each MAP-2 positive cell (Fig. 4a and c), indicating that ischemia-activated astrocytes from young and middle-aged animals retain similar abilities to promote neuronal differentiation. Finally we determined whether there were age differences in the recruitment of new neurons to the peri-ischemic site in vivo. Newly-born (postischemia) migrating neurons were identified by DCX, which is expressed by migrating neuroblasts and BrdU, a

DNA replication marker. As shown in Fig. 5a and b, BrdU-positive cells were seen in both the ischemic cortex and striatum and a small proportion of these were colabeled by DCX. The proportion of BrdU/DCX labeled cells were no different in young and middle-aged females (Fig. 5c), indicating that age does not affect recruitment of new neurons to the ischemic zone 2 weeks postischemia.

3.5. Effect of astrocyte-conditioned medium on PBMCs

Stroke also increases the recruitment of peripheral blood leukocytes to the ischemic site (Dirnagl et al., 1999; Stoll et al., 1998). In order to assess age-related changes in ACM on leukocyte migration, PBMCs were cultured with ACM from young and middle-aged female-derived astrocytes in a transwell plate. PBMCs grown in middle-aged ACM showed a 32% increase in migration as compared with those PBMCs cultured in young ACM ($F(1,8) = 25.9$, $p = 0.05$; Fig. 6a). To assess sex differences in ACM-induced migration, ACM was also collected from age-matched males. For these studies, the PBMC donors were either 6–8-month-old females or 6–8-month-old males. Interestingly, ACM from astrocytes derived from young or middle-aged males showed no differences in PBMC migration when PBMCs were derived from young females (Fig. 6b). However, when the PBMCs were derived from 6–8-month-old males, a similar age-related increase in PBMC migration was observed. Migration was increased by 1.5-fold when the ACM was derived from middle-aged astrocytes compared with ACM derived from younger astrocytes ($F(1,4) = 17.7$, $p = 0.05$; Fig. 6b).

3.6. Protein expression of cytokines/chemokines and inflammatory markers

Cytokine/chemokine expression was assessed in media from ischemia-activated cortical astrocytes derived from young and middle-aged females and age-matched males. The cytokines IL-1 β , IL-5, IL-17, IL-18, TNF- α , Regulated on Activation, Normal T Expressed and Secreted (RANTES), as well as the chemokines MCP-1, vascular endothelial growth factor (VEGF) and growth related oncogene (GRO-KC; also known as CXCL1) were detectable but not significantly different in astrocyte media derived from young or middle-aged females. Two inflammatory mediators were significantly regulated by age: IL-6 was significantly decreased by 29% in the media from middle-aged cortical astrocytes as compared with young astrocyte media (Fig. 7a; $F(1,4) = 11.5$, $p = 0.05$). Further, MIP-1 was significantly increased by 30% in middle-aged astrocyte media as compared with young astrocyte media (Fig. 7b; $F(1,6) = 7.3$; $p = 0.05$). Both IL-6 and MIP-1 were examined in ischemia-activated male astrocytes, and no significant differences were found in either case (Fig. 7c and d).

4. Discussion

Stroke culminates in several excitotoxic and inflammatory events (Benveniste et al., 1984; Dirnagl et al., 1999; Globus et al., 1988; Kim et al., 1995; Stoll et al., 1998) ultimately leading to neural cell death. With age, these excitotoxic and inflammatory events occur more quickly and lead to greater neurodegeneration (Popa-Wagner et al., 2007). Reproductive aging, which is a midlife event in women and female rats, also leads to increased neural damage following focal ischemia. Our laboratory has previously shown that in

reproductively senescent, middle-aged female rats, which mimic several aspects of the human menopause, cortical infarct volumes are significantly greater when compared with younger, normal cycling female rats (Selvamani and Sohrabji, 2010a). The present study supports the hypothesis that age-related severity in cortical infarction is associated with impaired cortical astrocyte function.

In human brain injury (Castejón, 1998; Hausmann et al., 2000), stroke (Martí-Fàbregas et al., 2010), and in vascular dementia (Delacourte and Buée, 1989; Vijayan et al., 1991) an increased presence of reactive, hypertrophic astrocytes has been observed. Moreover, dysfunction in the neurovascular unit, in which astrocytes play a prominent role, has also been suggested for vascular diseases (see review, Lecrux and Hamel, 2011). In this study we focused on cortical astrocytes because of their role in determining the extent of neuronal injury following focal ischemia (Grzybicki et al., 1998; Kim et al., 1995; Lau and Yu, 2001; Ordý et al., 1993; Petito et al., 1990; Ridet et al., 1997; Yamamoto et al., 1987). An early event after focal ischemia is neuronal and glial depolarization resulting in transporter dysregulation and excessive release of neurotransmitters such as glutamate (Benveniste et al., 1984; Globus et al., 1988). Astrocytes regulate glutamate concentrations through the glial glutamate transporters (Amara and Fontana, 2002; Pines et al., 1992; Storck et al., 1992), which can be impaired under adverse conditions such as reduced adenosine triphosphate (ATP), metabolic dysfunction and ionic disturbances (Liao and Chen, 2001), and hypoxia (Boycott et al., 2007). Aging coupled in the context of injury may further impair astrocyte trophic support. Using a model of in vitro aging, H₂O₂ treatment resulted in reduced glutamate uptake in 90-days in vitro (DIV) neonatal rodent astrocyte cultures as compared with 10-days in vitro cultures (Pertusa et al., 2007). Moreover, basal levels of reactive oxygen species increased, mitochondrial function decreased, and the ability of these “aged” astrocytes to support cortical neurons also declined (Pertusa et al., 2007). Similar findings were also reported by Gottfried et al. (2002). In the present study, a significant age-related reduction in glutamate uptake was observed in adult-derived cortical astrocyte cultures. Cortical astrocytes collected from aging females cleared 15% less glutamate per hour as compared with astrocytes collected from younger females. During the crucial first 3–4 hours after ischemia, this would be amplified to a 50%–61% reduction in glutamate clearance, resulting in a more toxic microenvironment in the older brain. Our initial examination of GLT-1/EAAT2 expression indicated no significant differences between young and middle-aged female astrocytes. Further studies will focus on expression of the EAAT1 transporter as well as the activity of both transporters, especially because the peptide hormone IGF-1, which is known to improve glutamate transporter activity (Suzuki et al., 2001), is significantly reduced in middle-aged female astrocytes as compared with young female astrocytes (Supplementary Fig. 3). In contrast to the effects observed in females, age-matched, ischemia-activated astrocytes from males showed no significant age effects on glutamate uptake, consistent with the observation that young and middle-aged males do not differ in cortical infarct volume. Taken together, our data indicate that impaired glutamate uptake is closely tied to severity of infarct volume.

Focal ischemia also leads to an increased cellular response in which astrocytes (Ordý et al., 1993; Petito et al., 1990; Yamamoto et al., 1987) and microglia (Morioka et al., 1993; Stoll et al., 1998) become activated and peripheral blood elements (Dirnagl et al., 1999; Stoll et

al., 1998) as well as neural stem cells (Arvidsson et al., 2002; Parent et al., 2002; Zhang et al., 2001) migrate to the injury site. Astrocytes actively participate in recruitment of peripheral blood elements and NPCs. In hypoxia-treated neonatal rat astrocytes (Xu et al., 2007) astrocyte-secreted vascular endothelial growth factor, stem cell factor, stromal-derived factor-1 α , and MCP-1 promoted NPC migration, while astrocyte-derived MIP-2 enhanced chemotaxis of human neutrophils (Otto et al., 2002). Our data show that ischemia-activated astrocytes from middle-aged females strongly promoted the migration of both NPCs and PBMCs. While elevated recruitment of PBMCs is consistent with the increased cell death seen in the older ischemic female, elevated migration of NPCs may indicate heightened capacity for repair and regeneration if these NPCs are new neurons. Coculture experiments however indicate that both young and middle-aged astrocytes are no different in their ability to promote neuronal fate, as estimated by MAP-2 positive cells. If the proportion of neuronal fate is similar between NPCs cultured with young and middle-aged astrocytes, then the greater migration of recruitment of NPCs may result in more new neurons in the peri-ischemic sites of the middle-aged brain. However, our in vivo studies indicate a similar proportion of new neurons in the peri-ischemic regions of the young and middle-aged brain. Collectively, these data suggest that while the aging brain has the potential to recruit more progenitors, it does not sustain this new population in the peri-ischemic region.

The injury itself may also modulate the astrocyte phenotype which in turn can influence the role these cells play in the repair process. The cortical astrocytes used in these studies proliferated readily in culture and immunolabeled for GFAP, a marker expressed in every subset of astrocyte, whether normal or reactive (Ridet et al., 1997). Some studies suggest that mature astrocytes are stimulated to enter the cell cycle following injury (Janeczko, 1991; Topp et al., 1989) while other studies indicate that these astrocytic GFAP-positive cells are themselves neural progenitor cells (Frisén et al., 1995; Holmin et al., 1997; Martí-Fàbregas et al., 2010; Takamiya et al., 1988). In the present study, cultured cells were immunopositive for GLT1, which is a marker of mature astrocytes (Tanaka et al., 1997). Our goal was to capture all astrocytes found in the ischemic cortex and it is likely that both mature, reactive astrocytes that re-entered the cell cycle and GFAP-positive neural progenitor cells were present. We hypothesize that the impairments observed in middle-aged ischemia-activated cortical astrocytes is due in part to the astrocyte phenotype in the ischemic cortex and future studies will address this question.

Aging female astrocytes also increased migration of PBMCs, potentially leading to local accumulation of inflammatory mediators which could influence the extent of neural damage in the ischemic brain. Astrocytes have been shown to secrete several cytokines (Kim et al., 1995; Lau and Yu, 2001; Ransohoff et al., 1993; Ridet et al., 1997), and in the current study MIP-1 α was significantly increased in the older female-derived astrocytes. This chemokine is involved in recruitment and activation of neutrophils, T-cells, and macrophages (Davatelis et al., 1988; Shi et al., 1996; Standiford et al., 1995; Taub et al., 1993; VanOtteren et al., 1994; Wolpe and Cerami, 1989) and MIP-1 α enhances the migration of human bone marrow stromal cells (Wang and Shuaib, 2002). Elevated MIP-1 α levels may not only mediate the increased migratory response by middle-aged female astrocytes, but also underlie the increased infarct volume observed in this group. In rodents, MIP-1 α exacerbated the systemic inflammatory response following trauma-hemorrhage (Hsieh et al., 2008) and

MIP-1 α as well as MCP-1 enhanced inflammatory cell accumulation following bleomycin-induced lung injury (Smith, 1996). Given the role of MIP-1 α in recruitment of hematogenous cells and its role in heightening the inflammatory response coupled with increased blood-brain barrier permeability in middle-aged females (Bake and Sohrabji, 2004), it is not surprising that middle-aged females show greater cellular damage after stroke as compared with younger females.

In the current study we also observed a significant increase in the proinflammatory cytokine IL-6 in the younger female astrocyte cultures. Known to be an important mediator of fever and the acute phase response (Morrone et al., 1988), IL-6 stimulates prostaglandin E₂ (PGE₂) synthesis in the hypothalamus (Fernandez-Alonso et al., 1996) and thermogenesis in muscle and fatty tissue (Li et al., 2002). IL-6 has been shown to be elevated in plasma of stroke patients, although correlating IL-6 levels with stroke severity is confounded by the fact that stroke patients present with other comorbid conditions such as vascular disease, and diabetes which also elevate IL-6. Remarkably, IL-6 also has anti-inflammatory properties including suppressing TNF- α and IL-1 in PBMCs (Schindler et al., 1990), suppressing lipopolysaccharide (LPS)-induced TNF- α in vivo and in cultured monocytes (Aderka et al., 1989) and inducing the interleukin-1 receptor antagonist (IL-1ra) in human plasma and cultured monocytes (Tilg et al., 1994). Hence elevated IL-6 expression by young astrocytes may regulate and suppress local inflammation.

The comparison between 6–8- and 10–12-month-old males versus the same age of females in this study provides a useful approach to pinpoint age-related impairments in astrocyte function. Although aged (20-month-old) male and female mice sustain a much larger infarct than young mice, the middle-aged group (10–12 months) has not received significant experimental attention. In our studies, middle-aged females sustain a much larger cortical infarct than younger females (Selvamani and Sohrabji, 2010a), however, these differences are not observed in comparably age-matched males. This sex difference may be related to the loss of ovarian hormones in females at middle age, especially estrogen, which is known to be neuroprotective to young females in experimental stroke models (Dubal et al., 1998; Rusa et al., 1999; Simpkins et al., 1997). Clinical data indicate that the peri-menopausal period confers unique vulnerability to cerebrovascular disease based on reports that the incidence for stroke among women 45–54 years of age is almost twice that of men and is also more likely to be fatal (Ayala et al., 2002; Bushnell, 2008; Towfighi et al., 2007). In young rats (Alkayed et al., 1998; Li et al., 1996; Zhang et al., 1998) and mice (Park et al., 2006), larger infarct volumes were observed in intact males as compared with the age-matched females, further supporting the idea that ovarian hormones confer neuroprotection. In the present study, we exploited this sex difference in stroke severity with aging to elucidate the role of astrocytes. In comparison with young female astrocytes, middle-aged female astrocytes display impaired glutamate clearance, heightened propensity for leukocyte recruitment, and elevated chemokine levels while young and middle-aged male astrocytes did not differ in these measures. Collectively, our data make a strong case that severity of tissue loss following experimental stroke in older females is associated with impaired ability of the astrocyte to detoxify and stabilize inflammation in the microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH AG027684, AG042189, and NS074895 to F.S. and a fellowship from the Texas Consortium in Behavioral Neuroscience NIH 5-T32-MH065728-07 to D.K.L. K.T.T. was supported by the John Warren Brain Tumor Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neurobiolaging.2011.11.007.

References

- Aderka D, Le JM, Vilcek J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J Immunol.* 1989; 143:3517–3523. [PubMed: 2584704]
- Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke.* 1998; 29:159–165. [PubMed: 9445346]
- Amara SG, Fontana AC. Excitatory amino acid transporters: keeping up with glutamate. *Neurochem Int.* 2002; 41:313–318. [PubMed: 12176072]
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med.* 2002; 8:963–970. [PubMed: 12161747]
- Ayala C, Croft JB, Greenlund KJ, Keenan NL, Donehoo RS, Malarcher AM, Mensah GA. Sex differences in US mortality rates for stroke and stroke subtypes by race/ethnicity and age, 1995–1998. *Stroke.* 2002; 33:1197–1201. [PubMed: 11988590]
- Badan I, Buchhold B, Hamm A, Gratz M, Walker LC, Platt D, Kessler Ch, Popa-Wagner A. Accelerated glial reactivity to stroke in aged rats correlates with reduced functional recovery. *J Cereb Blood Flow Metab.* 2003; 23:845–854. [PubMed: 12843788]
- Bake S, Sohrabji F. 17β -Estradiol differentially regulate blood-brain barrier permeability in young and aging female rats. *Endocrinologist.* 2004; 145:5471–5475.
- Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem.* 1984; 43:1369–1374. [PubMed: 6149259]
- Biernaskie J, Corbett D, Peeling J, Wells J, Lei H. A serial MR study of cerebral blood flow changes and lesion development following endothelin-1-induced ischemia in rats. *Magn Reson Med.* 2001; 46:827–830. [PubMed: 11590661]
- Boycott HE, Dallas M, Boyle JP, Pearson HA, Peers C. Hypoxia suppresses astrocyte glutamate transport independently of amyloid formation. *Biochem Biophys Res Commun.* 2007; 364:100–104. [PubMed: 17927959]
- Bramlett HM, Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab.* 2004; 24:133–150. [PubMed: 14747740]
- Brown AM, Baltan Tekkk S, Ransom BR. Energy transfer from astrocytes to axons: the role of CNS glycogen. *Neurochem Int.* 2004; 45:529–536. [PubMed: 15186919]
- Bushnell CD. Stroke in women: risk and prevention throughout the lifespan. *Neurol Clin.* 2008; 26:1161–1176. [PubMed: 19026906]
- Camarillo C, Kumar LS, Bake S, Sohrabji F, Miranda RC. Ethanol regulates angiogenic cytokines during neural development: evidence from an in vitro model of mitogen-withdrawal-induced cerebral cortical neuroepithelial differentiation. *Alcohol Clin Exp Res.* 2007; 31:324–335. [PubMed: 17250626]

- Castejón OJ. Morphological astrocytic changes in complicated human brain trauma. A light and electron microscopic study. *Brain Inj.* 1998; 12:409–427. [PubMed: 9591144]
- Davatelis G, Tekamp-Olson P, Wolpe SD, Hermesen K, Luedke C, Gallegos C, Coit D, Merryweather J, Cerami A. Cloning and characterization of a cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties. *J Exp Med.* 1988; 167:1939–1944. [PubMed: 3290382]
- Delacourte A, Buée L. Alzheimer's disease: the glial reaction is general and severe in all areas of the central nervous system. *CR Acad Sci III.* 1989; 308:359–365.
- Dinapoli VA, Benkovic SA, Li X, Kelly KA, Miller DB, Rosen CL, Huber JD, O'Callaghan JP. Age exaggerates proinflammatory cytokine signaling and truncates signal transducers and activators of transcription 3 signaling following ischemic stroke in the rat. *Neuroscience.* 2010; 170:633–644. [PubMed: 20633608]
- Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 1999; 22:391–397. [PubMed: 10441299]
- Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM. Estradiol protects against ischemic injury. *J Cereb Blood Flow Metab.* 1998; 18:1253–1258. [PubMed: 9809515]
- Fernandez-Alonso A, Benamar K, Sancibrian M, Lopez-Valpuesta FJ, Minano FJ. Role of interleukin-1beta, interleukin-6 and macrophage inflammatory protein-1 beta in prostaglandin-E2-induced hyperthermia in rats. *Life Sci.* 1996; 59:PL185–190. [PubMed: 8809217]
- Fontana A, Kristensen F, Dubs R, Gerns D, Weber E. Production of prostaglandin E and an interleukin-1 like factor by cultured astrocytes and C6 glioma cells. *J Immunol.* 1982; 129:2413–2419. [PubMed: 6982921]
- Frisén J, Johansson CB, Török C, Risling M, Lendahl U. Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. *J Cell Biol.* 1995; 131:453–464. [PubMed: 7593171]
- Garcia-Estrada J, Garcia-Segura LM, Torres-Aleman I. Expression of insulin-like growth factor I by astrocytes in response to injury. *Brain Res.* 1992; 592:343–347. [PubMed: 1280521]
- Gleeson JG, Lin PT, Flanagan LA, Walsh CA. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron.* 1999; 23:257–271. [PubMed: 10399933]
- Globus MY, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD. Effect of ischemia on the in vivo release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. *J Neurochem.* 1988; 51:1455–1464. [PubMed: 2902196]
- Gottfried C, Tramontina F, Goncalves D, Goncalves CA, Moriguchi E, Dias RD, Wofchuk ST, Souza DO. Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H₂O₂. *Mech Ageing Dev.* 2002; 123:1333–1340. [PubMed: 12297336]
- Grzybicki D, Moore SA, Schelper R, Glabinski AR, Ransohoff RM, Murphy S. Expression of monocyte chemoattractant protein (MCP-1) and nitric oxide synthase-2 following cerebral trauma. *Acta Neuropathol.* 1998; 95:98–103. [PubMed: 9452827]
- Hausmann R, Riess R, Fieguth A, Betz P. Immunohistochemical investigations on the course of astroglial GFAP expression following human brain injury. *Int J Leg Med.* 2000; 113:70–75.
- Hazell AS. Excitotoxic mechanisms in stroke: an update of concepts and treatment strategies. *Neurochem Int.* 2007; 50:941–953. [PubMed: 17576023]
- Holmin S, Almqvist P, Lendahl U, Mathiesen T. Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. *Eur J Neurosci.* 1997; 9:65–75. [PubMed: 9042570]
- Hsieh CH, Frink M, Hsieh YC, Kan WH, Hsu JT, Schwacha MG, Choudhry MA, Chaudry IH. The role of MIP-1 alpha in the development of systemic inflammatory response and organ injury following trauma hemorrhage. *J Immunol.* 2008; 181:2806–2812. [PubMed: 18684972]
- Janeczko K. The proliferative response of S-100 protein-positive glial cells to injury in the neonatal rat brain. *Brain Res.* 1991; 564:86–90. [PubMed: 1777824]
- Janzer RC, Raff MC. Astrocytes induce blood–brain barrier properties in endothelial cells. *Nature.* 1987; 325:253–257. [PubMed: 3543687]

- Jezierski MK, Sohrabji F. Region- and peptide-specific regulation of the neurotrophins by estrogen. *Brain Res Mol Brain Res*. 2000; 85:77–84. [PubMed: 11146109]
- Jezierski MK, Sohrabji F. Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. *Neurobiol Aging*. 2001; 22:309–319. [PubMed: 11182481]
- Kim JH, Kim JH, Park JA, Lee SW, Kim WJ, Yu YS, Kim KW. Blood-neural barrier: intercellular communication at gliovascular interface. *J Biochem Mol Biol*. 2006; 39:339–345. [PubMed: 16889675]
- Kim JS, Gautam SC, Chopp M, Zaloga C, Jones ML, Ward PA, Welch KM. Expression of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 after focal cerebral ischemia in the rat. *J Neuroimmunol*. 1995; 56:127–134. [PubMed: 7860708]
- Lau LT, Yu AC. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *J Neurotrauma*. 2001; 18:351–359. [PubMed: 11284554]
- Lecrux C, Hamel E. The neurovascular unit in brain function and disease. *Acta Physiol (Oxf)*. 2011; 203:47–59. [PubMed: 21272266]
- Lewis DK, Bake S, Thomas K, Jezierski MK, Sohrabji F. A high cholesterol diet elevates hippocampal cytokine expression in an age and estrogen-dependent manner in female rats. *J Neuroimmunol*. 2010; 223:31–38. [PubMed: 20435353]
- Lewis DK, Johnson AB, Stoehlgren S, Harms A, Sohrabji F. Effects of estrogen receptor agonists on regulation of the inflammatory response in astrocytes from young adult and middle-aged female rats. *J Neuroimmunol*. 2008b; 195:47–59. [PubMed: 18328572]
- Lewis DK, Woodin HR, Sohrabji F. Astrocytes from acyclic female rats exhibit lowered capacity for neuronal differentiation. *Aging Cell*. 2008a; 7:836–849. [PubMed: 18778412]
- Li G, Klein RL, Matheny M, King MA, Meyer EM, Scarpace PJ. Induction of uncoupling protein 1 by central interleukin-6 gene delivery is dependent on sympathetic innervation of brown adipose tissue and underlies one mechanism of body weight reduction in rats. *Neuroscience*. 2002; 115:879–889. [PubMed: 12435426]
- Li K, Futrell N, Tovar S, Wang LC, Wang DZ, Schultz LR. Gender influences the magnitude of the inflammatory response within embolic cerebral infarcts in young rats. *Stroke*. 1996; 27:498–503. [PubMed: 8610320]
- Liao SL, Chen CJ. Differential effects of cytokines and redox potential on glutamate uptake in rat cortical glial cultures. *Neurosci Lett*. 2001; 299:113–116. [PubMed: 11166951]
- Liesi P, Silver J. Is astrocyte laminin involved in axon guidance in the mammalian CNS? *Dev Biol*. 1988; 130:774–785. [PubMed: 3143614]
- Luke LM, Allred RP, Jones TA. Unilateral ischemic sensorimotor cortical damage induces contralesional synaptogenesis and enhances skilled reaching with the ipsilateral forelimb in adult male rats. *Synapse*. 2004; 54:187–199. [PubMed: 15472929]
- Martí-Fàbregas J, Romaguera-Ros M, Gómez-Pinedo U, Martínez-Ramírez S, Jiménez-Xarrié E, Marín R, Martí-Vilalta JL, García-Verdugo JM. Proliferation in the human ipsilateral subventricular zone after ischemic stroke. *Neurologist*. 2010; 74:357–365.
- Morioka T, Kalehua AN, Streit WJ. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *J Comp Neurol*. 1993; 327:123–132. [PubMed: 8432904]
- Morrone G, Ciliberto G, Oliviero S, Arcone R, Dente L, Content J, Cortese R. Recombinant interleukin 6 regulates the transcriptional activation of a set of human acute phase genes. *J Biol Chem*. 1988; 263:12554–12558. [PubMed: 2457587]
- Nordell V, Scarborough M, Buchanan A, Sohrabji F. Differential effects of estrogen in the injured forebrain of young adult and reproductive senescent animals. *Neurobiol Aging*. 2003; 24:579–588.
- Nowakowski RS, Lewin SB, Miller MW. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J Neurocytol*. 1989; 18:311–318. [PubMed: 2746304]
- Ordy JM, Wengenack TM, Bialobok P, Coleman PD, Rodier P, Baggs RB, Dunlap WP, Kates B. Selective vulnerability and early progression of hippocampal CA1 pyramidal cell degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of transient global ischemia. *Exp Neurol*. 1993; 119:128–139. [PubMed: 8432346]

- Otto VI, Gloor SM, Frentzel S, Gilli U, Ammann E, Hein AE, Folkers G, Trentz O, Kossmann T, Morganti-Kossmann MC. The production of macrophage inflammatory protein-2 induced by soluble intercellular adhesion molecule-1 in mouse astrocytes is mediated by src tyrosine kinases and p42/44 mitogen-activated protein kinase. *J Neurochem.* 2002; 80:824–834. [PubMed: 11948246]
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol.* 2002; 52:802–813. [PubMed: 12447935]
- Parish CL, Finkelstein DI, Tripanichkul W, Satoskar AR, Drago J, Horne MK. The Role of Interleukin-1, Interleukin-6, and Glia in Inducing Growth of Neuronal Terminal Arbors in Mice. *J Neurosci.* 2002; 22:8034–8041. [PubMed: 12223557]
- Park EM, Cho S, Fry KA, Glickstein SB, Zhou P, Anrather J, Ross ME, Iadecola C. Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *J Cereb Blood Flow Metab.* 2006; 26:392–401. [PubMed: 16049426]
- Pertusa M, García-Matas S, Rodríguez-Farré E, Sanfeliu C, Cristòfol R. Astrocytes aged in vitro show a decreased neuroprotective capacity. *J Neurochem.* 2007; 101:794–805. [PubMed: 17250685]
- Petito CK, Morgello S, Felix JC, Lesser ML. The two patterns of reactive astrocytosis in postischemic rat brain. *J Cereb Blood Flow Metab.* 1990; 10:850–859. [PubMed: 2211878]
- Pines G, Danbolt NC, Bjørås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI. Cloning and expression of a rat brain L-glutamate transporter. *Nature.* 1992; 360:464–467. [PubMed: 1448170]
- Popa-Wagner A, Badan I, Walker L, Groppa S, Patrana N, Kessler C. Accelerated infarct development, cytochrome c expression and apoptosis following transient cerebral ischemia in aged rats. *Acta Neuropathol.* 2007; 113:277–293. [PubMed: 17131130]
- Prock TL, Miranda RC. Embryonic cerebral cortical progenitors are resistant to apoptosis, but increase expression of suicide receptor DISC-complex genes and suppress autophagy following ethanol exposure. *Alcohol Clin Exp Res.* 2007; 31:694–703. [PubMed: 17374049]
- Ransohoff RM, Hamilton TA, Tani M, Stoler MH, Shick HE, Major JA, Estes ML, Thomas DM, Tuohy VK. Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *FASEB J.* 1993; 7:592–600. [PubMed: 8472896]
- Ransom BR, Fern R. Does astrocytic glycogen benefit axon function and survival in CNS white matter during glucose deprivation? *Glia.* 1997; 21:134–141. [PubMed: 9298856]
- Ridet J, Malhotra S, Privat A, Gage F. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci.* 1997; 20:570–577. [PubMed: 9416670]
- Robbins DS, Shirazi Y, Drysdale BE, Lieberman A, Shin HS, Shin ML. Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. *J Immunol.* 1987; 139:2593–2597. [PubMed: 3116087]
- Rusa R, Alkayed NJ, Crain BJ, Traystman RJ, Kimes AS, London ED, Klaus JA, Hurn PD. 17 β -estradiol reduces stroke injury in estrogen-deficient female animals. *Stroke.* 1999; 30:1665–1670. [PubMed: 10436119]
- Santillano DR, Kumar LS, Prock TL, Camarillo C, Tingling JD, Miranda RC. Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. *BMC Neurosci.* 2005; 6:59. [PubMed: 16159388]
- Schindler R, Clark BD, Dinarello CA. Dissociation between interleukin-1 β mRNA and protein synthesis in human peripheral blood mononuclear cells. *J Biol Chem.* 1990; 265:10232–10237. [PubMed: 2354999]
- Schousboe A, Svenneby G, Hertz L. Uptake and metabolism of glutamate in astrocytes cultured from dissociated mouse brain hemispheres. *J Neurochem.* 1977; 29:999–1005. [PubMed: 23414]
- Schwartz JP, Nishiyama N. Neurotrophic factor gene expression in astrocytes during development and following injury. *Brain Res Bull.* 1994; 35:403–407. [PubMed: 7532097]
- Selvamani A, Sohrabji F. Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. *Neurobiol Aging.* 2010a; 31:1618–1628. [PubMed: 18829137]

- Selvamani A, Sohrabji F. The neurotoxic effects of estrogen on ischemic stroke in older female rats is associated with age-dependent loss of insulin-like growth factor-1. *J Neurosci.* 2010b; 30:6852–6861. [PubMed: 20484627]
- Sensi SL, Rockabrand E, Canzoniero LM. Acidosis enhances toxicity induced by kainate and zinc exposure in aged cultured astrocytes. *Biogerontology.* 2006; 7:367–374. [PubMed: 16964527]
- Shi MM, Godleski JJ, Paulauskis JD. Regulation of macrophage inflammatory protein-1 α mRNA by oxidative stress. *J Biol Chem.* 1996; 271:5878–5883. [PubMed: 8621460]
- Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg.* 1997; 87:724–730. [PubMed: 9347981]
- Smith RE. Chemotactic cytokines mediate leukocyte recruitment in fibrotic lung disease. *Biol Signals.* 1996; 5:223–231. [PubMed: 8891198]
- Sohrabji F, Peeples KW, Marroquin OA. Local and cortical effects of olfactory bulb lesions on trophic support and cholinergic function and their modulation by estrogen. *J Neurobiol.* 2000; 45:61–74. [PubMed: 11018768]
- Standiford TJ, Kunkel SL, Lukacs NW, Greenberger MJ, Danforth JM, Kunkel RG, Strieter RM. Macrophage inflammatory protein-1 α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J Immunol.* 1995; 155:1515–1524. [PubMed: 7636213]
- Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol.* 1998; 56:149–171. [PubMed: 9760699]
- Storck T, Schulte S, Hofmann K, Stoffel W. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. *Proc Natl Acad Sci U S A.* 1992; 89:10955–10959. [PubMed: 1279699]
- Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, Swanson RA. Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316, p819 ([R-R*, S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide). *J Pharmacol Exp Ther.* 2007; 321:45–50. [PubMed: 17251391]
- Suzuki K, Ikegaya Y, Matsuura S, Kanai Y, Endou H, Matsuki N. Transient upregulation of the glial glutamate transporter GLAST in response to fibroblast growth factor, insulin-like growth factor and epidermal growth factor in cultured astrocytes. *J Cell Sci.* 2001; 114:3717–3725. [PubMed: 11707523]
- Takamiya Y, Kohsaka S, Toya S, Otani M, Tsukada Y. Immunohistochemical studies on the proliferation of reactive astrocytes and the expression of cytoskeletal proteins following brain injury in rats. *Brain Res.* 1988; 466:201–210. [PubMed: 3359311]
- Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science.* 1997; 276:1699–1702. [PubMed: 9180080]
- Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 α and MIP-1 β . *Science.* 1993; 260:355–358. [PubMed: 7682337]
- Thiele DL, Kurosaka M, Lipsky PE. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. *J Immunol.* 1983; 131:2282–2290. [PubMed: 6605384]
- Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood.* 1994; 83:113–118. [PubMed: 8274730]
- Topp KS, Faddis BT, Vijayan VK. Trauma-induced proliferation of astrocytes in the brains of young and aged rats. *Glia.* 1989; 2:201–211. [PubMed: 2526082]
- Towfighi A, Saver JL, Engelhardt R, Ovbiagele B. A midlife stroke surge among women in the United States. *Neurologist.* 2007; 69:1898–1904.

- Tzingounis AV, Wadiche JI. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat Rev Neurosci.* 2007; 8:935–947. [PubMed: 17987031]
- Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. Control of synapse number by glia. *Science.* 2001; 291:657–660. [PubMed: 11158678]
- VanOtteren GM, Standiford TJ, Kunkel SL, Danforth JM, Burdick MD, Abruzzo LV, Strieter RM. Expression and regulation of macrophage inflammatory protein-1 alpha by murine alveolar and peritoneal macrophages. *Am J Respir Cell Mol Biol.* 1994; 10:8–15. [PubMed: 8292385]
- Vijayan VK, Geddes JW, Anderson KJ, Chang-Chui H, Ellis WG, Cotman CW. Astrocyte hypertrophy in the Alzheimer's disease hippocampal formation. *Exp Neurol.* 1991; 112:72–78. [PubMed: 2013308]
- Volterra A, Trotti D, Tromba C, Floridi S, Racagni G. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. *J Neurosci.* 1994; 14:2924–2932. [PubMed: 7910203]
- Wang CX, Shuaib A. Involvement of inflammatory cytokines in central nervous system injury. *Prog Neurobiol.* 2002; 67:161–172. [PubMed: 12126659]
- Wolpe SD, Cerami A. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *FASEB J.* 1989; 3:2565–2573. [PubMed: 2687068]
- Wu H, Friedman W, Dreyfus C. Differential regulation of neurotrophin expression in basal forebrain astrocytes by neuronal signals. *J Neurosci.* 2004; 24:76–85.
- Wujek J, Akeson R. Extracellular matrix derived from astrocytes stimulates neuritic outgrowth from PC12 cells in vitro. *Dev Brain Res.* 1987; 34:87–97.
- Xu Q, Wang S, Jiang X, Zhao Y, Gao M, Zhang Y, Wang X, Tano K, Kanehara M, Zhang W, Ishida T. Hypoxia-induced astrocytes promote the migration of neural progenitor cells via vascular endothelial factor, stem cell factor, stromal-derived factor-1alpha and monocyte chemoattractant protein-1 upregulation in vitro. *Clin Exp Pharmacol Physiol.* 2007; 34:624–631. [PubMed: 17581219]
- Yamamoto K, Akai F, Yoshimine T, Yanagihara T. Immunohistochemical investigation of cerebral ischemia after middle cerebral artery occlusion in gerbils. *J Neurosurg.* 1987; 67:414–420. [PubMed: 3302131]
- Yu AC, Gregory GA, Chan PH. Hypoxia-induced dysfunctions and injury of astrocytes in primary cell cultures. *J Cereb Blood Flow Metab.* 1989; 9:20–28. [PubMed: 2562958]
- Zhang RL, Zhang ZG, Zhang L, Chopp M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience.* 2001; 105:33–41. [PubMed: 11483298]
- Zhang YQ, Shi J, Rajakumar G, Day AL, Simpkins JW. Effects of gender and estradiol treatment on focal brain ischemia. *Brain Res.* 1998; 784:321–324. [PubMed: 9518671]

Fig. 1.

Glutamate clearance by ischemia-activated primary cortical astrocytes derived from young females, middle-aged females, and age-matched males. Glutamate was added to cortical astrocytes at a concentration of 250 μ M and incubated with the cells for 1 hour. Middle-aged female cortical astrocytes were significantly impaired in their ability to clear glutamate as compared with young female cortical astrocytes (left panel). In male cortical astrocytes (right panel), no significant difference was observed between cortical astrocytes from young males as compared with older males. A total of 3–4 replicates were included in each analysis. Data were normalized to young astrocytes in each replicate. * $p < 0.05$. Gray bar: young animals (6–8 months); blue bar: middle-aged animals (10–12 months).

Fig. 2.

Expression of the glutamate transporter (GLT)-1/excitatory amino acid transporter 2 (EAAT2) in astrocyte cell lysates derived from young and middle-aged female astrocytes. (a) GLT-1/EAAT2 expression in primary cortical astrocytes treated with 250 μ M glutamate for 1 hour. GLT-1/EAAT2 expression was upregulated after exposure to glutamate, in both young and middle-aged astrocytes. (b) Immunoblot of GLT-1/EAAT2 expression: immunoblots confirmed the expression of GLT-1/EAAT2 in young and middle-aged astrocytes. Immunosignal for GLT-1/EAAT2, normalized to α -tubulin, showed no change with age. * p 0.05. Gray bar: young animals (6–8 months); blue bar: middle-aged animals (10–12 months).

Fig. 3.

Effect of astrocyte-conditioned media (ACM) from young and middle-aged females and age-matched males on migration of neural progenitor cells (NPCs). NPC migration was significantly increased when cocultured with ACM from middle-aged females (a). No significant differences were observed between ACM from young and middle-aged males (b). Bars represent mean $\pm$ standard error of the mean (SEM). A total of 3–4 replicates were included in each analysis. Data were normalized to young ACM in each replicate. * p 0.05. Gray bar: young animals (6–8 months); blue bar: middle-aged animals (10–12 months).

Fig. 4.

Neuronal differentiation of neural progenitor cells (NPCs) cocultured with ischemia-activated astrocytes from young and middle-aged females. Neuronal differentiation was assessed by microtubule-associated protein (MAP)-2 positive labeling (a) and approximately 40%–50% of the NPCs were MAP-2 positive. No significant differences were observed in either the number of MAP-2 positive cells (b) or in the number of processes on MAP 2 positive cells (c) when NPC were cocultured with young or middle-aged astrocytes. Bars represent mean $\pm$ standard error of the mean (SEM). A total of 3–4 replicates were included in each analysis. Gray bar: young animals (6–8 months); blue bar: middle-aged animals (10–12 months).

Fig. 5.

Recruitment of new neurons to the peri-ischemic site in young and middle-aged female rats. Young and middle-aged females were subject to endothelin (ET)-1-induced middle-cerebral artery (MCA) occlusion and later received intraperitoneal injections (IP) of BrdU 2 times daily for 7 days. Brain sections collected at 14 days post ischemia were double-labeled for BrdU (green) and doublecortin (DCX; red). BrdU-labeled cells were seen in the cortex and striatum of the ischemic hemisphere (a and b), and a small proportion of these cells were also DCX-positive (indicated by white arrows). The number of single and double-labeled cells were counted and normalized to age-matched, sham-stroked females. No significant differences in BrdU-positive, DCX-positive, or BrdU/DCX-positive cells were observed in the ischemic cortex or striatum (c) of young or middle-aged animals after stroke. Bars represent mean $\pm$ standard error of the mean (SEM). $n = 5-6$. Gray bar: young, 6-8 mos.; blue bar: middle-aged, 10-12 mos.

Fig. 6.

Effect of astrocyte-conditioned media (ACM) on migration of peripheral blood monocyte cells (PBMCs). PBMC migration was significantly increased in cocultures exposed to ACM from middle-aged females (a). However, in males an age-related increase in PBMC migration was only observed when the PBMC donor was male not female (b). Bars represent mean $\pm$ standard error of the mean (SEM). A total of 3–4 replicates were included in each analysis. Data were normalized to young astrocytes in each replicate. * $p < 0.05$. Gray bar: young, 6–8 months, using young female-derived PBMCs; blue bar: middle-aged, 10–12 months using young female-derived PBMCs; yellow bar: young, 6–8 months using young male-derived PBMCs; green bar: middle-aged, 10–12 Months using young male-derived PBMCs.

Fig. 7.

Interleukin-6 (IL-6) and macrophage inflammatory protein-1 (MIP-1) protein expression in astrocyte media. Secreted IL-6 was significantly higher in astrocyte media from young females as compared with middle-aged females, while the inverse was observed for MIP-1. MIP-1 was significantly increased in middle-aged female astrocytes as compared with young astrocytes. In male astrocyte cultures, no significant differences were observed for either IL-6 or MIP-1. Bars represent mean $\pm$ standard error of the mean SEM. A total of 3–4 replicates were included in each analysis. Data were normalized to young astrocyte-conditioned media (ACM) in each replicate. * $p < 0.05$. Gray bar: young, 6–8 months; blue bar: middle-aged, 10–12 months.