Live Imaging of Neuroinflammation Reveals Sex and Estrogen Effects on Astrocyte Response to Ischemic Injury

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Background and Purpose—We sought to develop a model system for live analysis of brain inflammatory response in ischemic injury.

Methods—Using a reporter mouse-expressing luciferase gene under transcriptional control of the murine glial fibrillary acidic protein (GFAP) promoter (GFAP-luc mice) and biophotonic/bioluminescent imaging as tools, we developed a model system for in vivo analysis of astrocyte activation/response in cerebral ischemia.

Results—Analysis of photon emissions from the brains of living animals revealed marked sex differences in astrocyte response to ischemic injury. The increase in GFAP signals was significantly higher in female mice in the metestrus/diestrus period compared with male transgenic mice ($1.71 \times 10^7 \pm 0.19 \times 10^7$ vs $0.92 \times 10^7 \pm 0.15 \times 10^7$, $P < 0.001$). Similar results were obtained by quantitative immunohistochemistry (males vs females: 13.4 ± 0.5 vs 16.96 ± 0.64, $P < 0.0001$). However, astrocyte activation/GFAP signals showed cyclic, estrus-dependent variations in response to ischemic injury. Physiologically higher levels of estrogen and application of pharmacologic doses of estrogen during replacement therapy attenuated GFAP upregulation after stroke. Interestingly, contrary to a positive correlation between the intensities of GFAP signals and infarct size in male mice, no such correlation was observed in any of the experimental groups of female GFAP-luc mice.

Conclusions—Our results suggest that GFAP upregulation in ischemic injury may have different functional significance in female and male experimental animals and may not directly reflect the extent of ischemia-induced neuronal damage in female GFAP-luc mice. Using a novel live imaging approach, we demonstrated that the early-phase brain inflammatory response to ischemia may be associated with sex-specific biomarkers of brain damage. (***Stroke***. 2008;39: 935-942.)

Key Words: estrogen ■ glial fibrillary acidic protein ■ focal ischemia ■ gliosis ■ inflammation ■ biophotonic/bioluminescent imaging ■ transgenic mice

Reactive astrogliosis is 1 of the key components of the cellular response to brain injury, and the passage from quiescent to reactive astrocytes is associated with strong upregulation of the intermediate-filament, glial fibrillary acidic protein (GFAP).[1–3] An increase in GFAP expression has been found to be a hallmark of many neurodegenerative conditions, such as Parkinson disease, Alzheimer disease,[4,5] and amyotrophic lateral sclerosis.[6,7] In cerebral ischemia, GFAP has been widely used as an alternative marker of neuronal damage.[8–10]

Recent studies of the GFAP-knockout mouse have suggested that the role of this intermediate-filament protein in brain injury may be more complex than previously thought.[11] Contrary to expectations, an absence of GFAP protein has been associated with increased susceptibility to ischemic brain damage after middle cerebral artery occlusion (MCAO)[12] and marked alterations in posttraumatic glial scarring and tissue healing.[13] In cerebral ischemia, intensively stained GFAP-positive astrocytes accumulate in large numbers in the areas around the ischemic lesion. The functional significance of such an astrocytic response surrounding focal cerebral infarcts is still unclear.[14,15] To further investigate the role of astrocyte response to ischemia, we took the advantage of GFAP-luc transgenic mice.[16] In this mouse model, the luciferase gene (firefly luciferase, luc) is driven by the GFAP promoter, and luciferase expression (bioluminescence photon emission) can be followed longitudinally in live animals by biophotonic imaging and a high-resolution CCD camera.[16,17]

We report herein a marked sex difference in the astrocyte response to ischemic injury. Furthermore, our results suggest that the functional significance of postischemic GFAP upregulation may differ in female versus male mice.

Materials and Methods

Transgenic Mice

The transgenic GFAP-luc mice (FVB/N background and heterozygous for the *luc* gene) were obtained from Xenogen (Caliper Life Sciences, Hopkinton, Mass). In this mouse model, the luciferase reporter (firefly luciferase) is driven under transcriptional control of the 12-kb fragment of the murine GFAP promoter.[16] The GFAP-luc transgenic mice were genotyped by polymerase chain reaction with the HotStar Taq Master Mix kit (Quiagen, Mississauga, Canada) in 15 mmol/L $MgCl_2$ polymerase chain reaction buffer with the following primers: 5′GAAATGTCCGTTCGGTTGGCAGAAGC and 5′ CCAAAACCGTGATGGAATGGAACAACA. The polymerase chain reaction conditions were as follows: 95°C for 15 minutes and 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute, and 72°C for 7 minutes. All experimental procedures were approved by the Laval University Animal Care Ethics Committee and are in accordance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care.

Surgical Procedures

As previously described,[18,19] transient focal cerebral ischemia was induced by unilateral left MCAO. The surgery was performed on 2- to 3-month-old male and female GFAP-luc mice and their wild-type (FVB/N) littermates. Unilateral transient focal cerebral ischemia was induced by intraluminal filament occlusion of the left MCA with a 6-0 silicone-coated monofilament suture for 1 hour followed by reperfusion times of 24 and 72 hours, 5 and 7 days after surgery. To avoid cooling, body temperature was maintained at 37°C with a heating pad. All animals were allowed free access to water and food before and after surgery.

Ovariectomy (OVX) was performed on anesthetized female mice at 10 weeks of age by bilateral removal of the ovaries. MCAO was induced 14 and 40 days after OVX. To investigate the effects of estrogen replacement therapy, as previously described,[20] 1 group of OVX mice was anesthetized and subcutaneously implanted with a silicone elastomer implant of cholesterol/17β-estradiol, 100:1. The other group was sham operated. Seven days after surgery, the mice were subjected to MCAO and imaged at different time points after experimental ischemia.

Vaginal Smears and Hormone Detection

Vaginal epithelial cells were obtained from female mice and smeared onto precleaned slides. Slides were then evaluated for the presence of white blood cells and the morphology of epithelial cells to determine the stage of the estrus cycle. Estradiol (pg/mL) levels were determined from frozen serum samples and run in duplicate on a gas chromatograph/mass spectrometer. Estradiol levels were nondetectable in OVX mice (vs 119.03 pg/mL in OVX mice on estrogen therapy. (The lower limit of quantification for estradiol is 2 to 3 pg/mL.)

In Vivo Bioluminescence Imaging

The images were gathered with an IVIS 200 imaging system (Xenogen, Alameda, Calif). Before the imaging session, the mice received an injection of D-luciferin (150 mg/kg IP, Xenogen), a luciferase substrate, dissolved in 0.9% saline. The mice were then anesthetized with 2% isoflurane in 100% O_2 at a flow rate of 2 L/min and placed in a heated, light-tight imaging chamber. Images were collected with a high-sensitivity CCD camera with wavelengths ranging from 300 to 600 nm. Exposure time for imaging was 1 to 2 minutes with different fields of views and an f/1 lens aperture. As previously described,[17,19] bioluminescence emission was normalized and displayed in physical units of surface radiance (photons $\cdot$ s^{-1} $\cdot$ cm^{-2} $\cdot$ steradian^{-1} [sr]). Light output was quantified by determining the total number of photons emitted per second with the use of Living Image 2.5 acquisition and imaging software (Xenogen). Region-of-interest measurements on the images were used to convert surface radiance (photons $\cdot$ s^{-1} $\cdot$ cm^{-2} $\cdot$ sr^{-1}) to source flux or the total flux of photons expressed in photons/second. The

data are represented as pseudocolor images indicating light intensity (red and yellow, most intense), which were superimposed over gray-scale reference photographs. For acquisition of 3D images, we acquired gray-scale photographs and structured light images, followed by a series of bioluminescent images at different wavelengths (560 to 660 nm). Three-dimensional images were created with diffuse luminescent imaging tomography algorithms to reconstruct the position, geometry, and strength of the internal light sources. The modifiable parameters were analysis across wavelengths, source spectrum, and tissue properties (Living Image 3D Analysis Software, Xenogen).

Infarct Size

At day 8, mice were humanely killed by an overdose of anesthetic and transcardially perfused with phosphate buffer solution followed by 4% paraformaldehyde at pH 7.4. The brains were then cut into 35-μm-thick slices and stained with cresyl violet histologic stain.[21] Infarct volume (mm^3) was calculated for each section of brain and quantified by using the Neurolucida program (MBF Bioscience, Williston, Vt). Approximately 150 sections were analyzed per brain per mouse. Nine to 15 mice were used for each experimental group.

Immunocytochemistry and Tissue Collection

The animals were anesthetized and transcardially perfused with phosphate buffer solution followed by 4% paraformaldehyde at pH 7.4. Tissue sample were postfixed overnight in 4% paraformaldehyde and then cryopreserved in phosphate-buffered 30% sucrose. As previously described,[19] immunofluorescence was performed as follows. Thirty-five-micron brain sections were blocked in Tris-buffered saline containing 5% goat serum and 0.20% Triton X-100 for 30 minutes. The sections were then incubated overnight at room temperature in a primary mouse monoclonal anti-GFAP antibody (Sigma, Oakville, Canada), followed by incubation with fluorescent goat Alexa Fluor 488 secondary antiserum (Invitrogen, Eugene, Ore). GFAP immunoreactivity was quantified with the Metamorph Imaging System by measuring the intensity of fluorescence per unit of surface area (arbitrary units). Ten sections per mouse were used for this analysis. For Figure 3, the data were averaged and analyzed by a Mann–Whitney test.

Statistical Analysis

All data are presented as mean±SEM. Statistical analysis was performed by 1-way ANOVA followed by a post hoc comparison test (Bonferroni test). $P \leq 0.05$ was considered statistically significant.

Results

Real-Time Imaging Reveals Estrogen-Dependent Modulation of GFAP Signals in Female Mice

Astrocyte response to ischemic injury in live animals was studied in GFAP-luc mice. Previous work by Zhu and colleagues[16] demonstrated that an increase in GFAP signal is correlated with astrogliosis in GFAP-luc mice. As shown in Figure 1, the animals were imaged 24 and 72 hours and the 5 and 7 days after surgery. A robust signal arising from the ischemic part of the brain was detected in all animals subjected to MCAO. The intensity of the signal peaked at 24 and 72 hours and showed a decline at days 5 and 7 (Figure 1, A–L). Our preliminary analysis revealed that photon emission from the ischemic part of the brain was not detectable on day 0, 6 to 8 hours after surgery (data not shown). To our surprise, statistical analysis revealed that the signal intensities of total photon emission per second of the GFAP signals in the first 24 to 72 hours were significantly higher in female mice when they were out of estrus compared with male, age-matched littermates (24 hours after MCAO: metestrus/diestrus [M/D]
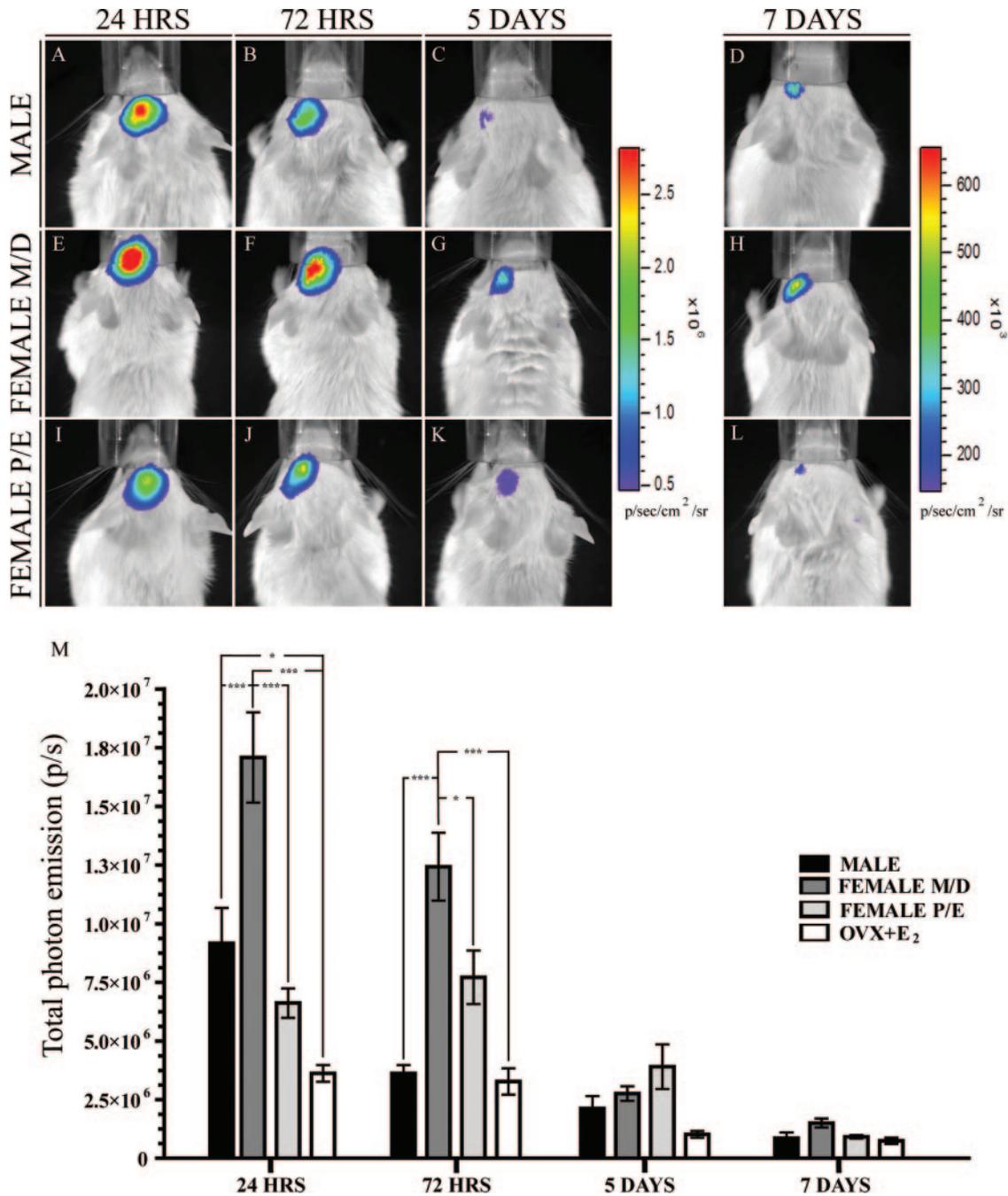
Figure 1. Real-time visualization of bioluminescent GFAP signals after cerebral ischemia. Representative images of male (A–D), female in M/D (E–H), and female in P/E (I–L) GFAP-luc mice imaged at 24 and 72 hours for 5 and 7 days after 60 minutes of MCAO. The images were longitudinally recorded from the same experimental animal and reveal the dynamics of astrocyte activation throughout the 7 days. Scales on the right are the color maps for photon counts. M, Quantification of luciferase signals by LivingImage software (Xenogen) revealed a higher GFAP signal induction in female M/D mice.

females vs males, $1.708 \times 10^7 \pm 0.192 \times 10^7$ vs $0.9170 \times 10^7 \pm 0.151 \times 10^7$; $P < 0.001$, n=7 to 9; 72 hours after MCAO: M/D females vs males, $1.243 \times 10^7 \pm 0.145 \times 10^7$ vs $0.362 \times 10^7 \pm 0.0357 \times 10^7$; $P < 0.001$, n=7 to 9; Figure 1M). To investigate whether cyclic changes in estrogen levels have an effect on astrocyte response to injury, we monitored vaginal smears daily to determine the stage of the estrus cycle. In addition, to further confirm the effects of higher levels of estrogen on astrocyte activation, we investigated GFAP

signal induction after stroke in mice treated with pharmacologic doses of estrogen (replacement therapy). Compared with female mice in M/D, the female mice in estrus and the OVX mice on estrogen replacement therapy (pharmacologic doses) showed a significant decrease in GFAP signal induction after stroke. At 24 hours after MCAO, the values were OVX+estrogen, $0.362 \times 10^7 \pm 0.036 \times 10^7$; at 72 hours after MCAO, the corresponding value was $0.327 \times 10^7 \pm 0.562 \times 10^7$ (n=7; Figure 1M).
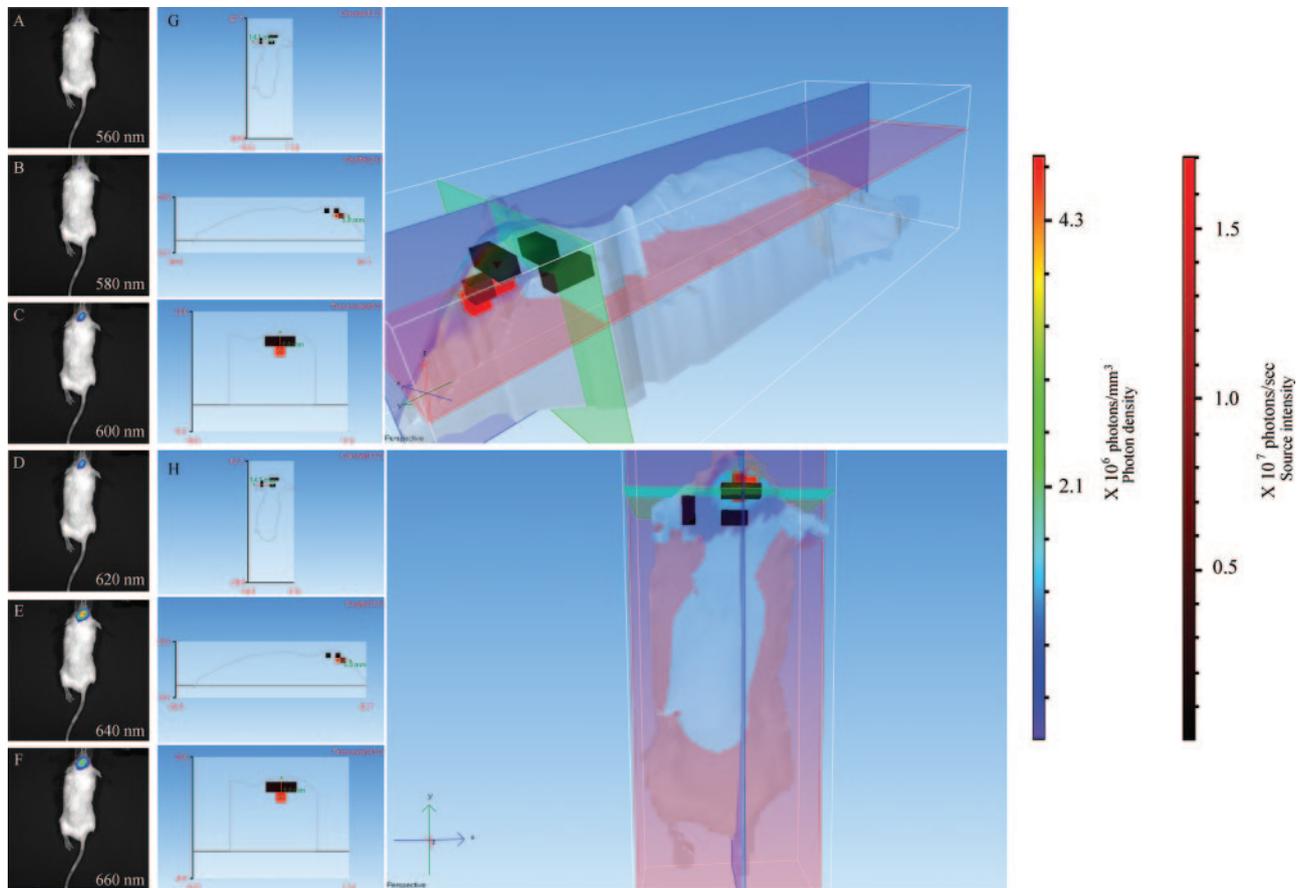
Figure 2. Three-dimensional reconstruction of bioluminescent signals emitted from the brains of poststroke GFAP-luc mice 72 hours after MCAO. A–F, Representative images show sample collection at 6 different wavelengths across the emission spectrum of the bioluminescent source, with a substantial fraction of the light at 600 nm (D and E). With the use of diffuse luminescent imaging tomography algorithms and structural images (Living Image 3D software, Xenogen), the data were transformed to 3D images (G and H). Red and brown rectangles (concentrated around the predicted ischemic lesion) represent areas of the brain with the highest intensity of photon emission. Localization of the highest-intensity area was measured and presented along 3 axes (*x*, *y*, and *z*) in millimeters from the skull surface (small panels on the left, G and H). Scales on the right are the color maps for photon density and source intensity.

Spatiotemporal Distribution of Bioluminescent Signals in GFAP-luc Mice Is Correlated With GFAP Immunoreactivity

To determine whether photons detected by the CCD camera after cerebral ischemia were being emitted from the appropriate brain regions, we performed a 3D reconstruction of the recorded signals. In transient MCAO, 2 to 3 days after reperfusion, intensively stained GFAP-positive astrocytes are situated in the areas surrounding the ischemic lesion.[14,18] A 3D reconstruction of the imaging samples (by diffuse luminescent imaging tomography) at 6 wavelengths (560 to 660 nm) across the emission spectrum of the source (Figure 2, A–F), revealed that the areas of highest photon-emission density (red and brown rectangles) were indeed situated in the areas surrounding the site of the ischemic lesion (Figure 2, G and H). Because our imaging data revealed a significant difference in GFAP signals 24 to 72 hours after stroke (7 days of longitudinal imaging), we investigated whether our in vivo imaging results could be confirmed by immunocytochemistry. Consistent with the bioluminescent signal data, 72 hours after transient MCAO ischemic/reperfusion, injury was characterized by a robust increase in GFAP immunoreactivity in the areas at the periphery of the ischemic lesion (Figure 3,

A–C). Further analysis of high-magnification photomicrographs revealed that GFAP-labeled astrocytes in female mice displayed a more ramified morphology than did GFAP-positive astrocytes surrounding the ischemic lesion in male mice (Figure 3, B–D) suggesting a higher activation state of the astrocytes.[22] Quantitative analysis of immunofluorescent labeling in the brain sections of poststroke mice revealed a significant increase in GFAP immunoreactivity in the female compared with male mice (male, 13.40 ± 0.55, n=7; female, 16.96 ± 0.64, n=9; $P<0.0001$, Figure 3E).

Chronic Lack of Estrogen Increases Astrocyte Response to Ischemic Injury in Female Mice

Because physiologically and pharmacologically higher doses of estrogen attenuated GFAP upregulation, we hypothesized that a lack of estrogen in females should then be associated with a higher GFAP signal induction in response to cerebral ischemia. Therefore, the same experimental protocol was performed in OVX mice. To our surprise, the signal intensities after brain ischemia in OVX GFAP-luc mice when MCAO was performed 14 days after OVX were significantly smaller compared with intact female littermates out of estrus (Figure 4). However, when MCAO was performed 40 days
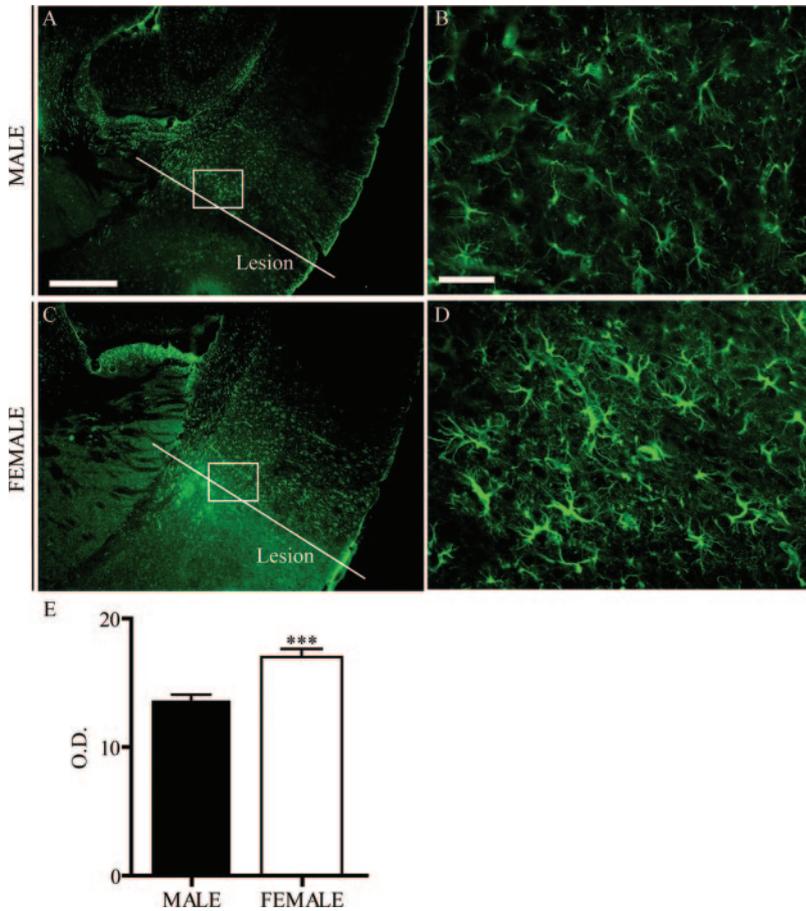
Figure 3. Representative photomicrographs of male and female GFAP-stained brain sections 72 hours after MCAO. Immunofluorescence labeling showed higher GFAP immunoreactivity in areas at the periphery of the ischemic lesion (A and C). Higher-magnification photomicrographs revealed a more ramified morphology of activated astrocytes in female compared with male mice (B and D). Densitometry quantification revealed significantly higher levels of GFAP expression in the brain sections of female mice (males, 13.40±0.55, n=7; females, 16.96±0.64, n=9; $P<0.0001$). Scale bars=500 μm for A and C, 50 μm for B and D.

after OVX, we observed a significant increase in astrocyte response (GFAP upregulation) to ischemic injury, suggesting that chronic estrogen deprivation may change astrocyte activation and inflammatory response in the brain (Figure 4).

GFAP Upregulation in Cerebral Ischemia Is Differentially Correlated With Neuronal Damage in Male and Female Mice

Previous reports revealed a strong correlation between the increase in GFAP levels and neuronal damage in cerebral
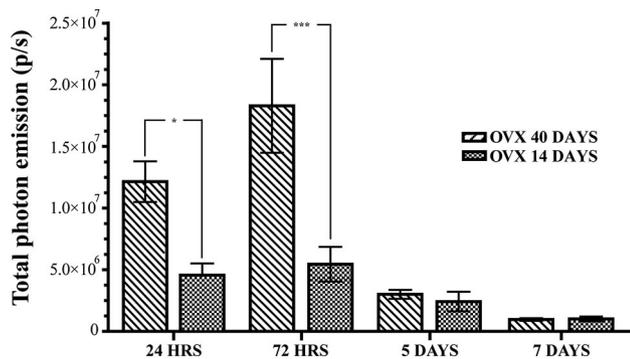

Figure 4. Histograms represent total photon emission (quantification of GFAP signals) measured in GFAP-luc mice after 60 minutes of MCAO and at40 (OVX 40 days) and 14 days (OVX 14 days) of estrogens deprivation. A higher GFAP induction signal was observed in the experimental group, OVX 40 days, at 24 and 72 hours (24 hours, OVX 40 days vs OVX 14 days: $1.214\times10^7\pm0.165\times10^7$ vs $0.457\times10^7\pm0.095\times10^7$; 72 hours: $1.83\times10^7\pm0.38\times10^7$ vs $0.545\times10^7\pm0.014\times10^7$, n=5).

ischemia.[23] Thus, one can potentially predict the severity of the ischemic lesion on the basis of the intensity of detected GFAP signals. Because in our experiments we observed a different astrocyte response in female and male mice, we wanted to investigate whether GFAP upregulation would be a good biomarker and predictor of ischemic injury in both sexes. As shown in Figure 5A, quantitative analysis of cresyl violet–stained brain sections (at the end of the imaging protocols) revealed that the size of the ischemic lesion was in general significantly higher in male compared with female mice (males, 12.11±1.361, n=7; M/D females, 8.366±1.522, n=5; proestrus/estrus [P/E)] females, 5.295±0.900, n=6; OVX at 14 days, 10.25±1.706, n=7; OVX+estrogen, 4.598±0.970, n=5; males versus P/E females, $P=0.0004$; males vs OVX+estrogen, $P=0.0003$). In addition, female GFAP-luc mice in estrus and OVX mice on high-dose estrogen therapy had significantly smaller lesions compared with other experimental groups. Furthermore, we investigated the correlation between the levels of photon emission/GFAP signal induction and infarct size. In all experimental protocols, the same animal was imaged for 7 days, and stroke area was measured at the end of the experimental protocol on day 8. As demonstrated in Figure 5B, bioluminescence signal intensities in GFAP-luc male mice showed a positive correlation with the size of the ischemic lesion ($r^2=0.5427$; $P=0.0063$). To our surprise, contrary to the findings in male mice, no correlation between bioluminescent signal intensity/GFAP induction and infarct
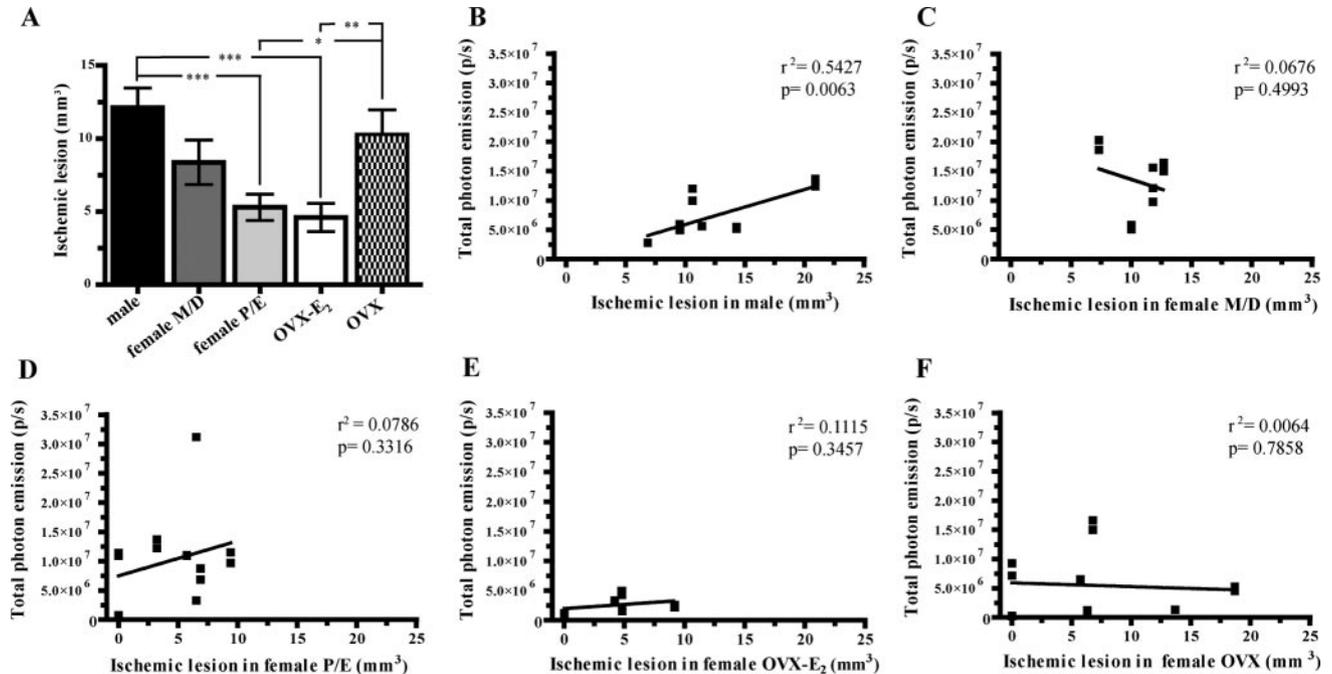
Figure 5. The histogram represents the size (volume) of the ischemic lesion in (mm³) measured 8 days after 60 minutes of MCAO (A). Ischemic lesions were significantly smaller in P/E, OVX, and OVX+estradiol mice compared with males. B, Correlation analysis between lesion size and GFAP intensity signals revealed a positive correlation in male GFAP-luc mice, whereas no such correlation was observed in female M/D (C), P/E (D), OVX (E), and OVX+estradiol (F) GFAP-luc mice (D). Note that at day 8, after 60 minutes of transient MCAO, the ischemic lesions were generally small, because spontaneous recovery processes had already been initiated.

size was observed in any of the experimental groups of female GFAP-luc mice (Figure 5, C–F). Interestingly, although the intensities of photon emission/GFAP signal induction differed within the different groups of female GFAP-luc mice, depending on the levels of estrogen, GFAP signal induction/astrocyte activation was not a good predictor of ischemic injury in any of the experimental groups of female mice.

Discussion

We report here a novel live imaging approach to study astrocyte response to ischemic injury in the brains of living mice. Our results revealed marked effects of sex and estrogen on astrocyte response to ischemic injury. We report here that: (1) bioluminescent signal intensities/GFAP induction were significantly higher in female mice (out of estrus) compared with males (confirmed by immunohistochemistry); (2) in female mice, astrocyte response to ischemia/GFAP upregulation was strongly dependent on the estrus cycle and serum estrogen level; and (3) contrary to the findings in male mice, there was no correlation between bioluminescent signal intensity/GFAP upregulation and size of the ischemic lesion in female GFAP-luc mice.

GFAP is a 50-kDa intermediate filament, predominantly expressed by mature astrocytes in the central nervous system.[24,25] Reactive astrogliosis is a key component of the inflammatory cellular response to central nervous system injury, including ischemia.[2,26] It is characterized by astrocyte hypertrophy and hyperplasia and the strong transcriptional upregulation of GFAP expression.[3,26] The astrocyte activation after brain ischemia is initiated within the first few days and

may persist up to 7 days (or longer) after stroke.[27,28] This concept was confirmed by our live imaging results, suggesting that the GFAP-luc mouse represents a reliable model system for in vivo analysis of astrocyte response to ischemic injury (Figures 3 and 4).

At present, little is known about estrogen modulation of astrocyte activation/GFAP upregulation in cerebral ischemia. Analysis of in vivo GFAP signals in our experiments showed that photon emissions 24 to 72 hours after stroke were significantly higher in female compared with male GFAP-luc mice. (Similar differences in GFAP activation were confirmed by immunohistochemistry.) Importantly, this finding changed when female mice entered estrus and/or when they were administered a high pharmacologic dose of estrogen, suggesting that estrogen may affect astrocyte response (ie, GFAP upregulation) to ischemic injury. This is in agreement with a recent report from Martinez and de Lacalle,[29] who demonstrated a direct influence of gonadal hormones on the morphology and functional response of glial cells, particularly astrocytes, to brain injury.

The question that arises here is what are the possible explanations for this estrogen-dependent astrocytes response to ischemic injury? Previous studies of noninjured astrocytes demonstrated cyclic, estrus-dependent variations in GFAP expression in certain nuclei of the rat brain.[30–32] In addition, a putative estrogen-responsive element binding site has been detected in the 5′-upstream region of the human and rat GFAP promoter,[33] thus suggesting that the level of circulating gonadal hormones would predict or modulate the glial response to brain injury. The importance of gonadal hormones in the modulation of astrocyte response to injury was further

confirmed in our hormone-deprivation experiments. At the early time point after OVX, we did not observe an increase in astrocyte response after MCAO, which may in part be explained by compensatory mechanisms and/or higher P450 aromatase activity in astrocytes[34] as a source of nongonadal estrogens. In contrast, chronic deprivation of gonadal hormones was associated with a strong increase in astrocyte response to ischemia. A similar time course of astrocyte responsiveness to injury in OVX mice was observed by McAsey et al.[35]

However, the most striking result that emerged from our live imaging study was the marked sex difference in astrocyte response to cerebral ischemia. Although a sex effect is a known factor in stroke studies, previous reports have mostly focused on the direct neuroprotective effects of estrogens.[36–39] For example, physiologic levels of estrogens have been shown to confer neuroprotection against stroke-related injury in young and middle-aged rats.[37,40] Conversely, treatment with estrogen receptor antagonists exacerbated ischemic injury in female mice.[41] In addition, a recent study demonstrated that estradiol treatment after cerebral ischemia enhanced neurogenesis.[39] Our results are in line with previous evidence: namely, that ischemic lesions were generally smaller in female mice than in GFAP-luc males. Moreover, the infarcts were significantly smaller in females during estrus and in the females on estrogen replacement therapy (pharmacologic doses), thus confirming a direct neuroprotective effect of estrogen in ischemia (Figure 5A). However, contrary to the findings in male mice, where a positive correlation was observed between bioluminescent signal intensities/GFAP upregulation and infarct size, there was no correlation between GFAP upregulation/astrocyte response and infarct size in any of the experimental groups of female GFAP-luc mice, thus suggesting that GFAP upregulation/astrocyte response to ischemic injury may not have the same functional significance in male and female mice.

In conclusion, our results revealed marked sex and estrogen effects on astrocyte response to ischemic injury. In addition, the results of our study revealed that GFAP upregulation may have a different functional significance in female and male experimental animals and may not directly reflect the extent of ischemic brain damage in female GFAP-luc mice. Using a novel live imaging approach, we demonstrated that the early-phase inflammatory response in ischemia may be associated with sex-specific injury markers.

Disclosures

None.

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