DECREASED INFARCT SIZE AFTER FOCAL CEREBRAL ISCHEMIA IN MICE CHRONICALLY INFECTED WITH *TOXOPLASMA GONDII*

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Abstract—To determine whether *Toxoplasma gondii* infection could modify biological phenomena associated with brain ischemia, we investigated the effect of permanent middle cerebral artery occlusion (MCAO) on neuronal survival, inflammation and redox state in chronically infected mice. Infected animals showed a 40% to 50% decrease of infarct size compared with non-infected littermates 1, 4 and 14 days after MCAO. The resistance of infected mice may be associated with increased basal levels of anti-inflammatory cytokines and/or a marked reduction of the MCAO-related brain induction of two pro-inflammatory cytokines, tumor necrosis factor-alpha and interferon-gamma (IFNγ). In addition, potential anti-inflammatory/neuroprotective factors such as nerve growth factor, suppressor of cytokine signaling-3, superoxide dismutase activity, uncoupling protein-2 and glutathione (GSH) were upregulated in the brain of infected mice. Consistent with a role of GSH in central cytokine regulation, GSH depletion by diethyl maleate inhibited *Toxoplasma gondii* lesion resistance by increasing the proinflammatory cytokine IFNγ brain levels. Overall, these findings indicate that chronic toxoplasmosis decisively influences both the inflammatory molecular events and outcome of cerebral ischemia.

Key words: toxoplasmosis, cerebral ischemia, cytokines, redox factors, rodent.

One of the possible biological consequences of infection is a change of the redox status in both CNS and peripheral tissues (Arsenijevic et al., 2001). Although both animal models and clinical studies showed that infections promote neuronal death following cerebral ischemic injury (Sacco, 2001; Emsley and Tyrrell, 2002), activation of the immune system can also result in neuroprotection (Bordet et al., 2000). In fact, the effect of infection on ischemic damage may largely depend on the regulation of reactive oxygen species by pro-inflammatory and anti-inflammatory cytokines as well as by the main antioxidant state regulators, namely superoxide dismutase (SOD) (Guegan et al., 1998; Murakami et al., 1998), glutathione (GSH) (Nicholls and Budd, 2000; Schulz et al., 2000; Droge, 2002), uncoupling protein-2 (UCP2) (Arsenijevic et al., 2000b; Mattiasson et al., 2003) and nerve growth factor (NGF) (Brodie, 1996; Guegan et al., 1999; Villoslada et al., 2000). In addition, the newly described suppressor of cytokine signaling (SOCS) proteins are induced in peripheral and central models of inflammation (Lebel et al., 2000; Bates et al., 2001; Larsen and Ropke, 2002; Wang and Campbell, 2002; Huang et al., 2003; Park et al., 2003; Jo et al., 2005). SOCS possibly interact with cellular redox determinants (Park et al., 2003) and transgenic SOCS expression has been shown to inhibit inflammation and apoptosis following lipopolysaccharide (LPS) injection (Jo et al., 2005).

Chronic murine toxoplasmosis may be of particular interest in the study of infection-related effects on brain ischemia since it is associated with a tissue-specific regulation of the oxidative state (Arsenijevic et al., 2001) as well as activation of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ) and interleukin-2 (IL-2), but also anti-inflammatory cytokines such as interleukin-10 (IL-10) (Arsenijevic et al., 1997, 1998). Some of these cytokines are known to enhance neurodegeneration following ischemia (Arsenijevic et al., 2006). In order to determine how chronic infection influences the main biological phenomena associated with acute cerebral ischemia, the present study explores the impact of chronic *Toxoplasma gondii* infection on brain ischemic injury and inflammatory/antioxidant processes induced by permanent middle cerebral artery occlusion (MCAO).

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Abbreviations: cDNA, complementary DNA; DEM, diethyl maleate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; IFNγ, interferon-gamma; IL-2, interleukin-2; IL-10, interleukin-10; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; NGF, nerve growth factor; SOCS, suppressor of cytokine signaling; SOCS-3, suppressor of cytokine signaling-3; SOD, superoxide dismutase; TNFα, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; UCP2, uncoupling protein-2.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Veterinary Office of the Canton of Zurich Health Directorate and the Veterinary Office of Geneva in accordance with the Swiss Animal Care Guidelines. All efforts were made to minimize the number of animals used in this study and every effort was taken to reduce any suffering.

Mice and diets

Male Swiss Webster mice of 4 months of age from Charles River Laboratories (Wilmington, MA, USA) were used. Mice were chronically infected by i.p. injection of 10 cysts of *Toxoplasma gondii* (Me49 strain obtained from Dr A. Hemphill, University of Bern, Switzerland) (Arsenijevic et al., 1997). Less than 5% of mice died due to infection during weeks 2 and 3. After this time point all infected mice survived. Acute infection with *Toxoplasma gondii* results in anorexia and body weight loss (Arsenijevic et al., 1997). We followed infected mice body weight and food intake 7 days before infection and up to 28 days (chronic phase) after infection ($n=10$). In the chronic phase of infection, some of these mice may show a partial weight regain (50%) or no weight regain (50%) (Arsenijevic et al., 1997). For all experiments, we used only the latter type of mice since these animals had higher basal brain cytokine levels and were expected to maximally respond to a new inflammation (Arsenijevic et al., 1998). A group of non-infected mice ($n=18$) was chronically underfed to mimic the food intake level of infected mice from days 1–28. This group was used to determine: 1) if the reduced food intake of the infected mice may influence basal brain GSH levels ($n=6$); 2) the effect of underfeeding on brain GSH levels after MCAO ($n=6$) and 3) the effect of underfeeding on ischemic lesion size ($n=6$). All mice were individually weighed and food intake was measured daily. For the MCAO study, infected and non-infected mice with and without MCAO ($n=18$ mice per group) were monitored daily from 7 days prior and up until 3 days after operation. Daily food intake (g/mouse/day) and body weight changes after MCAO were measured, and food intake changes after MCAO were calculated as a percentage of pre-ischemia average food intake for each mouse group.

Histology of infected brains compared with non-infected controls

Histological analysis was performed in infected (28 days following infection) ($n=4$) and non-infected control brains ($n=4$) prior to MCAO. Brain slices (20 μm) were stained with hematoxylin and eosin for histological identification of *Toxoplasma gondii* cysts and infiltrating immune cells (Frenkel and Escajadillo, 1987; Arsenijevic et al., 2007a). Detection of apoptosis in the brain of chronically infected mice was made using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) labeling as previously described (de Bilbao et al., 2000). The suppressor of cytokine signaling-3 (SOCS-3) mRNA expression was determined by *in situ* hybridization in the brains of these mice ($n=6$ per group). Riboprobe preparation and *in situ* hybridization histochemistry were kindly performed by Dr S. Rivest (Laval University, Canada). The rat SOCS-3 complementary DNA (cDNA) fragment that was initially inserted in a pEF-FLAG-1 vector (provided by Dr. Doug Hilton, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) was extracted with *Xba*I and reinserted into a pCRII (Invitrogen, Carlsbad, CA, USA). The new construct was then linearized with *Xho*I. 35S-UTP was used to label the probe (for the complete *in situ* hybridization protocol conditions see reference by Lebel et al., 2000).

Induction of permanent focal cerebral ischemia and volume of the infarct

Mice were operated 28 days after infection, when their body weight and food intake had stabilized (Arsenijevic et al., 1998). We performed permanent MCAO in infected and non-infected control mice ($n=6$ for each group and post-MCAO time) as described in details elsewhere (de Bilbao et al., 2000). All mice survived and showed infarction after MCAO. One day, 4 days and 14 days later, the animals were perfused through the ascending aorta with a solution of paraformaldehyde 4% in phosphate-buffered saline

(PBS, pH 7.35). Brains were removed and processed for paraffin embedding. Sections (7 μm) of the whole infarct area were cut on slides pretreated with 3-aminopropyltriethoxy-silane (Sigma, MO, USA), counterstained with Cresyl Violet for the histological identification of the nuclear boundaries and peri-infarct areas and mounted in Eukitt. For each animal, quantification of the infarcted area was performed on the Cresyl Violet–stained sections at five representative levels throughout the rostro-caudal extent of the lesion (A 0.26, -0.22, -0.40, -0.70 and -1.2–4 mm relative to Bregma) (Franklin and Paxinos, 1997). The rostro-caudal extent of the infarct was the same in both groups of mice. The infarcted area of each section was calculated by the subtraction of healthy tissue areas of the contralateral to the ipsilateral side of the section in order to compensate for the effect of brain edema (Guegan et al., 1998) using a computer-assisted image analyzing system (Software Morphometry, Samba 2005 TITN, Alcatel). Volumes of infarct (mm^3) were calculated for each animal after integration of areas with the distance between each level (de Bilbao et al., 2000).

To evaluate whether local alterations in cerebral vascular anatomy contribute to different susceptibility to injury in infected mice, an additional series of five non-infected and five infected mice were killed on day 1 after ischemia. Cerebral vasculature was studied in non-infected and infected mice (non-operated and on day 1 after ischemia) after intracardial perfusion of a mixture of an equal proportion of gelatinous water (5%) and China ink (Sennelier, France) warmed at 40 °C (1 ml). Brains were removed and immersed for 24 h in 4% paraformaldehyde at 4 °C (Chen et al., 2005). Cerebral vasculature was observed with a Zeiss stereo zoom microscope. The absence of cerebral blood flow in the infarct area was assessed visually and by transcranial measurements of cerebral blood flow that were made using laser Doppler flowmetry (Oxford Optronix Ltd., UK) just before and after MCAO. Animals were placed under a stereotactic head frame and then a fine needle probe (MNP110XP, 0.48 mm diameter) was lowered onto the temporal bone surface 0.5–1 mm dorsal to the opening giving access to the MCA and wetted with a small amount of physiological saline.

Physiological parameters

Physiological parameters including arterial blood pressure (Kent mouse tail blood pressure system RTBP2000, Kent Scientific Corporation, Torrington, USA), plasma glucose (using Roche Glucotrend Active, Rotkreuz, Switzerland) and hematocrit were measured daily ($n=5$ for each type of mice) before MCAO and on day 1 and day 4 after injury. During surgery, mice were placed on a warm mat and rectal temperature was measured. During the operation, all mice had a body temperature of 38 °C.

Northern blot for UCP2 mRNA

Infected and non-infected mice subjected or not to ischemia (1 day post-MCAO) ($n=6$ mice per group) were anesthetized i.p. with xylazine (20 mg/kg)/ketamine (100 mg/kg) in 0.9% NaCl (100 μl/ 10 g body weight). They were intracardially perfused without delay with ice-cold isotonic saline. At the end of the perfusion the whole brains were quickly dissected out and frozen. Total RNA was prepared as described before (Arsenijevic et al., 1997). Northern blot analyses were performed using the mouse UCP2 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA labeled with ^{32}P under standard conditions. A similar amount of total RNA (20 μg) was used in every lane.

Cytokines and NGF levels in brain

We measured TNFα, IFNγ, IL-10, IL-2 and NGF in the brain of infected and non-infected mice subjected or not to ischemia (1 day post-MCAO; $n=6$ mice per group). Brains were analyzed 1 day

after ischemia since it is well established that cytokines return to basal levels after this time point (Guegan et al., 1998). They were aseptically removed, immediately placed in dry ice and then put in CHAPS solution and homogenized (Arsenijevic et al., 2000a). The supernatant was collected and frozen at -20 °C. TNFα, IFNγ, IL-10, and IL-2 were measured by using immunoassay kits from Amersham (Switzerland) (Arsenijevic et al., 2006). An immunoassay kit was also used to determine NGF (Catalys, Switzerland) (Arsenijevic et al., 2006).

SOD activity

We investigated SOD activity using a biochemical assay (1 day post-MCAO; $n=6$ mice per group) (Ewing and Janero, 1995). Brains were homogenized in phosphate-buffered saline and frozen immediately in liquid nitrogen. Aliquots of brain supernatants were processed as previously described (Ewing and Janero, 1995). Brain supernatants were added (25 μl) to a 125 μl solution containing 50 mM phosphate buffer (pH 7.4, 0.1 mM EDTA, 50 μM NBT, 78 μM NADH). To start the reaction, 25 μl of 3.3 μM PMS phenazine methosulfate (Sigma) (final concentration) was added and the absorbance at 560 nm was measured continuously using a microplate reader (DynaTech MR5000). The readings were every minute for 10 min. The first 5 min were used to determine the rate of superoxide production by the samples.

GSH levels

Tissues were collected from infected (1, 3, 7, 14 and 28 days post-infection) and non-infected control mice ($n=6$ mice per group). The consequences of MCAO for brain GSH levels were also determined in both types of mice 1 day post-ischemia ($n=6$ for each group). Tissues were homogenized in phosphate buffer (50 mM, pH 7.4). Total GSH levels were measured using a method based on the formation of a chromophoric product resulting from the reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma) and GSH (Sigma) (de Bilbao et al., 2004). The absorbance was immediately measured at 412 nm. GSH contents were calculated by using a calibration curve established with standard samples.

To test whether elevated brain GSH levels in infected mice 1) were involved in the lack of cytokine induction 1 day after MCAO and 2) may influence lesion size response after ischemia, GSH was depleted by a s.c. injection of diethyl maleate (DEM) (24 μl/ 30 g body weight and was made up to 300 μl with saline) (Pileblad and Magnusson, 1990). To determine if DEM depleted brain GSH, non-infected ($n=3$) and infected mice ($n=3$) were treated with DEM and killed 2 h later and GSH in brain was measured as described above. To determine whether GSH depletion may alter TNFα and IFNγ levels 1 day after MCAO, infected ($n=4$) and non-infected mice ($n=4$) were treated with DEM, underwent MCAO 2 h later and were killed 1 day post-ischemia. Estimates of brain cytokine levels were obtained as described above. For all experiments, mice treated with saline were used as controls for the DEM groups.

Brains of 28-day infected mice (see Histology section) were stained for GSH with the fluorescent histochemical dye phthaldialdehyde (10 mM) and then processed for microglia immunohistochemistry using the anti-Iba-1 antibody (1 mg/ml, from Dr. Y. Imai, National Center of Neurology and Psychiatry, Japan) which was detected by the fluorescent goat-anti-rabbit immunoglobulin Alexa Fluor 568 (Molecular Probes) (de Bilbao et al., 2004). Since we have previously shown that chronic toxoplasmosis results in an increased brain expression of UCP2 mRNA which is specific for microglia associated with inflammatory loci (Arsenijevic et al., 2007), we also explored whether there was an association between GSH and UCP2 in this cell type. Mounted brain sections were immunostained for GSH using rabbit polyclonal anti-GSH (1/10 dilution provided by Prof. Ottersen, University of Oslo, Nor-

Table 1. Physiological parameters before and after MCAO in non-infected and infected mice

Group and treatment	1 Day before MCAO	D1 after MCAO	D4 after MCAO
Hematocrit (%)			
Non-infected	42±4	44±5	45±5
Infected	48±4	50±4	51±4
Plasma glucose (mmol/L)			
Non-infected	8.2±0.2	7.9±0.3	8.1±5
Infected	7.7±0.3	7.6±0.2	7.8±4
Arterial pressure (mm Hg)			
Non-infected	94±8	96±8	95±5
Infected	105±12	98±9	101±6

Data are means±S.E.M. of 5 animals per group. See text for details.

way) which was revealed by ABC kit peroxidase and subsequently processed for *in situ* hybridization using a rat UCP2 probe (de Bilbao et al., 2004).

Data analyses

All data are presented as means±S.E. Statistical analyses were performed using Kruskal-Wallis non-parametric test. P values of less than 0.05 were considered significant.

RESULTS

### Physiological parameters before and after MCAO

Arterial pressure, plasma glucose and hematocrit levels before and after MCAO on day 1 and day 4 were not significantly different between non-infected and infected mice at a given time point as shown in Table 1. After MCAO, both non-infected and infected mice showed an absence of cerebral blood flow in the infarct area.

Brain histology and SOCS-3 mRNA induction in infected mice

Unlike non-infected mice, the brains of the infected mice showed many randomly located inflammatory loci (including neutrophils, macrophages/monocytes and lymphocytes) 28 days after infection. These loci were not colocalized with intact *Toxoplasma gondii* cysts (Fig. 1A, B) and also occurred around blood vessels, but not within the vessels' walls (Fig. 1C). There was no evidence of neuronal apoptosis as determined by TUNEL labeling in these mice (data not shown). *In situ* hybridization showed SOCS-3 basal expression mainly in the hypothalamic region (Fig. 1D) (Lebel et al., 2000). Following infection, SOCS-3 mRNA labeling was randomly induced throughout the cerebral cortex as well as in areas associated with brain immune cell infiltration and blood vessels (Fig. 1D). This expression was associated with neurons, microglia and infiltrating phagocytes as previously described (data not shown) (Lebel et al., 2000).

Ischemic brain injury is reduced in *Toxoplasma gondii* infected mice

One day, 4 days and 14 days after MCAO, infarct volume was decreased by 42%, 50% and 48% respectively in
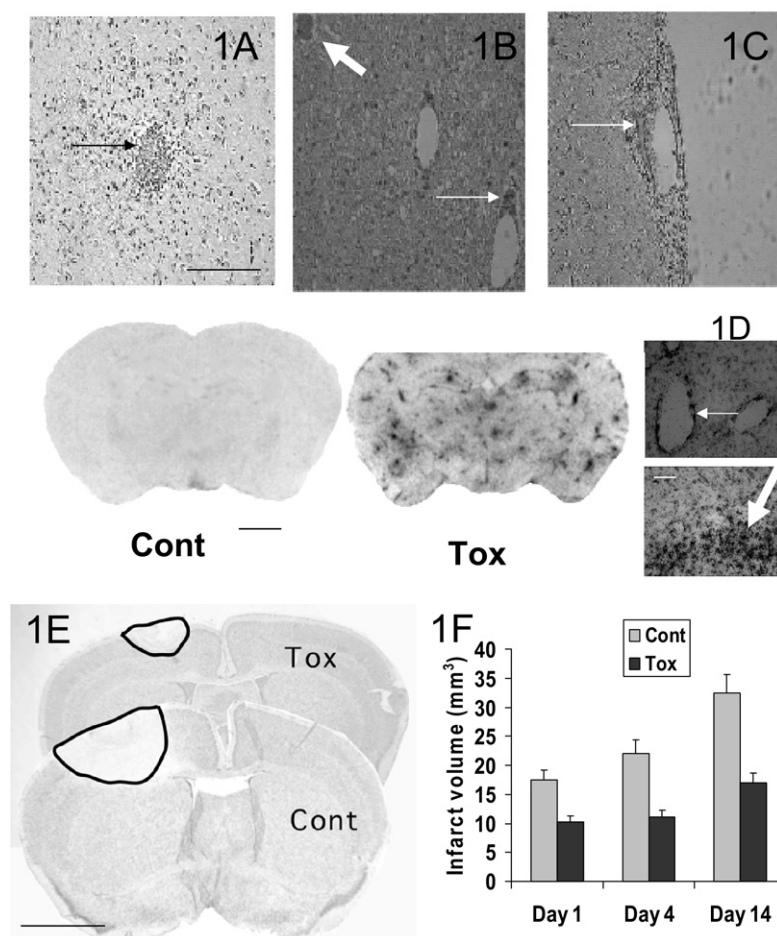
Fig. 1. Brain histology, *in situ* hybridization for SOCS-3 mRNA expression and brain infarct size in *Toxoplasma gondii* infected mice. (A–C) Coronal cross-sections of a *Toxoplasma gondii*–infected (for 28 days) mouse brain stained with hematoxylin and eosin (scale bar=15 μm). Note that infection resulted in inflammatory loci found in brain tissue (A) (thin black arrow) and in association with blood vessels (B, C) (thin black arrows). Note also that the presence of *Toxoplasma* cysts was not associated with inflammatory loci (B, thick arrow). (D) Representative *in situ* hybridization for SOCS-3 mRNA in non-infected control (Cont) and infected mice (Tox) brains (scale bar=120 μm). SOCS-3 mRNA basal expression was found in particular in hypothalamic region. Twenty-eight days following infection, SOCS-3 mRNA expression was randomly located and induced throughout the brain of infected mice in particular in the cortical area (Tox) (n=4 for each group). The right column shows a higher magnification of selected areas specific for SOCS-3 mRNA expression in infected brains. SOCS-3 mRNA was associated with microglia/infiltrated immune cells (thick arrow) and blood vessels (thin arrow). Cells are visualized with Thionin staining and SOCS-3 silver grains are black (scale bars=15 μm). (E, F) Effect of *Toxoplasma gondii* infection on infarct size. (E) Representative coronal sections showing ischemic lesion size 4 days after MCAO in control (Cont MCAO) and chronically infected mice (Tox MCAO). Sections were stained with Cresyl Violet. The surrounded areas denote the size of the ischemic area (scale bars=120 μm). (F) Infarct brain volumes were reduced in infected mice (Tox) at 1, 4 and 14 days after MCAO compared with non-infected mice (Cont).

Toxoplasma gondii infected mice when compared with non-infected control mice (P<0.01) (Fig. 1E, F), suggesting that chronic infection with *Toxoplasma gondii* may protect neocortical areas from ischemic damage.

Transient hyperphagia occurs early in *Toxoplasma gondii*–infected mice in response to MCAO

On the day of lesion, MCAO resulted in a significant reduction of food intake in both infected and non-infected control mice when compared with their pre-MCAO basal food intakes ($-26\pm0.5\%$ and $-39\pm0.7\%$ respectively, n=18, P<0.01). The day after ischemia, both non-infected and infected mice started to regain appetite. From this time point, infected mice increased food intake more rapidly

than the non-infected group ($-3\pm0.2\%$ and $-28\pm2\%$, respectively, n=18, P<0.01). Infected mice showed significantly enhanced hyperphagia on days 2 and 3 postischemia compared with their non-operated controls ($28\pm1.2\%$ and $1\pm0.2\%$ respectively, n=18, P<0.01). Body weight loss induced by ischemia followed a similar temporal pattern to that of food intake indicating that *Toxoplasma gondii*–infected mice had attenuated negative energy balance in response to MCAO.

### Brain specific alterations in GSH levels due to infection

There was a 45% decrease in brain GSH levels on day 7 postinfection when compared with non-infected control

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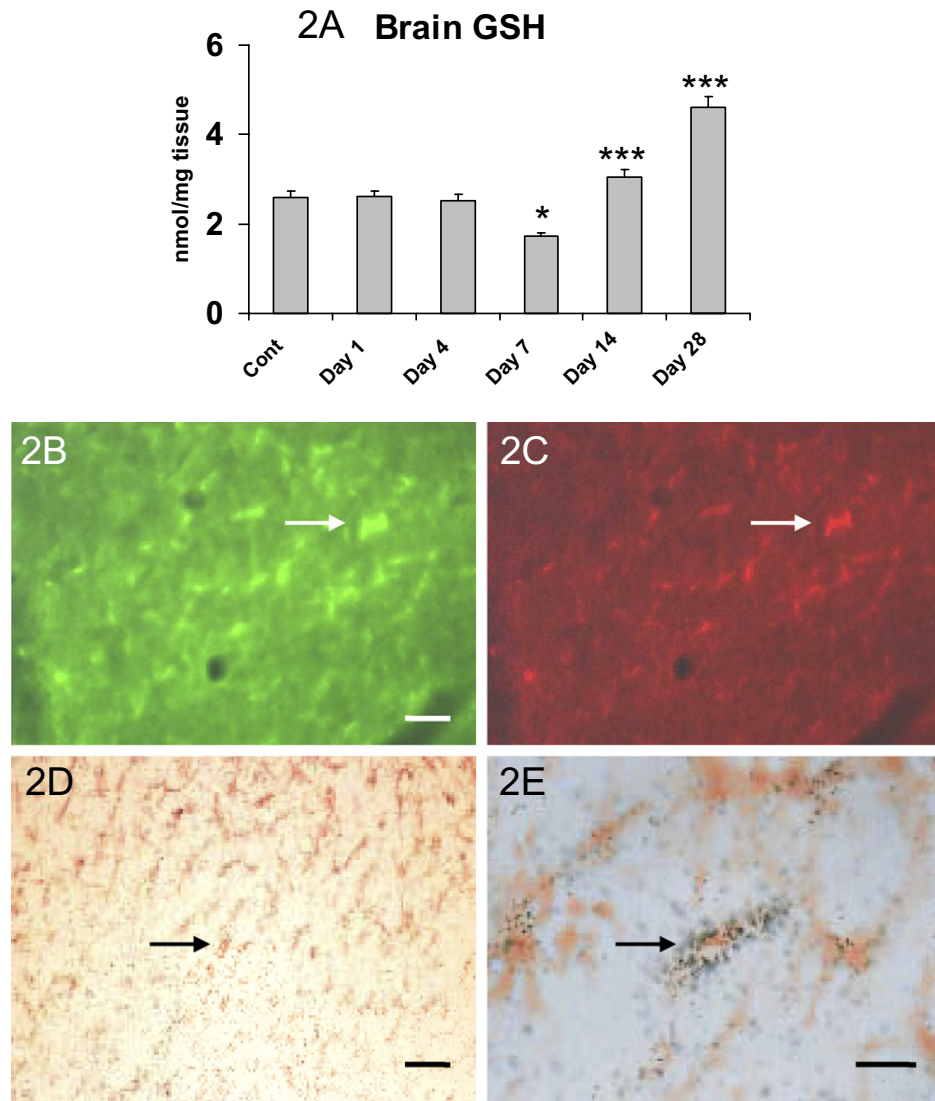
Fig. 2. Changes in brain GSH levels from day 1 to day 28 following murine *Toxoplasma gondii* infection. (A) Toxoplasmosis resulted in a slight reduction in brain GSH levels on day 7. From day 14 to day 28 after infection, GSH was significantly increased in the brain of infected mice compared with non-infected control mice (Cont). Values (nmol/mg tissue) are presented as mean±S.E. (* $P<0.05$, *** $P<0.001$, comparison to saline control). Each group represents six mice. (B) GSH (fluorescent staining, white arrow) is specifically increased in microglia in infected mice (red, white arrow) (C). Only microglia cells that have increased UCP2 mRNA (E, black arrow) show an increase in GSH (immunohistochemical detection, orange, black arrow) (D). Scale bars = 15 μm (B, C, D); 7.5 μm (E). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

mice ($P<0.05$). On day 14, as mice started to regain appetite, there was a progressive increase in brain GSH levels in infected mice ($P<0.001$) up to 28 days after infection ($P<0.001$) (Fig. 2A). The increase in GSH in the brains of 28-day infected mice was associated with a specific increase in microglia (Fig. 2B, C); only microglia with elevated UCP2 mRNA levels showed GSH labeling (Fig. 2D, E). In non-infected control mice, MCAO resulted in a 80% decrease of brain GSH levels (from 0.52 ± 0.02 nmol/mg tissue to 2.60 ± 0.18 nmol/mg tissue) ($P<0.001$) (Fig. 3A). Interestingly, this decrease due to ischemia was only 11% in infected animals (from $4.60\pm 0.80-4.11\pm0.31$ nmol/mg tissue) ($P<0.01$). In addition, GSH brain content in infected animals having undergone

MCAO was significantly higher compared with non-infected MCAO animals ($P<0.001$) (Fig. 3A). Chronically underfed non-infected mice did not show altered brain GSH levels compared with non-infected controls (2.47 ± 0.19 versus 2.60 ± 0.07 nmol/mg tissue) (Fig. 3A) suggesting that anorexia does not explain the increased brain GSH levels in infected mice. Although 1 day after ischemia, GSH levels were reduced in the chronically underfed non-infected mice, this decrease was significantly less pronounced compared with the non-infected control group ($P<0.01$) (Fig. 3A). This was associated with a reduced infact size in the chronically underfed group compared with the non-infected control group (15.0 ± 0.9 mm^3 and 19.3 ± 1.1 mm^3, respectively, $n=6$ mice, $P<0.05$). Al-

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3A **Brain GSH**

3B **Brain SOD**
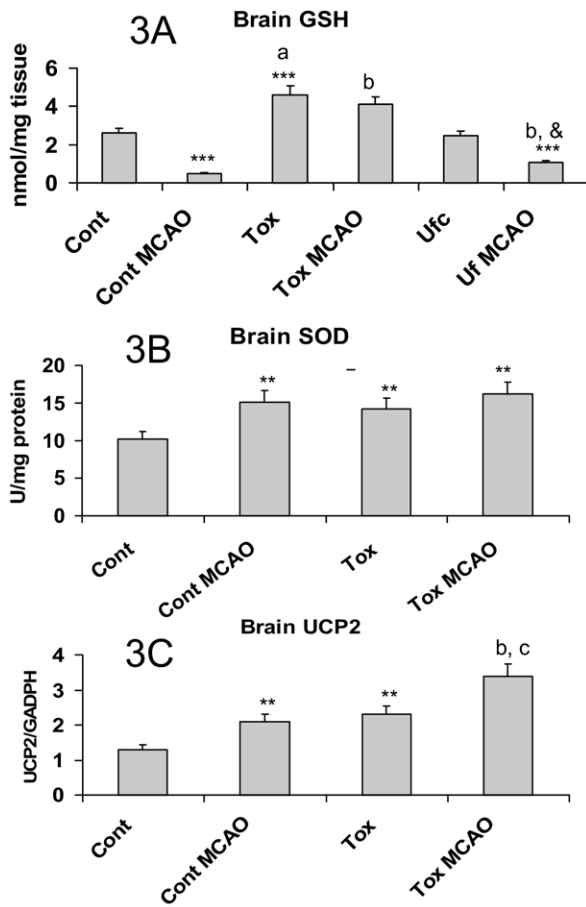
3C **Brain UCP2**

Fig. 3. Brain GSH, brain SOD activity and brain UCP2 mRNA expression. (A) One day after MCAO, we observed a reduction in brain GSH levels in non-infected mice (Cont MCAO) compared with their non-operated controls (Cont). After MCAO, brain levels of GSH in infected mice were markedly elevated compared with non-infected animals (Cont MCAO). Underfeeding (Ufc) results in no change in brain GSH levels compared with control mice (Cont). One day after MCAO, GSH level in underfed mice (Ufc MCAO) was reduced, however this reduction was less than that found in Cont MCAO mice. (B) Brain SOD activity was increased during chronic infection (Tox) when compared with non-infected animals (Cont). One day post-MCAO, SOD activities were similarly increased in both groups compared with non-infected controls (Cont). (C) Brain UCP2 mRNA expression was increased following infection (Tox) compared with non-infected controls (Cont). MCAO increased brain UCP2 mRNA levels in both infected and non-infected mice, but levels were higher in the former group. Values are presented as mean$\pm$S.E. for the various groups. Each group represents six mice. * Indicates the statistical comparison with non-infected controls (Cont), ^{a} indicates the statistical comparison between Tox and Ufc mice, ^{b} indicates the statistical comparison between Tox MCAO and Cont MCAO mice, ^{c} indicates the statistical comparison between Tox and Tox MCAO mice, & indicates comparison between Cont MCAO and Ufc MCAO (* $P<0.05$, *** $P<0.001$, ** $P<0.01$, *** $P<0.001$, ^{a} $P<0.001$, ^{b} $P<0.001$, ^{c} $P<0.05$, & $P<0.01$).

though a contributing effect cannot be excluded, the underfeeding effect on reduced lesion size could not totally explain the much greater reduction of infarct size observed in the infected mice (10.2 ± 0.4 mm^3) (Fig. 1F) ($P<0.05$).

Infection increases brain SOD activity

SOD activity (Fig. 3B) was higher in the brains of infected mice (14.2 ± 1.2 units/mg protein) compared with non-infected mice (10.2 ± 0.8 units/mg protein) ($P<0.01$). This difference did not persist 1 day after MCAO (Fig. 3B) as SOD activities were similarly increased in both groups (17.2 ± 1.5 and 15.1 ± 1.1 units/mg protein respectively).

Upregulation of brain UCP2 mRNA levels in infected mice after ischemia

Toxoplasmosis resulted in a 76% increase in brain UCP2/GAPDH mRNA ratio compared with non-infected mice (2.3 ± 0.2 versus 1.3 ± 0.1) ($P<0.01$) (Fig. 3C). One day post-MCAO, there was a 62% increase in UCP2/GADPH mRNA ratio in infected mice compared with MCAO non-infected mice (3.4 ± 0.2 versus 2.1 ± 0.2) ($P<0.001$) (Fig. 3C).

Infection does not result in enhanced cytokine levels after MCAO

Basal levels of proinflammatory cytokines (TNFα, IL-2, IFNγ) were increased in *Toxoplasma gondii* infected mice on day 28 postinfection compared with non-infected controls ($P<0.001$) (Table 2). One day post-MCAO, infected mice had higher TNFα, IL-2 and IFNγ levels compared with MCAO non-infected animals ($P<0.001$). However, these higher levels did not significantly differ from those observed in infected mice not subjected to ischemia. This sharply contrasts with the results obtained in non-infected mice which showed a marked induction of cytokine levels 1 day post-MCAO compared with non-operated animals. Infected mice also had significantly higher brain levels of IL-10 and NGF compared with non-infected control mice ($P<0.001$) (Table 2). NGF and IL-10 significantly increased in non-infected mice that underwent MCAO ($P<0.001$); this induction was not observed in infected mice suggesting an attenuated immune response in the brains of infected mice (Table 2). Following MCAO, NGF levels in infected animals were doubled compared with non-infected controls ($P<0.001$). This was not the case for IL-10 suggesting that NGF may be a more important anti-inflammatory agent in infected mice.

Table 2. Basal and one day post-MCAO levels of brain TNFα, IFNγ, IL-2, IL-10 and NGF in non-infected and infected mice

	Cont	Cont MCAO	Tox	Tox MCAO
TNFα pg/ml	100 ± 4	800 ± 12***	993 ± 51***	1093 ± 50^a
IFNγ pg/ml	25 ± 2	175 ± 4***	330 ± 8***	358 ± 9^a
IL-2 pg/ml	44 ± 2	750 ± 23***	1622 ± 64***	1804 ± 61^a
IL-10 pg/ml	24 ± 2	492 ± 38***	504 ± 40***	560 ± 19
NGF pg/ml	40 ± 3	300 ± 12***	600 ± 22***	624 ± 20^a

Values (pg/ml tissue) are presented as mean$\pm$S.E.
* Indicates statistical comparison with non-infected animals (Cont) (*** $P<0.001$). Each group included six mice. See text for details.
^{a} Indicates statistical comparison between Tox MCAO and Cont MCAO mice (^{a} $P<0.001$). Each group included six mice. See text for details.
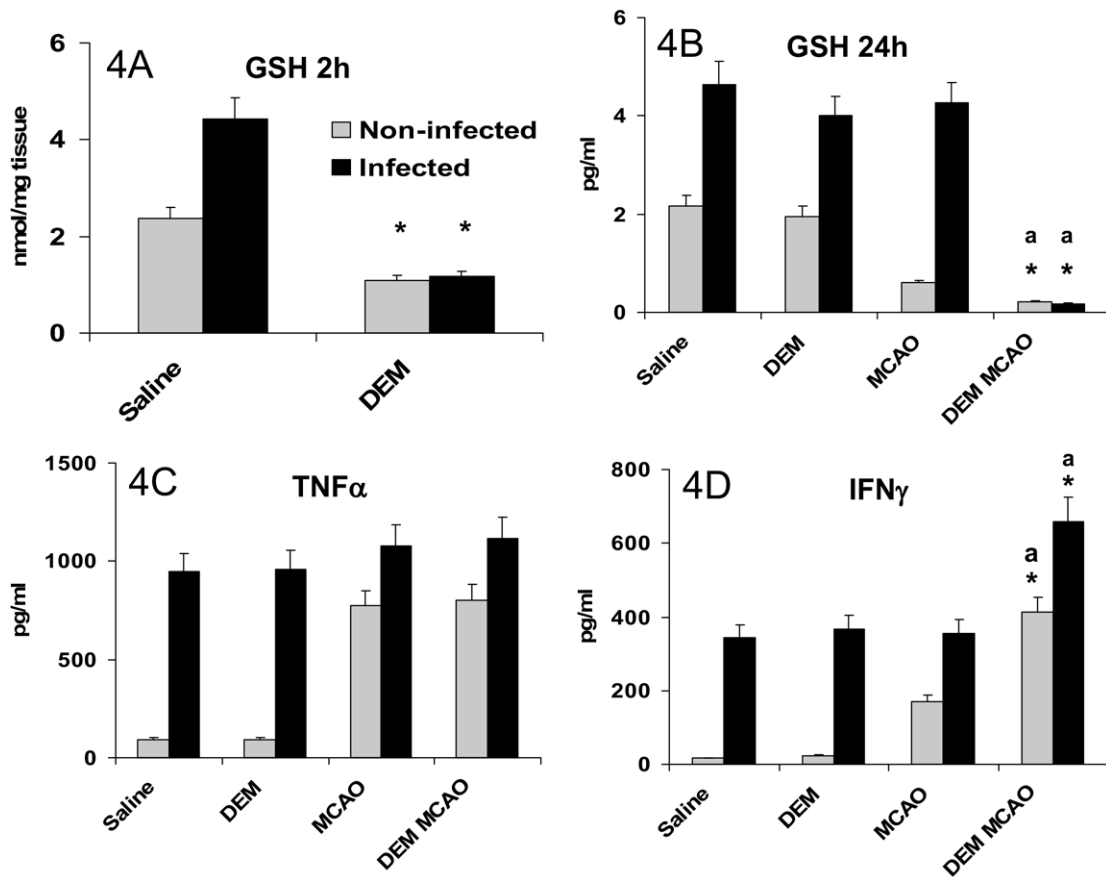
Fig. 4. GSH, TNFα and IFNγ were measured in non-infected and infected mice after ischemia; 2 h prior to MCAO, mice were treated with DEM (or saline). (A) DEM significantly reduced brain GSH in non-infected and infected mice 2 h after treatment. (B) Twenty-four hours after ischemia, DEM significantly reduced brain GSH levels in infected and non-infected mice. Note that, consistent with the known transitory effect of DEM on GSH levels (Gupta et al., 2000), GSH returned to basal levels 24 h after DEM treatment in the absence of ischemic damage in both infected and non-infected mice. (C, D) DEM did not significantly alter brain TNFα (C) but significantly increased brain IFNγ levels (D) 24 h after ischemia. Data are means$\pm$S.E. of three to five animals per group. * $P<0.001$, comparison with saline controls; [a] $P<0.001$, comparison with MCAO controls.

Depletion of brain GSH by DEM reversed neuroprotection by increasing lesion size and central IFNγ levels after MCAO in infected mice.

Brain GSH levels in both non-infected and infected mice were markedly decreased 2 h after an s.c. injection of DEM as seen in Fig. 4A. One day after ischemia (Fig. 4B), brain GSH levels were further reduced by DEM when compared with non-treated MCAO groups. This reduction was associated with an increased lesion size in infected mice when compared with infected mice not treated with DEM (22.2$\pm$1.7 mm^3 versus 12.8$\pm$0.5 mm^3, $n=6$ per group, $P<0.01$). Non-infected mice also showed an increase in lesion size when treated with DEM (15.8$\pm$1.5 mm^3 for saline versus 20.3$\pm$1.0 mm^3 for DEM, $n=6$ per group, $P<0.05$). After ischemia, DEM treatment resulted in induction of central IFNγ levels but not TNFα in both types of mice (Fig. 4C, D). Note that, consistent with the known transitory effect of DEM on GSH levels (Gupta et al., 2000), GSH returned to basal levels 24 h after DEM treatment in the absence of ischemic damage in both infected and non-infected mice (Fig. 4C, D).

DISCUSSION

This study revealed a marked resistance of *Toxoplasma gondii*–infected mice to acute cerebral ischemia characterized by a marked decrease of infarct size one, and 4 but also 14 days post-MCAO. The reverse effect of DEM on brain infarct size implies that GSH up-regulation may play a pivotal role in the observed ischemic resistance. Our results make it also possible to propose additional biological mechanisms surrounding this phenomenon, such as marked differences in pre- and/or post-ischemic cytokine status as well as pre- and/or post-ischemic GSH, SOCS-3 mRNA and UCP2 mRNA levels in infected compared with non-infected mice.

Following the acute phase of toxoplasmosis, the initially produced pro-inflammatory cytokines will subsequently induce a counter-regulatory anti-inflammatory response (Arsenijevic et al., 1997). During the chronic phase of infection, apart from the role that the adaptive specific immune systems (CD4, CD8 T cell activation) could play, the consequence of a second inflammation caused by

cerebral ischemia may be determined by the balance between these two antagonistic systems. The basal up-regulation of antioxidant molecules such as GSH, SOD, UCP2, SOCS-3 mRNA and anti-inflammatory cytokines NGF and IL-10 may represent the two first lines of defense against ischemic damage in infected mice. One could postulate that the observed changes in redox status may be a simple epiphenomenon of the underfeeding observed in the chronically infected mice. In fact, it has been shown that underfeeding may result in protection from cerebral injury by modifying redox status (Yu and Mattson, 1999). Our findings show that both the steady increase of brain GSH levels by day 7 to day 28 post-infection as well as the brain resistance to ischemia are specific to *Toxoplasma gondii* infection and did not depend on energy intake. Actually, although we did find that post-ischemic GSH was partially dependent on energy intake, this component represented only 13% of the post-ischemic GSH levels of infected mice. We also show that energy intake does influence lesion size, but alone cannot account for the degree of resistance observed in infected animals. A possible causal relationship between redox status and cytokine production has been previously suggested in that increased basal GSH levels and SOD activity may result from high NGF levels (Guegan et al., 1998, 1999; de Bilbao et al., 2004; Arsenijevic et al., 2006). Consistent with their possible role in neuroprotection (Guegan et al., 1999; Mattiasson et al., 2003), the differences in GSH, UCP2 mRNA and NGF levels persisted between infected and non-infected mice after MCAO. In the absence of a specific antibody for brain UCP2, it was not possible to assess the impact of UCP2 mRNA changes on protein levels. However, previous data using UCP2 transgenic and UCP2 KO mice have shown that UCP2 plays a central role in brain neuroprotection following ischemia (Mattiasson et al., 2003; de Bilbao et al., 2004).

An additional molecular mechanism involved in this context may involve the absence of pro-inflammatory cytokine induction observed in infected mice after ischemia. This phenomenon could be partly due to the already elevated basal levels of these cytokines in the brains of these mice (i.e. counter-regulatory mechanisms). Although one could argue that this finding might reflect the presence of a threshold in cytokine levels reached in *Toxoplasma*-infected mice, this is an unlikely scenario since we have previously demonstrated that an i.p. injection of LPS led to a marked upregulation of pro-inflammatory cytokines in these mice (Arsenijevic et al., 1998). Importantly, infected mice showed feeding behavior concordant with the relative stunting of brain cytokine response after ischemia. In fact, transient hyperphagia started earlier in infected mice compared with non-infected mice after MCAO. This is consistent with the attenuated post-ischemic induction of anorectic cytokines (TNFα, IFNγ) in the brain.

In agreement with previous contributions, our data suggest that both SOCS-3 and GSH are plausible candidate molecules for the regulation of secondary inflammatory response and cytokine production in *Toxoplasma gondii*–infected mice (Bjorkbaek et al., 1999; Auerhammer and Melmed, 2001; Park et al., 2003). The absence of pro-inflammatory cytokine induction could be partly due to the parallel elevation of the SOCS-3 mRNA after infection. The SOCS-3 is a potent inhibitor of cytokine signaling and its transgenic over-expression may inhibit inflammation and associated apoptosis *in vivo* (Auerhammer and Melmed, 2001; Jo et al., 2005). Accordingly, although infected mice had elevated basal pro-inflammatory cytokine levels (i.e. TNFα, IFNγ and IL-2) (Eizenberg et al., 1995; Bate et al., 2006; Lee et al., 2006; Yu et al., 2006), there was no evidence of neuronal apoptosis in their brain tissue. A key role for GSH is suggested by the fact that the decreased infarct size in MCAO mice chronically infected is no longer observed when brain GSH levels have been depleted by DEM treatment. This phenomenon was specifically associated with the central up-regulation of pro-inflammatory IFNγ but not TNFα in response to MCAO. Previous studies have also shown that DEM may not have a marked effect on TNF induction in response to an inflammatory response (Kang et al., 1999; Wang et al., 1999). Further work is needed to get a better understanding of the complex interactions of molecules regulating the inflammatory response in this context. As recently proposed, the observed elevated basal SOCS-3 and GSH levels could act synergistically as GSH can interact in SOCS-3 pathways to inhibit cytokines (Cisowski et al., 2002).

Besides its direct effect against oxidative stress, GSH may also act via the suppression of glia-mediated inflammation (Wang et al., 2006). In the same line with this idea, our contributions have shown that both UCP2 and SOCS-3 mRNA levels in brains infected with *Toxoplasma gondii* were elevated in microglia (Arsenijevic et al., 2007a). Although we cannot exclude a possible participation of infiltrated leukocytes, our evidence suggests that GSH, UCP2 and SOCS-3 may attenuate ischemic injury in infected mice by modifying microglia activity.

Despite a widely disseminated idea, only rare studies have shown that pre-existing infections can enhance brain pathology in response to a subsequent new inflammatory process (Arsenijevic et al., 1998). In contrast, several studies have shown that chronic infection can attenuate some peripheral and central pathological processes: complete Freund's adjuvant prevents the onset of experimental allergic encephalomyelitis (Bach, 2001), schistosomiasis can protect against asthma (Yang et al., 2007), coxsackievirus B3 can inhibit cardiomyopathy (Huber et al., 2006) and various infections can attenuate murine models of multiple sclerosis (Sewell et al., 2002). In some conditions, a pre-existing inflammation can induce brain neurodegeneration. This is the case for the acute peripheral and/or central injection of LPS (Nguyen et al., 2004; Cunningham et al., 2005; McColl et al., 2007; Qin et al., 2007; Spencer et al., 2007). However, it appears that the LPS dose used in these models may be a determinant in the observed degeneration. In fact, although high LPS doses may increase cytokines and oxidative stress, low doses of peripheral LPS have a neuroprotective effect (Bordet et al., 2000). This is consistent with our recent study in which we observed an increase in brain NGF and

GSH levels 3 days after peripheral LPS treatment (Arsenijevic et al., 2007b; Hernadfalvi et al., 2007). In this context, the present study provides a new model to explore the effect of a pre-existing chronic inflammatory status on the outcome of brain ischemic insult. Since each bacterium or parasite produces different cytokine and leukocyte activation profiles and, ultimately, patterns of neurodegeneration, it is crucial to investigate the molecular mechanisms surrounding the beneficial or deleterious effect of various models of chronic inflammation on cerebral ischemia.

CONCLUSION

This study revealed a marked resistance of *Toxoplasma gondii*–infected mice to acute cerebral ischemia that is characterized by a decreased lesion size 1 day, 4 days and 14 days post-MCAO. Marked differences in pre- and/or post-ischemic cytokines and antioxidant molecules could explain this phenomenon. In addition, our findings imply that GSH may play a pivotal role in these processes since pharmacological depletion of GSH resulted in increased lesion size and increased brain IFNγ levels. Functional neurological data could be useful to confirm further the protective effect of *Toxoplasma gondii* infection on the outcome of cerebral ischemia.

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