In vivo imaging induction of heat-shock protein-70 gene expression with fluorescence reflectance imaging and intravital confocal microscopy following brain ischemia in reporter mice

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Abstract

Purpose: Stroke induces strong expression of the 72-kDa heat-shock protein (HSP-70) in the ischemic brain, and neuronal expression of HSP-70 is associated with the ischemic penumbra. This study was aimed to image induction of Hsp-70 gene expression *in vivo* after brain ischemia using reporter mice.

Methods: A genomic DNA sequence of the *Hspa1b* promoter was used to generate an Hsp70-mPlum far-red fluorescence reporter vector. The construct was tested in cellular systems (NIH3T3 mouse fibroblast cell line) by transient transfection and examining mPlum and Hsp-70 induction under a challenge. After construct validation, mPlum transgenic mice were generated. Focal brain ischemia was induced by transient intraluminal occlusion of the middle cerebral artery and mice were imaged *in vivo* with fluorescence reflectance imaging (FRI) with intact skull, and with confocal microscopy after opening a cranial window.

Results: Cells transfected with the Hsp70-mPlum construct showed mPlum fluorescence after stimulation. One day post-ischemia, reporter mice showed a FRI signal located in the HSP-70 positive zone within the ipsilateral hemisphere, as validated by immunohistochemistry. Live confocal microscopy allowed visualizing brain tissue at the cellular level. mPlum fluorescence was observed *in vivo* in the ipsilateral cortex 1 day after ischemia in neurons, where it is compatible with penumbra and neuronal viability, and in blood vessels in the core of infarction.

Conclusions: This study shows the *in vivo* induction of Hsp-70 gene expression in the ischemic brain using reporter mice. The fluorescence signal showed *in vivo* the induction of hsp-70 in penumbra neurons and in the vasculature within the ischemic core.

Key words: Hsp-70, reporter mice, molecular imaging, *in vivo* imaging, stroke

Introduction

The inducible 72-kDa heat-shock protein (HSP-70) is a chaperone that binds to nascent polypeptides and supports protein folding and protein trafficking through intracellular compartments [1]. HSP-70 is induced in response to several types of stress situations [2] and it is regarded as a survival molecule that is coupled with functional protein translation [3]. Although it is not expressed in the adult brain under physiological conditions, the expression of HSP-70 is strongly induced following ischemia [4-6]. Natural induction of HSP-70 after cerebral ischemia is associated with neuronal survival [7-9]. Very intense expression of HSP-70 is seen in neurons at the periphery of the ischemic core 24 hours after occlusion of the middle cerebral artery (MCA), while this protein is mainly expressed in blood vessels within the core [4-6]. The dynamic features of regional HSP-70 induction after ischemia suggested that it is expressed by viable penumbral neurons [4-10]. Demonstration of HSP-70 induction in the brain zones with suppressed protein synthesis but preserved ATP validated that HSP-70 is strongly induced in the ischemic penumbra [11-12]. The extension of the penumbra region is gradually reduced with increasing time as brain infarction progresses [13]. Short periods of MCA occlusion induce HSP-70 in neurons to a larger extent than more prolonged episodes or than permanent ischemia [5-6], which is consistent with the notion that early reperfusion rescues penumbral neurons from death [14-16].

The aim of this study was to develop reporter transgenic mice expressing the far-red fluorescent protein mPlum under the transcriptional control of the Hsp-70 gene promoter to visualize Hsp-70 gene expression *in vivo* after transient cerebral ischemia. We examined the living mouse brain by fluorescence reflectance imaging (FRI) and by intravital confocal microscopy. We selected a far-red fluorescent protein, mPlum, as the fluorescent reporter to minimize fluorescence absorption and scattering by the cerebral parenchyma.

Methods

Plasmid construction

The Hsp70-mPlum reporter plasmid contains a mini-gene that expresses the far-red fluorescent protein mPlum (the excitation and emission peaks are at 590 and 649nm, respectively) under the control of the mouse *Hspa1b* gene proximal promoter. The mini-gene was assembled by using the multisite gateway three-fragment vector construction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. This required prior construction of three entry clones that provided the elements for the construction of the reporter. The first clone, pDONR-P4-P1R-hspa1b, contained a 516 bp-DNA fragment of the mouse *Hspa1b* gene comprising positions 21304395 and 21304910 in the C57BL/6J mouse chromosome 17 genomic contig (NCBI accession number NT_039649.8, the gene runs on the complementary strand). This represents 284 bp of sequence upstream of the transcription initiation site and the complete 5'-UTR of *Hspa1b*. The insert was amplified by PCR from genomic DNA isolated from a Balb/C mouse, with primers that furnished 5' attB4 and 3' attB1 sites, and was cloned into pDONR-P4-P1R. The second entry clone (pDONR221-mPlum) contained the mPlum ORF, which was amplified by PCR from plasmid pBAD-mPlum (a kind gift of Dr Roger Y. Tsien, UCSD) with primers that provided 5' attB1 and 3' attB2 sites, and was cloned into pDONR221. Finally, a cassette containing the SV40 early poly-adenylation signal was amplified from pCDNA3.1 by PCR with primers that furnished 5' attB2 and 3' attB3 sites, and was subsequently cloned into vector pDONR-P2R-P3. All inserts were amplified through two-step PCR with two sets of primers that sequentially furnished the appropriate attB sites for each clone. Primer sequences are provided in Supplementary Table 1. PCR-amplified inserts were cloned into the corresponding vectors through BP clonase-mediated reactions. Clones containing the correct inserts were checked by sequencing. The Hsp70-mPlum reporter vector was obtained as a result of a multi-site LR

Clonase-mediated recombination reaction between the promoter-less destination vector pDEST-R4-R3 and each of the three entry clones. Clones were analyzed by sequencing to ensure the integrity of the mini-gene's constituents. The Hsp70-mPlum vector was further modified by the introduction of a Kozak sequence (ACCACC), and a rabbit β-globin intron 5' relative to the initiation codon in the mPlum ORF (Norsk Transgen Senter, Oslo, Norway), in order to improve cellular processing of the mRNA expressed from the mini-gene.

Animals and generation of the reporter transgenic mice

Purified DNA containing the minigene was injected into (C57BL/6J x CBA/J)F2 mouse blastocyst at the Norsk Transgen Senter. Four female founders that carried the mPlum minigene, as deduced by PCR-genotyping, were obtained. Three of the founder females produced offspring in crosses with wild type (wt) C57BL/6J males. Transgenic female offspring were backcrossed to C57BL/6J wt males for 7 generations. Litters were genotyped in order to identify transgenic (Tg) male mice to be used in the experiments. A colony of homozygous transgenic mice was maintained in the animal house of the School of Medicine of the University of Barcelona. Double transgenic C57BL/6J mice were obtained by crossing the Hspa1b/mPlum (+/-) mice with Hspa1b/lucF (+/+) strain [17], and were reared at University of Bordeaux transgenic facilities. Animal work was conducted in the animal house of the School of Medicine of the University of Barcelona under approval of the ethical committee of this University (CEEA) and the *Generalitat of Catalunya* according to the directives of the Spanish law on this matter, and at the University of Bordeaux under the directives of the French Research Ministry; all in compliance with the regulations of the European Union.

PCR genotyping of transgenic mice

DNA from tail clips was extracted and subjected to PCR amplification by using the "Extract'N'Amp" tissue PCR kit (Sigma-Aldrich), according to the manufacturer's instructions. A 524 bp-fragment in the Hsp70-mPlum vector was amplified with oligonucleotide primers specifically recognizing the Hsp70-mPlum transgene: 5'-CCTGGGCAACGTGCTGGTTATTGT-3' (forward) and 5'-CGCGCACCTTCACCTTGTAGAT-3' (reverse). The conditions for the PCR were: initial denaturation at 94 ºC for 5 min, 30 cycles of amplification with denaturation at 94 ºC for 30 s, annealing at 61 ºC for 30 s and extension at 72 ºC for 45 s. A final extension step was carried out at 72 ºC for 5 min. Primers ICAMfw (5'-CTGAGCCAGCTGGAGGTCTCG-3') and ICAMrv (5'-GAGCGGCAGAGCAAAAGAAGC-3', which amplify a 178 bp DNA fragment from the mouse ICAM gene were included in the PCR master mix in order to provide an internal positive control for each sample. After completion, the PCR reactions were run on 1% agarose gels and visualized by staining with SYBR safe (Invitrogen). A representative result is shown in Supplementary figure 1.

Induction of HSP-70 and mPlum in cell cultures.

The NIH3T3 mouse fibroblast cell line was transiently transfected with the Hsp70-mPlum reporter vector. Briefly, 10^5 cells per well seeded on 12-well dishes were transfected with 1.5 µg of Hsp70-mPlum vector and 7.5 µL of Superfect transfection reagent (Qiagen, Crawley West Sussex, UK), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were exposed for 4 hours to 50 µM sodium arsenite ($NaAsO_2$) [18]. mPlum fluorescence was examined 24 hours later, under an Olympus IX70 inverted epifluorescence microscope (Olympus, Barcelona, Spain). Total cellular extracts were prepared right after observation and used in Western blotting experiments. Expression of mPlum (~27 kDa) and HSP-70 (~70 kDa) was detected by using specific antibodies (see below).

Induction of cerebral ischemia

Focal brain ischemia was produced by transient intraluminal occlusion of the right MCA, as reported [19], in Tg (n=22) and wt (n=12) mice. Anesthesia was induced with 4 % isoflurane in a mixture of 30% oxygen and 70% nitrogen protoxide) and maintained with 1-1.5 % isoflurane in the same a mixture by the aid of a facial mask. A longitudinal cut was produced in the ventral middle line of the neck to expose the right common carotid artery (CCA). Next the submaxilar glands and the omohyoid and sternohyoid muscles were separated, exposing the carotid vessels. A filament (nylon monofilament 6/0, Suturas Arago, Spain) was introduced through the external carotid artery to the level where the MCA branches out. Cerebral blood flow was assessed with laser Doppler flowmetry (Perimed AB, Järfälla Sweden). After 60 min, the filament was cautiously removed, and the suture of the ipsilateral CCA was taken off to allow reperfusion. Animals were allowed to recover and were killed 1-2 days later. Non-ischemic controls (n=8) were carried out by sham-operation where all surgical and anesthetic procedures were carried out but the filament was only briefly introduced through the external and internal carotid arteries, but it was immediately removed to avoid ischemia.

Fluorescence and bioluminescence imaging of live mice

Two days before the study the posterior part of the mice was shaved with a clipper and a depilatory cream to facilitate light propagation. Fluorescence reflectance imaging (FRI) was performed in the mPlum/lucF (n=6) mice before and 30 hours after induction of ischemia (n=6) or in controls (n=2). Fluorescence images of mice were acquired using a NightOWL II LB 983 system equipped with a NC 100 CCD deep-cooled camera (Berthold Technologies, Bad Wildbad, Germany). Mice were sedated with isoflurane (2% in air) and fluorescence images (exposure = 1 s, binning = 1×1, field of view= 12.5 x 12.5 cm^2) and photographs (100ms exposure) were taken in prone position. Excitation wavelengths of 590nm/20nm

were used and the fluorescence emission was detected at 680nm/30nm in a Berthold chamber. Grey-scale body-surface reference images were collected for superposition of FRI images on anatomical maps. Pseudo-color fluorescent images representing the spatial distribution of emitted photons were generated using IndiGO 2 software (Berthold Technologies). Analysis of the FRI was done manually by placing a small region of interest (ROI) at the level of interest area in the ipsilateral and contralateral hemisphere. Within this ROI, the mean light intensity (Photons s^{-1} cm^{-2}) was measured. Images were processed with IndiGO 2 software, and measures were taken from the ROIs. At the end point after *in vivo* imaging, animals were killed and the brain was quickly removed from the skull and it was imaged again *ex vivo* for comparison purposes.

Bioluminescence images of mice were acquired using a NightOWL II LB 983 system equipped with a NC 100 deep-cooled CCD camera (Berthold Technologies). Mice received an i.p. injection of D-luciferin (Promega, Madison, WI, USA) (2.9 mg in 100 µL sterile phosphate buffer). Then, mice were sedated with isoflurane (2% in air), bioluminescence images (2 minutes integration period, 4×4 binning) and photo (100 ms exposure) were taken 10 minutes after the luciferin injection. During acquisitions mice were anesthetized with isoflurane (2% in air). A low light-emitting standard (Glowell, LUX biotechnology, UK) was placed next to the animal during each image acquisition to provide a constant reference for the resulting images. Grey-scale body-surface reference images were collected for superposition of BLI images on anatomical maps. Control experiments of reporter gene expression by BLI (luciferase activity) and FRI (mPlum expression) in double transgenic mice were carried out by heating the mouse leg (45 ºC for 8 min, n=8) to activate the Hspa1b promoter using the water bath method as described before [20]. In these experiments, FRI time-course studies of fluorescence induction were carried out showing a maximum at 30 hours, which was the time chosen for FRI in the present work (see Supplementary Fig. 2).

Intravital confocal microscopy

For intravital confocal microscopy a cranial window was opened in the skull of the ipsilateral hemisphere either immediately after ischemia/reperfusion in mPlum Tg (n=6) and wt (n=3) animals. Mice were anesthetized with isoflurane and the cranial window was opened avoiding breaking the dura matter and was then sealed with a coverslip, following a previously reported procedure [21]. In brief, a circular groove of about 5 mm in diameter was drilled in the skull and the island of cranial bone was removed. A cover glass was placed in the hole and dental cement was applied to seal the glass to the skull. Live observation of the cerebral cortex was carried out under a high-speed confocal microscope (Leica TCS SP5 confocal ressonant scan spectral microscope, Leica Microsystems Heidelberg GmbH) using a 25× glycerol water immersion objective (NA 0.95). For microscopic observations, mice were anesthetized with an i.p. injection of 100 µL of a solution of ketamine (0.1 mg/g) xylazine (0.01 mg/g). A solution of sodium fluorescein (250 mM; 2 µM/Kg) was injected i.v. for live observation of the blood vessels under the confocal microscope. Some mice also received an i.p. injection of a solution of Hoechst 33342 (10 mg/mL; 12.5 µg/g) to visualize cell nuclei. mPlum was observed at 594nm ex, 649nm em; sodium fluorescein was detected at 490nm ex, 515nm em.; and Hoechst was detected at 405nm ex, 455nm em. Images were scanned at 8000 lines/second.

Real Time RT-PCR

Hsp-70 and mPlum mRNA was studied at 24 hours post-ischemia in the ipsilateral and contralateral hemisphere. Total RNA was extracted using TRIzol (Invitrogen) from the brain of Tg (n=6) and wt (n=6) ischemic mice and from controls (n=2 per group). RNA quantity and purity were determined using ND-1000 micro-spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (500 ng) was reverse-transcribed using a mixture of

random primers (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT-PCR analysis was carried out by SYBR green I dye detection (#11761500, Invitrogen) using the iCycler iQTM Multicolor Real-Time Detection System (BIO-RAD, Herculesm CA, USA). PCR primers were designed with the aid of Primer3 software to bridge the exon–intron boundaries within the gene of interest to exclude amplification of contaminating genomic DNA. Primers were purchased from IDT (Laboratorios Conda, Torrejon de Ardoz, Spain) and the following sequences were used for Hsp-70: F:5'-GGCTGATCGGCCGCAAGTT-3', R:5'-GGAAGGGCCAGTGCTTCAT-3', and for mPlum: F: 5'-CCCCGTAATGCAGAAGAAGA-3', R:5'-GTCCAGCTTGATGTCGGTCT-3'. Quantification was performed by normalizing Ct (Cycle threshold) values with the RPL14 (F:5'-GGCTTTAGTGGATGGACCCT-3', R:5'-ATTGATATCCGCCTTCTCCC-3') control gene Ct, and analysis was carried out with the 2-ΔΔCT method [22].

Assessment of brain infarction

To verify the presence of brain infarction, mice were anesthetized with isoflurane and killed. The brain was sliced in 1-mm thick coronal sections. After FRI imaging of the sections, they were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 10 min at 37 ºC.

Immunohistochemistry

Ischemic mice (24 hours) (Tg n=6 and wt n=3) and control mice (Tg n=3 and wt n=2), were anesthetized with isoflurane, and perfused through the heart with saline followed by paraformaldehyde (PFA, 4%) in phosphate buffer (pH 7.4). The brain was removed, post-fixed with PFA overnight and then kept in phosphate buffer, washed in the same buffer before slicing it in a vibratome to obtain 30 µm-thick coronal sections. In a separate set of experiments 1-mm thick TTC stained sections (see above) were also used for immunohistochemistry. To this end the TTC brain sections were fixed in PFA overnight,

washed in phosphate buffer and sliced in the vibratome. The vibratome brain sections were cryoprotected in a solution containing glycerol and were kept frozen at -20 ºC. Immunohistochemistry was performed free-floating as previously reported [10]. Endogenous peroxidases were blocked with 3% hydrogen peroxide and 10% methanol in PBS, for 25 min. Sections were incubated for 2 hours in 3% normal horse or goat serum for mouse monoclonal or rabbit polyclonal antibodies, respectively, to block unspecific binding sites, washed in T-PBS (PBS containing 0.5% Triton X-100), and incubated overnight at 4 ºC with either mouse monoclonal antibody against HSP-70 (#HSP01, Calbiochem, San Diego, CA, USA) diluted 1:500, or rabbit polyclonal anti-red fluorescence protein antibody (RFP, #ab34771, Abcam, Cambridge, UK) diluted 1:400. Thereafter, the sections were rinsed in T-PBS and incubated for 1 hour with a biotinylated secondary antibody (1:200, Vector Laboratories), followed by incubation with 1% avidin–biotin–peroxidase complex (ABC kit, Vector Labs, Burlingame, CA, USA). The reaction was visualized with 0.05% diaminobenzidine or with 0.01% benzidine dihydrochloride and 0.025% sodium nitroferricyanide in 0.01 M sodium phosphate buffer, pH 6. The reaction was developed after addition of 0.005% H_2O_2. Immunoreaction controls included omission of the first or second primary antibodies. Alternatively, immunofluorescence staining was carried out using the same primary antibodies as above, followed by Alexa Fluor®488 and/or Alexa Fluor®546 secondary antibodies (Invitrogen). Hoechst was used to highlight the cell nuclei. Fluorescence was examined under an inverted fluorescence microscope (IX70 Olympus).

Western Blotting

After a brief wash in PBS, cells were scraped on the wells in 100 µl of 1X loading buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10 mM phosphate buffer pH 7, 10% glycerol, 2.5% SDS, 0.5 mg/ml DTT, 0.16 mg/ml bromophenol blue). The lysates were spun through Nucleospin filters (Macherey-Nagel, Düren, Germany) in order to

break denatured chromatin. Ten percent of the sample was denatured at 100 ºC for 5 min and then loaded in 10% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were incubated overnight at 4 ºC with the mouse monoclonal antibody against HSP-70 and the rabbit polyclonal antibody against RFP (see immunohistochemistry section) both diluted 1:1,000. On the following day, membranes were incubated for 1 hour with an anti-mouse Ig peroxidase-linked secondary antibody (1:2,000) (Amersham, GE Healthcare UK Ltd, Buckinghamshire, UK). The reaction was visualized using a chemiluminescence detection system based on the luminol reaction. Membranes were then reacted with a control antibody to check for equal protein loading in each lane. Mouse monoclonal antibody against actin (Sigma-Aldrich), diluted 1:50,000; was used as the loading control.

Statistical analyses

Comparisons by surgery (sham vs ischemia) and by brain hemisphere (ipsilateral to MCA occlusion vs contralateral) or time point were carried out with two-way ANOVA followed by the post-hoc Bonferroni test.

Results

In vitro characterization of the mPlum construct

The constructed Hsp-70 (*Hspa1b*)-*mPlum* reporter vector is shown in Fig. 1A. NIH3T3 mouse fibroblast cells were transfected with the reporter vector and showed mPlum fluorescence after exposure to sodium arsenite, a chemical that induces Hsp-70 gene expression [18]. Total cellular extracts were prepared 24 hours after exposure to sodium arsenite for 4 hours, and were used in Western blotting experiments to detect induction of mPlum and of HSP-70 expression using antibodies against the red fluorescence protein (RFP) and against HSP-70,

respectively. Induction of RFP (~27 kDa) and HSP-70 were detected after treatment (Fig. 1B). In addition, after transfecting the cells with the mPlum reporter vector, fluorescence was observed by microscopy in cells treated with arsenite, but not in untreated cells (Fig. 1C).

Expression of mPlum and Hsp-70 after brain ischemia in transgenic mPlum mice

The construct described above was used to generate Hsp-70-mPlum transgenic mice. Brain ischemia was induced in Tg mice and the expression of *Hsp-70* and *mPlum* mRNA was examined at 24 hours. Induction of these mRNAs was detected in the ipsilateral but not in the contralateral hemisphere of the Tg mice. The level of expression (fold induction versus control) of *mPlum* mRNA showed a very good correlation (linear regression, $r^2 = 0.95$) with the level of expression of *Hsp-70* mRNA in the different mice. Control or sham-operated mice showed no expression of *Hsp-70* or *mPlum* mRNA (not shown). Ischemia in wt mice induced *Hsp-70* mRNA in the ipsilateral hemisphere, but no expression of *mPlum* mRNA was detected.

Immunohistochemical studies established that 24 hours after ischemia, HSP-70 protein was expressed in cortical neurons located at the border of infarction and in blood vessels within the ischemic core (Fig. 2). This cellular and regional pattern of HSP-70 expression agrees with that previously reported [4-6]. Immunohistochemistry and immunofluorescence against red fluorescent protein (RFP) with specific antibodies showed expression of mPlum in cells with the morphology of neurons located surrounding the ischemic core (Fig. 3A,B) and in blood vessels within the core (Fig 3C,D). Co-localization of RFP and HSP-70 was observed after double-immunohistochemistry (Fig. 3E-J). These findings demonstrate HSP-70 and mPlum proteins located in the same cells, and therefore the mPlum transgene is a good reporter of HSP-70 expression.

In vivo imaging mPlum by FRI and BLI

For imaging the head of living mice (with intact skull) we used the Hsp70/mPlum/luciferase reporter mouse. FRI showed an increase in fluorescence signal intensity in the ipsilateral hemisphere of living double-transgenic mice the day (time 30 hours) after 60-min MCA occlusion in relation to the signals obtained before ischemia (time 0 hour) (Fig. 4A), while no changes were observed in sham-operated mice. *Ex vivo* imaging of the brain, after removal from the skull, of the same animals showed similar results, but the intensity of the signal was higher than *in vivo* (Fig. 4A). Quantification of fluorescence intensity (Photons s^{-1} cm^{-2}) signals obtained *in vivo* was carried out in the ipsilateral and the contralateral hemispheres before and after ischemia. An increase in fluorescence signal intensity was detected in the ipsilateral hemisphere of ischemic mice compared to sham operated mice ($p<0.01$) (Fig. 4B). For each mouse the ratio was calculated between the measure taken after ischemia (time 30 hours) and that before ischemia (time 0 hour). The results showed a statistically significant increase in the ipsilateral hemisphere of the ischemic mice ($p<0.001$, two-way ANOVA by condition, i.e. ischemia versus sham-operation, and hemisphere, i.e. ipsilateral versus contralateral hemispheres) (Fig. 4C). Quantification of the signal intensity of the *ex vivo* images showed again a statistically significant increase ($p<0.001$) in the ipsilateral hemisphere of the ischemic mice compared to the contralateral hemisphere and to the signal in sham-operated mice (Fig. 4D). The brain of these mice was also imaged *ex vivo* the day after MCA occlusion after slicing the post-mortem tissue into 1-mm thick coronal sections. After FRI imaging, the tissue sections were stained with TTC to visualize the infarcted core (pale zone). A good correspondence was found between the location of infarction and the location of fluorescence emission (Fig. 5). A strong signal intensity focus was observed within the infarcted region. By carrying out HSP-70 immunohistochemistry on thinner slices (30 μm-thick vibratome sections) obtained from each TTC section, it became apparent that the zones with mPlum fluorescence grossly corresponded to the HSP-70

immunoreactive zones (Fig. 5). Nevertheless, it must be emphasized that in reporter mice technology any differences between the expression and/or localization of reporter fluorescent protein (mPlum) and the endogenous protein (HSP-70) are attributable to the fact that the reporter fluorescent protein traces induction of gene expression (Hsp-70 gene). However, the fate, half-life, and cellular trafficking of the reporter (mPlum) and the endogenous (HSP-70) proteins in the cells are not necessarily identical.

The double-transgenic mice were also imaged for *in vivo* bioluminescence (BLI) after brain ischemia following administration of luciferin. However, no increases in signal intensity were observed in the brain after ischemia (not shown), despite the transgenic animals showed an intense BLI signal in control experiments where the paw of the mice was heated to induce Hsp-70 expression (see Supplementary Fig. 2). Bioluminescence emission by the firefly luciferase reaction after injection of luciferin relies on the presence of oxygen and ATP, which is the limiting factor in this reaction [23]. Absence of BLI signal in the ischemic zone suggests that ATP or luciferin availability after ischemia was insufficient for the luciferase reaction to take place.

Confocal microscopy in the living mice

Intravital confocal microscopy was carried out to image the brain of living mice at the cellular level after opening a cranial window in the skull of the ipsilateral hemisphere (see Methods). The brain was examined 24 hours post-ischemia. Prior to live observation under the microscope, mice received an intravenous injection of sodium fluorescein to visualize blood vessels and fluorescence extravasation, as an indicator of alterations in the permeability of the blood-brain barrier (BBB). This dye remains intravascular under normal conditions but extravasates to the brain parenchyma when the permeability of the BBB is altered [24]. Red fluorescence was observed in the ipsilateral cortex of mPlum transgenic

(Tg) mice after ischemia (Fig. 6 C-G), but not in ischemic wt mice (Fig. 6A, B) or in non-ischemic wt or Tg mice (not shown). The red fluorescence spectrum was verified to approximately correspond to the wavelength of mPlum emission (Fig. 6I).

mPlum positive cells were occasionally observed in the ipsilateral cortex following ischemia (Fig. 6C). The size and morphology of these cells suggested that they were neurons. In addition, mPlum fluorescence was often observed in the brain vasculature (Fig. 6D-E, J). mPlum fluorescence was restricted to certain segments of the blood vessels located surrounding vessel branching points (Fig. 6D-E, J). The size of some of the imaged vessels (20-30 μm) corresponded to arterioles. Fluorescein extravasation was seen in several areas (Fig. 6J) suggesting alterations of BBB permeability.

Discussion

We imaged cerebral Hsp-70 gene expression *in vivo* after brain ischemia in reporter transgenic mice expressing the far-red fluorescent protein mPlum under the promoter of Hsp-70. After validating the Hsp-70-mPlum DNA construct in transfected cells exposed to challenges inducing Hsp-70 in cellular systems, the transgenic mice were generated and were subjected to transient MCA occlusion for imaging Hsp-70 induction in the ischemic brain. Hsp-70 expression is undetectable in the brain under physiological conditions but it is strongly induced after cerebral ischemia [4-10]. Accordingly, fluorescence reflectance imaging (FRI) of the mouse brain with intact skull allowed the detection of a fluorescence signal in the ipsilateral hemisphere the day following MCA occlusion, and the affected zone corresponded to the zone of Hsp-70 induction, as revealed by immunohistochemistry in the postmortem brain of the same mice. FRI after the brain was removed from the skull gave a more intense signal and a better signal-to-noise ratio than in the living mice, since the

signals obtained *in vivo* suffered attenuation by the presence of the skull. This experiment shows that post-ischemic induction of Hsp-70 gene expression in the brain can be followed non-invasively *in vivo* using the reporter mice and FRI technology. Cerebral Hsp-70 induction was also monitored *in vivo* at the cellular level by using intravital confocal microscopy after opening a cranial window. This technique allowed visualizing mPlum positive neurons and blood vessels.

Numerous studies found that Hsp-70 over-expression or pharmacological induction exerts neuroprotective actions against ischemic neuronal cell death [25-33]. Also, astrocyte targeted over-expression of Hsp-70 reduces neuronal vulnerability to transient global ischemia [34]. Moreover, systemic administration of recombinant HSP-70 protein linked to cell penetrating peptides reduced infarct volume and improved the neurological function after transient focal cerebral ischemia in mice [35] and rats [36]. In spite of these beneficial effects, one study reported that the Hsp-70 transgenic mice were not protected in an experimental model of global cerebral ischemia [37], and viral delivery of Hsp-70 was not protective in a model of transient MCA occlusion, in contrast to delivery of Hsp-27 that was found to be beneficial [38]. In line with most of the above findings, the natural induction of Hsp-70 in neurons after ischemia is also thought to exert protective effects [7-9]. Hsp-70 is induced in viable neurons of the penumbra region and it is a molecular marker of the penumbra after stroke [11-12]. Therefore, mPlum fluorescence in neurons indicated neuronal survival. Expression of Hsp-70 in the vasculature might also have protective effects since it is associated with attenuation of ischemia-reperfusion induced microvascular injury in other organs [39]. The size of some of the imaged fluorescent vessels was compatible with that of arterioles. By following the vessel tree under the confocal microscope we noticed that mPlum fluorescence was apparent not along the full length of the vessels but in particular vessel segments only. The latter were often located adjacent to branching points

and might reflect shear stress in these zones. Shear stress has been reported to induce Hsp-70 in synovial cells [40]. Therefore, induction of Hsp-70 in certain arteriolar segments after brain ischemia might be due to increased shear stress in these zones. Further studies will be needed to address whether the segmented vascular induction of Hsp-70 gene expression is due to vascular stress, and whether it might be related to changes in local blood flow and vascular autoregulation.

The benefits of Hsp-70 induction described in the literature [25-33] make it a good target for pharmacological intervention. Several drugs inducing Hsp-70 expression, like geldanamycin [41-42], exert protective effects against ischemic brain damage. The Hsp-70 reporter mice described here will allow monitoring Hsp-70 gene induction *in vivo* after pharmacological intervention and will contribute to validate its use as an imaging biomarker in preclinical studies. New developments, including functionalized magnetic/optical nanoparticles or PET tracers targeting Hsp-70 [43-44], are needed to guarantee readily application of Hsp-70 imaging in humans.

Overall, this study shows the *in vivo* expression of Hsp-70 gene in the ischemic brain with fluorescent reporter mice using FRI and intravital confocal microscopy. FRI illustrated Hsp-70 induction in the ischemic region of the whole mice, while confocal microscopy after opening a cranial window allowed visualizing it in neurons and in the brain vasculature. Neuronal Hsp-70 is taken as a marker of penumbra and indicates neuronal viability whereas Hsp-70 in vessels is a marker of the ischemic core. We conclude that the Hsp-70-mPlum reporter mice are good tools to study *in vivo* the induction of Hsp-70 gene expression.

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FIGURE LEGENDS

Fig. 1. A) Genomic structure of the mouse Hspa1b gene. The intron-less Hspa1b gene sequence lies on the complementary strand of chromosome 17, near the almost identical Hspa1a gene. Arrows point at positions in the sequence of mouse strain C57BL/6J chromosome 17 genomic contig (NT_039649.8). The Hspa1b coding sequence is represented by closed rectangles, while the 5'- and 3'-UTRs are represented by open rectangles. Positions of the PCR primers used for the amplification of the proximal promoter and 5'UTR up to the initiation codon of Hspa1b, are represented by little dashes above (forward primer) and below (reverse primer) the gene. This fragment (double headed arrow) was used to drive transcription of the Hsp70-mPlum minigene inserted into the transgenic mice. The structure of the minigene is depicted below (see details under Methods). B) Western blotting using antibodies against HSP-70 or RFP illustrates induction of both proteins in NIH3T3 cells transfected with the Hsp70-mPlum reporter vector after stimulation with sodium arsenite ($NaAsO_2$). β-actin is shown as the loading control. C) NIH3T3 cells (all transfected with the Hsp-70-mPlum reporter vector) express mPlum fluorescence after stimulation with sodium arsenite (bottom row), but not in the absence of stimulation (upper row). Bar scale: 20 μm.

Fig. 2 Brain ischemia induces HSP-70 as shown in postmortem tissue at 24 hours by immunohistochemistry. HSP-70 immunoreactivity is seen in the ipsilateral (B-F) but not in the contralateral (A) hemisphere. Several immunoreactive neurons are seen (arrowheads in B, F) at the borders of the ischemic core, which mainly shows immunoreactive vessels (arrows in B-E). Bar scale: A-B = 60 μm; C-E= 30 μm; F= 15 μm.

Fig. 3 Immunohistochemistry (A, C) and immunofluorescence (B, D) with antibodies against HSP-70 and RFP (to label mPlum), respectively, reveals the presence of positive neurons (A,

B) and blood vessels (C, D). Double immunofluorescence against mPlum (F, I) and HSP-70 (E, H) shows co-localization (G, J). Bar scale: A-D= 30 μm; E-G= 20 μm; H-J = 15 μm.

Fig. 4 FRI whole body imaging (A) reveals mPlum fluorescence both *in vivo* (left panels) and *ex vivo* (right column) in the ischemic hemisphere (upper row), but not in sham-operated mice (bottom row). Color scale indicates intensity (maximum is red and minimum is purple). Insets are magnifications of the surface of the brain to illustrate the fluorescent region. (B-D) Measures of fluorescence signal intensity in a region of interest placed in the ipsilateral or the contralateral brain hemispheres show signal intensity increases in the ipsilateral hemisphere at 30 hours after ischemia (n=4), both *in vivo* (B-C) and *ex vivo* (D), but not after sham-operation (n=2). Values are expressed either as absolute fluorescence units (Photons s^{-1} cm^{-2}) (B,D) or as the ratio between the time 30 hours and the time 0 hours in the same mice (C). ** $p<0.01$, *** $p<0.001$.

Fig. 5 *Ex vivo* FRI in coronal brain sections (1-mm thick) of a representative ischemic mouse (30 hours) (left column) show fluorescence in the ischemic hemisphere demonstrating induction of Hsp-70 gene expression. Fluorescence intensity is illustrated in the color-coded scale (red is maximum, purple is minimum). The same brain sections stained with TTC after FRI imaging are shown (middle column) to illustrate brain infarction (pale zone). Corresponding immunoreactivity against HSP-70 in 30-μm thick sections obtained after slicing the previous sections is shown (right column).

Fig. 6 *In vivo* confocal microscopy of wt (A,B) and mPlum Tg (C-G) mice 24 hours after MCA occlusion. (A-C) Fluorescein-positive vessels (green) and Hoechst-positive cell nuclei (blue) are shown together with mPlum fluorescence (red), which is seen in the Tg mice only. (D-G) mPlum fluorescence (red) is seen in certain vessels. (I) Spectrum of the red fluorescence

zone indicated in E. (G) A large mosaic area generated from multiple consecutive images is shown to illustrate the presence of fluorescence in certain vessel segments. Fluorescein extravasation indicative of alterations in blood-brain barrier permeability is seen in certain areas (green in D and G). Bar scale: A-C= 60 μm; D= 85 μm; E= 80 μm; G= 250 μm.

Figure 1

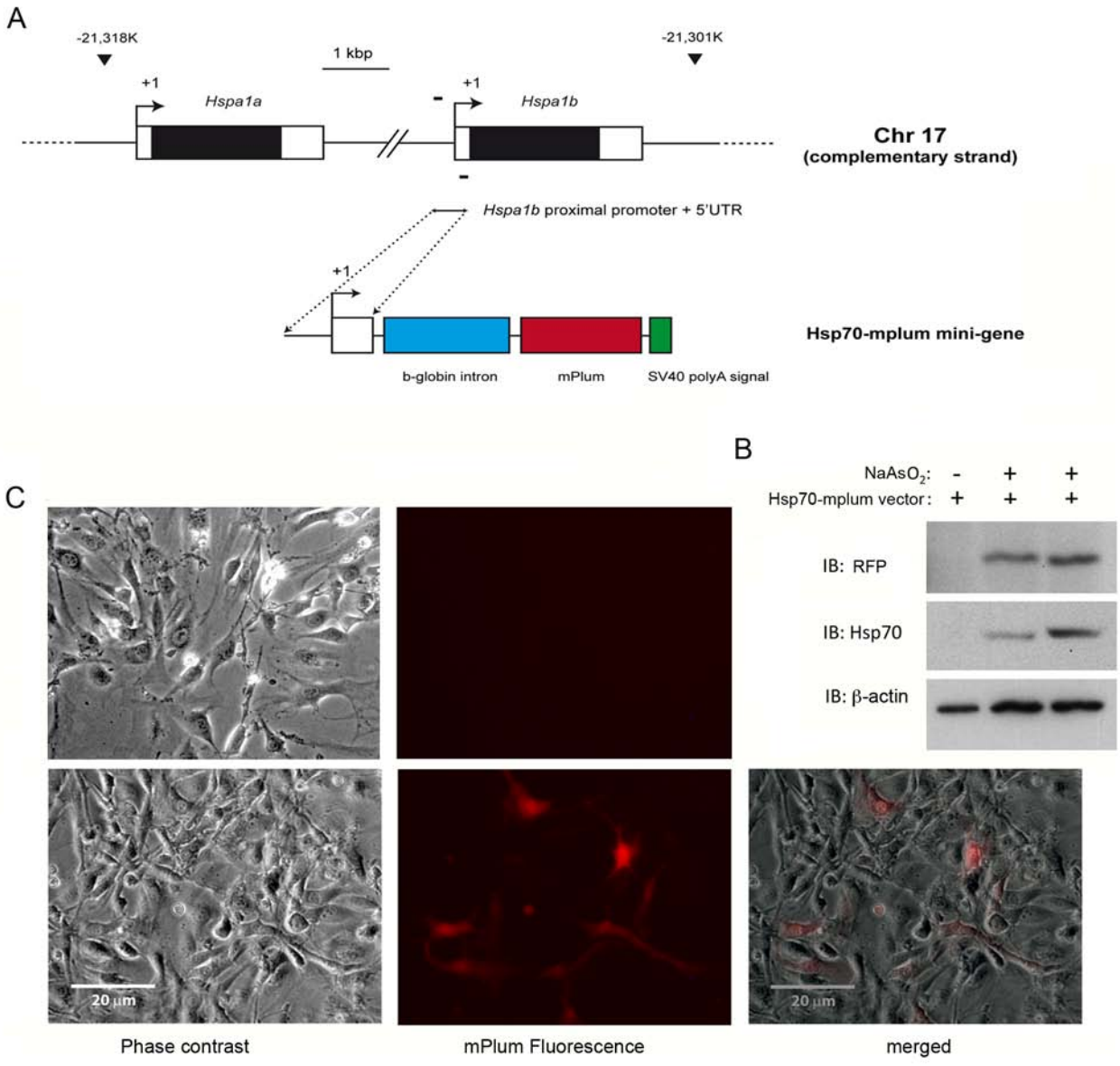
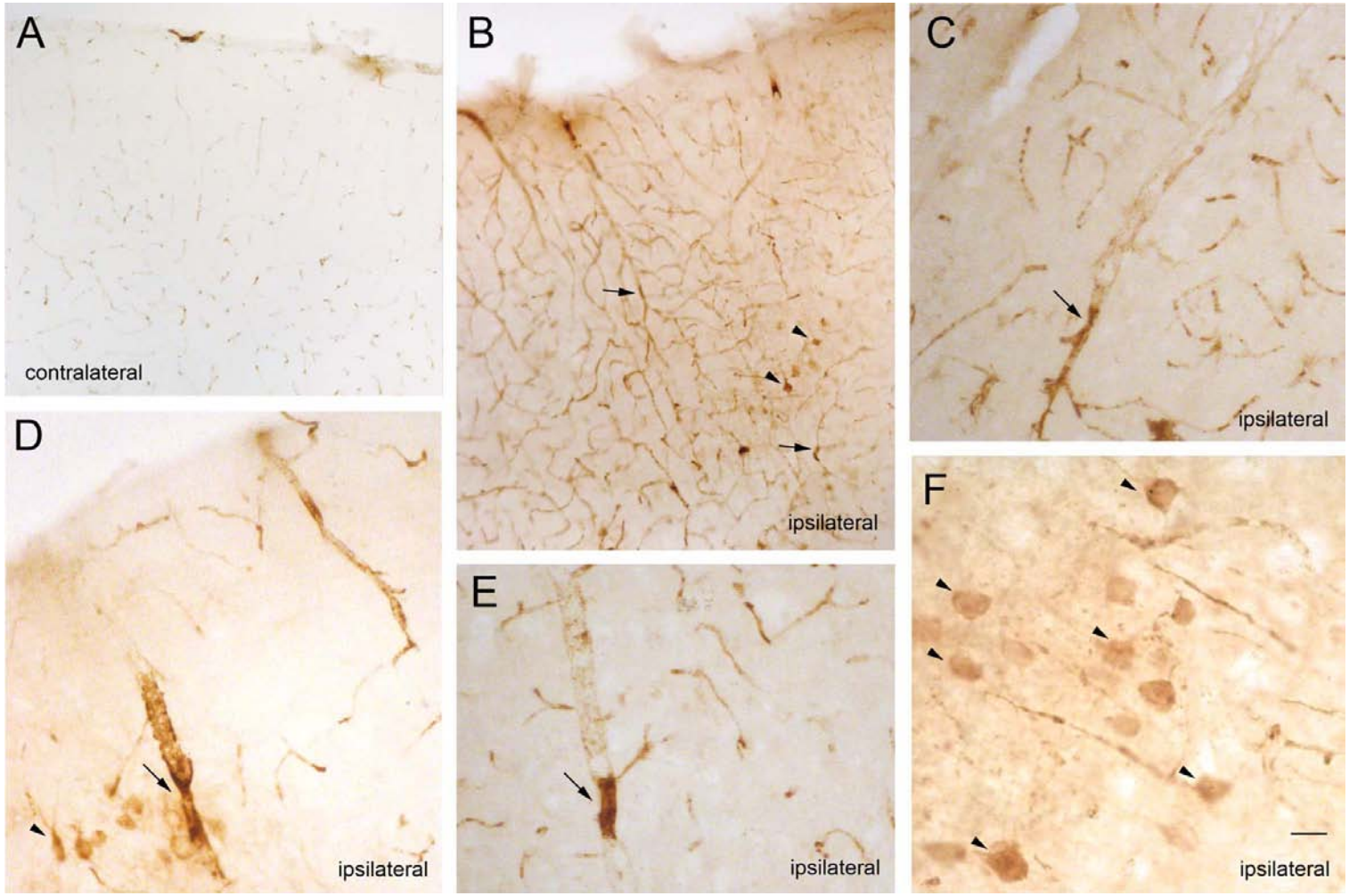

A
-21,318K
1 kbp
-21,301K
+1
Hspa1a
+1
Hspa1b
Chr 17
(complementary strand)
Hspa1b proximal promoter + 5'UTR
+1
Hsp70-mplum mini-gene
b-globin intron
mPlum
SV40 polyA signal
B
NaAsO2: - + +
Hsp70-mplum vector: + + +
IB: RFP
IB: Hsp70
IB: β-actin
C
20 μm
20 μm
Phase contrast
mPlum Fluorescence
merged

Figure 2



Hsp-70 immunoreactivity (mice Bordeaux)

A, B x10
C-E x20
F x40

Bar scale = 15 um in F, 30 um in C-E, 60 um in A-B

Figure 3

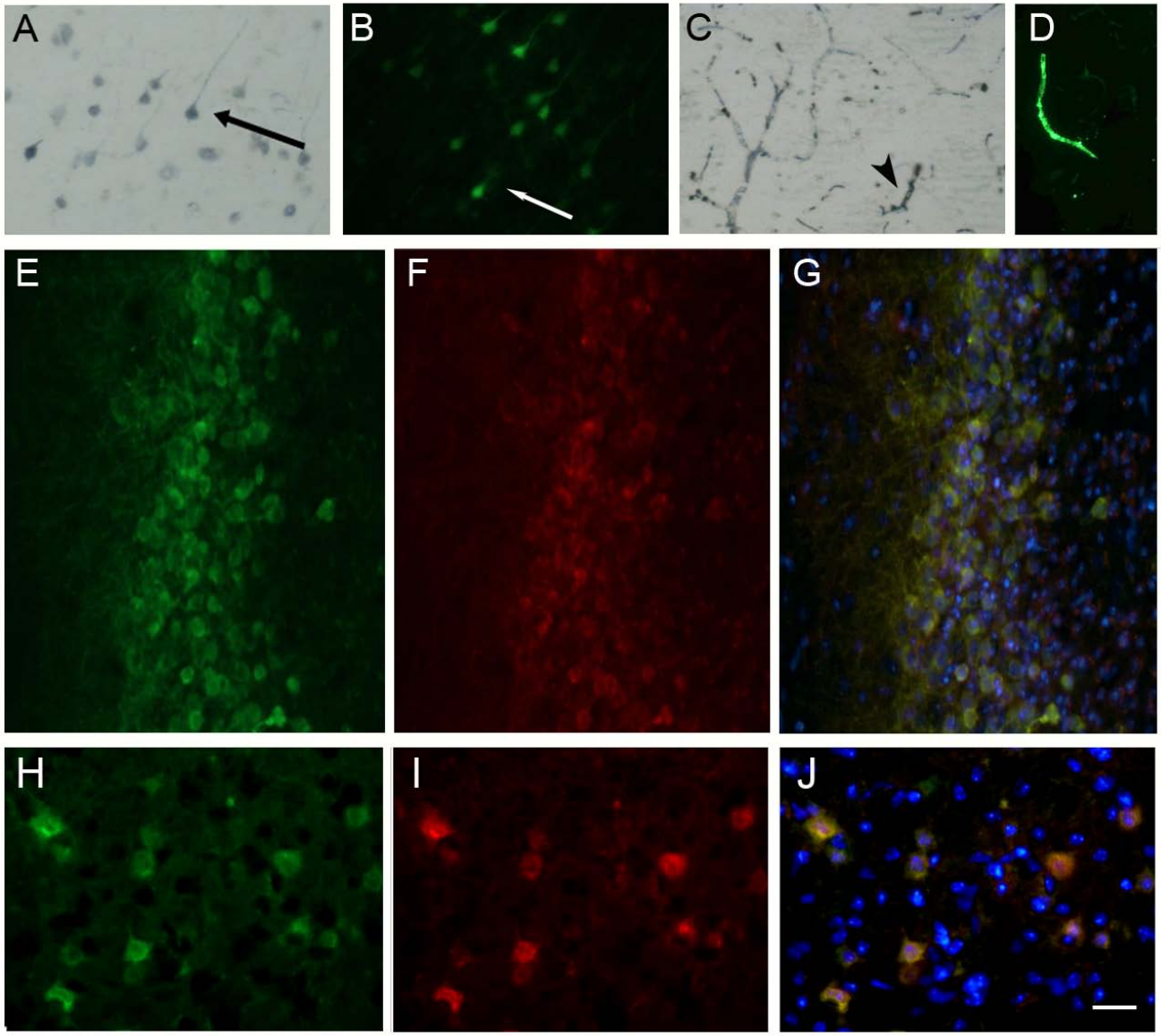


Figure 4

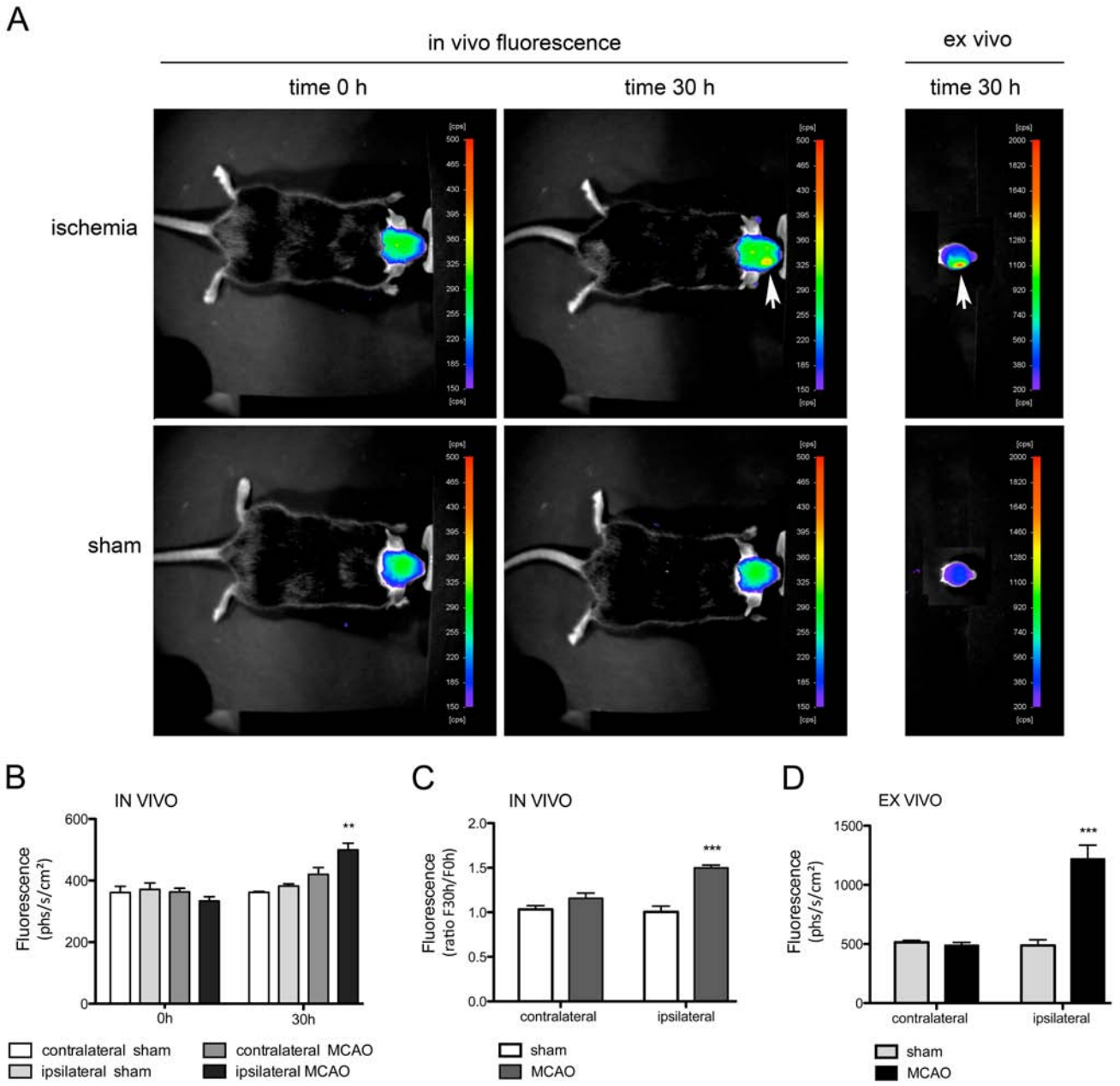

A
in vivo fluorescence
ex vivo
time 0 h
time 30 h
time 30 h
ischemia
sham
B
IN VIVO
Fluorescence (phs/s/cm²)
600
400
200
0
0h
30h
**
contralateral sham
ipsilateral sham
contralateral MCAO
ipsilateral MCAO
C
IN VIVO
Fluorescence (ratio F30h/F0h)
2.0
1.5
1.0
0.5
0.0
***
contralateral
ipsilateral
sham
MCAO
D
EX VIVO
Fluorescence (phs/s/cm²)
1500
1000
500
0
***
contralateral
ipsilateral
sham
MCAO

Figure 5

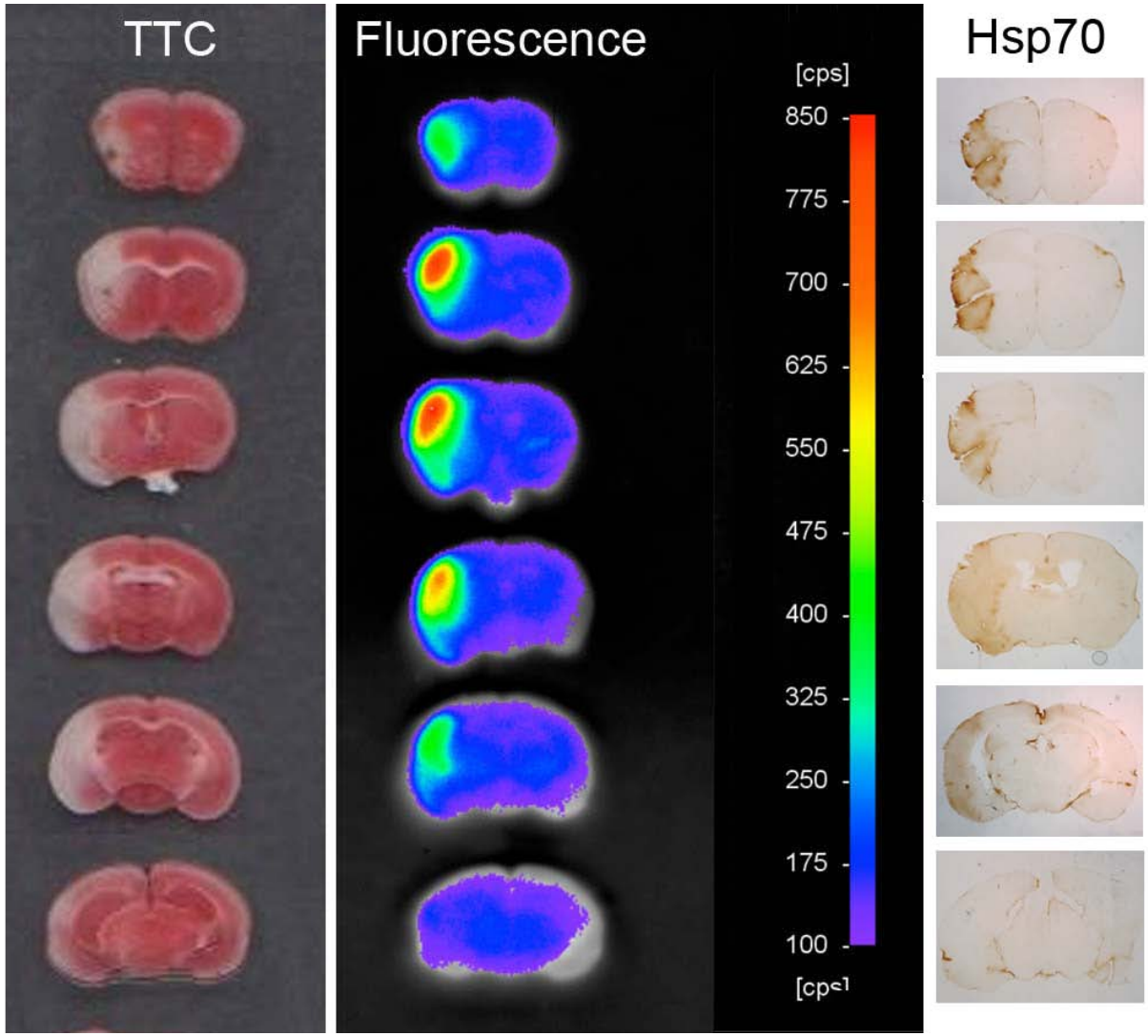
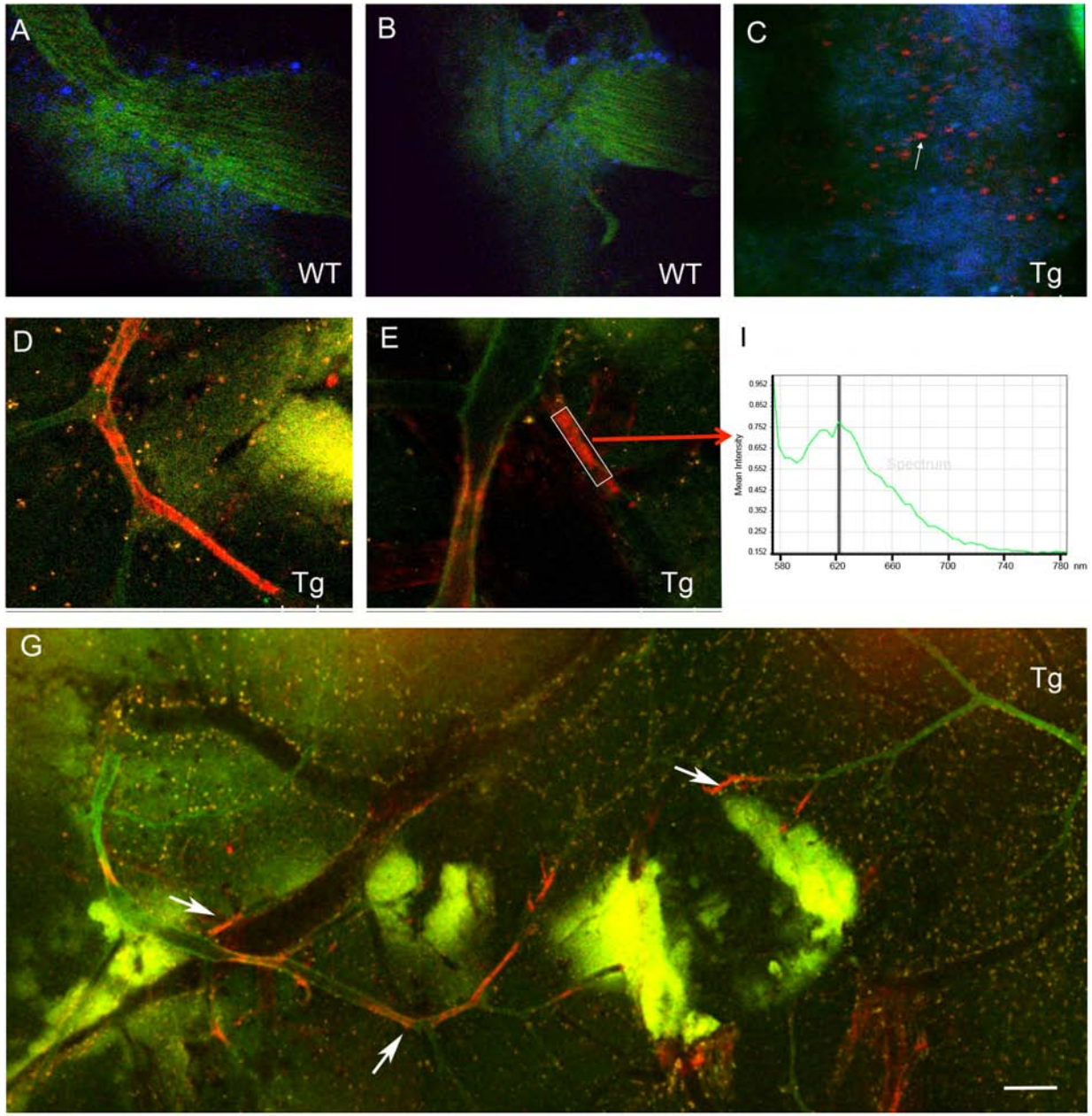

TTC
Fluorescence
[cps]
850
775
700
625
550
475
400
325
250
175
100
Hsp70

Figure 6


A
WT
B
WT
C
Tg
D
Tg
E
Tg
I
Mean Intensity
0.962
0.852
0.752
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0.552
0.452
0.352
0.252
0.152
Spectrum
580
620
660
700
740
780 nm
G
Tg