Heat Shock Protein 72 Overexpression Prevents Early Postoperative Memory Decline after Orthopedic Surgery under General Anesthesia in Mice

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ABSTRACT

Background: Problems with learning and memory are common after surgery in the elderly and are associated with high morbidity. Heat shock protein 72 (Hsp72) confers neuroprotection against acute neurologic injury. We hypothesized that overexpression of Hsp72 would prevent the development of postoperative memory loss.

Methods: C57BL/6 wild-type and Hsp72 overexpressing transgenic mice were randomly allocated to the following: control, isoflurane anesthesia alone, or tibial fracture during isoflurane anesthesia. Animals were trained 24 h before surgery using a fear conditioning protocol and assessed in their training environment and in a novel context on posttreatment days 1, 3, and 7. Microglial activation was assessed by immunostaining.

Results: Adult male C57BL/6 wild-type mice exhibited reduced memory evidenced by a decreased percentage freezing time on days 1 and 3 after anesthesia alone (58.8 ± 5, 46.5 ± 5 mean ± SEM) and after surgery (53.4 ± 6, 44.1 ± 7), compared with controls (78.8 ± 5, 63.4 ± 6; $P < 0.05$ and $P < 0.001$, respectively). Hsp72 mice showed no difference by treatment on any day. Similarly, nonhippocampal-dependent memory was significantly impaired on days 1 and 3 after surgery and day 3 after anesthesia. The genotype effect was significant on days 1 and 7. CD68-immunopositive activated microglia in the hippocampus varied modestly with subregion and time; on day 7, there was a significant treatment effect with no genotype effect, with more activated microglia after surgery in all regions.

Conclusion: Hsp72 overexpression is associated with prevention of postoperative hippocampal-dependent and -independent memory deficit induced by anesthesia and/or surgery. Memory deficit is not correlated with numbers of activated hippocampal microglia.

What We Already Know about This Topic

- Postoperative cognitive dysfunction is more common in the elderly
- Studies in mice have shown heat shock protein 72 (Hsp72) can protect the brain from stroke and ischemic insults

What This Article Tells Us That Is New

- In mice, Hsp72 overexpression was associated with prevention of postoperative hippocampus-dependent and -independent memory deficit induced by anesthesia alone or with orthopedic surgery

POSTOPERATIVE cognitive dysfunction (POCD) is characterized by long-term cognitive, including memory, impairment persisting at least 3 months after a major surgical intervention.[1,2] When POCD manifests soon after surgery, it may be referred to as early POCD.[3,4] POCD is defined as a change in performance on neuropsychological tests after surgery, compared with before, whereas postoperative delirium that manifests in the early postoperative period is diagnosed on the basis of the *Diagnostic and Statistical*

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*Manual of Mental Disorders*** which include fluctuating mental status, disturbance in attention, and may include memory deficits.[5] The relationship between these two is not clear. Early POCD may be linked to anesthesia,[6–8] as well as fluid and analgesic regimen.[3,8] Early POCD is seen more frequently after general anesthesia and is associated with higher mortality.[6,9,10] Steinmetz *et al.* showed that patients with early POCD are at higher risk of leaving the labor market prematurely because of disability or voluntary early retirement.[1] This condition has been noted in a diverse group of patients, particularly those undergoing cardiac and orthopedic procedures,[10,11] although others have suggested that the burden of POCD has been overestimated owing to statistical and methodological difficulties.[12]

Since its initial description, the worsened outcomes associated with POCD have stimulated a broad range of investigations to ascertain the prevalence, long-term consequences, and appropriate diagnostic criteria.[11,13] There is some evidence suggesting that the level of stress response, which depends on the type of insult and duration, may contribute to the development of POCD.[14,15] Despite the identification of risk factors for POCD,[16–18] the molecular mechanisms responsible for its development largely remain elusive, although central inflammatory response, specifically increased cytokines in the hippocampus, after surgery has been reported in a rat model of POCD,[19] and increased hippocampal interleukin (IL)-1β was suggested to play a role in memory deficit after infection.[20,21]

At the level of individual cells, heat shock proteins are induced by many forms of stress, and their expression is associated with resistance to a subsequent stress. Heat shock protein 72 (Hsp72) is a highly stress-inducible cytosolic heat shock protein that performs many cell-protective functions, including acting as a protein chaperone, regulating inflammation, and regulating cell death and survival pathways.[22] Reduced induction of heat shock proteins has been reported in aged rodents,[23] and under some circumstances, induced levels of Hsp72 may be insufficient to prevent the injurious effect of stress.[23–25] We have previously shown, in animal and cell models of stroke, that injury is reduced in animals or brain cells overexpressing Hsp72,[26] and that this is associated with reduced activation of the proinflammatory transcription nuclear factor κ light-chain enhancer of activated B cells (NFκB).[27] In addition to its roles in stress, survival, and inflammation, Hsp72 has also been implicated in learning and plasticity, with upregulation of heat shock cognate 70 reported in a learning paradigm[28] and changes in Hsp72 noted in the hippocampus of animals trained in a radial maze task.[29]

Only a few animal models of POCD have been developed to date.[19] To better study cellular and molecular mechanisms responsible for POCD, we developed a model using a surgery akin to arthroplasty, the most common major elective surgical procedure in the cohort of patients of an age in which POCD is common, and used it to test the hypothesis that Hsp72 overexpression can prevent or reduce POCD after orthopedic surgery under general anesthesia.

Materials and Methods

Animals

Experiments were performed in accordance with a protocol approved by the Stanford Animal Care and Use Committee of Stanford University (Stanford, California) and following the National Institutes of Health guidelines (National Institutes of Health, Bethesda, MD). All animals were supplied with food and water *ad libitum*. Temperature, humidity, and night-day cycle were maintained according to the standards set up by the research animal services at Stanford University. Adult male mice expressing a chimeric transgene of the rat-inducible Hsp72 gene (Hsp72-Tg) under control of the chicken-actin promoter and human cytomegalovirus enhancer were originally produced by Dillmann *et al.*[30] and back bred into the C57BL/6 background and maintained as heterozygous transgenics by always crossing with C57BL/6 wild-type (C57BL/6-WT) females, so littermates could be used for experiments after genotyping by polymerase chain reaction analysis of tail DNA. Brain levels of Hsp72 were previously shown to be increased approximately 10-fold in this strain.[27] C57BL/6-WT mice were purchased from Charles River Laboratories (Wilmington, MA).

Adult male mice were randomly allocated to one of three treatment groups: control (naïve animals without treatment (C), anesthesia alone (A), and tibial fracture surgery (S). Within each treatment group, separate groups were used for each assessment time point: days 1, 3, and 7. A total of four mice died in the postoperative period; all were from the surgery groups, two of each genotype, and were excluded from analysis. Animal ages were 3–14 months, with a median of 6 (range, 3–14) for C57BL/6-WT and 7 (3–14) for the Hsp72-Tg group. Animals weighed from 21 to 51 g, with a median of 37.2 (range, 23–51) for C57BL/6-WT and 32.0 (range, 21–49) for the Hsp72-Tg group.

Surgical Model

Animals from both genotypes either had anesthesia alone or anesthesia plus tibial fracture with pinning. General anesthesia, which lasted for 20 min, consisted of induction with 5% isoflurane in 30% oxygen in air at a flow rate of 1 l/min (vaporizer, model 100F; Ohio, Louisville, KY); anesthesia was maintained with 2.0 $\pm$ 0.4% isoflurane, approximately 1.4 minimal alveolar concentration for mice. The concentrations of isoflurane and oxygen were continuously monitored (VetEquip, Inc., Pleasanton, CA). Temperature was monitored throughout the surgical procedure by use of a rectal probe. Five minutes after induction of anesthesia, supplemental analgesia was provided by buprenorphine (0.3 mg/kg in saline) intraperitoneally in less than 1 ml given over 5 min.

** Diagnostics and Statistical Manual of Mental Disorders, fourth editon criteria. Available at: http://www.dsmivtr.org. Accessed April 9, 2010.

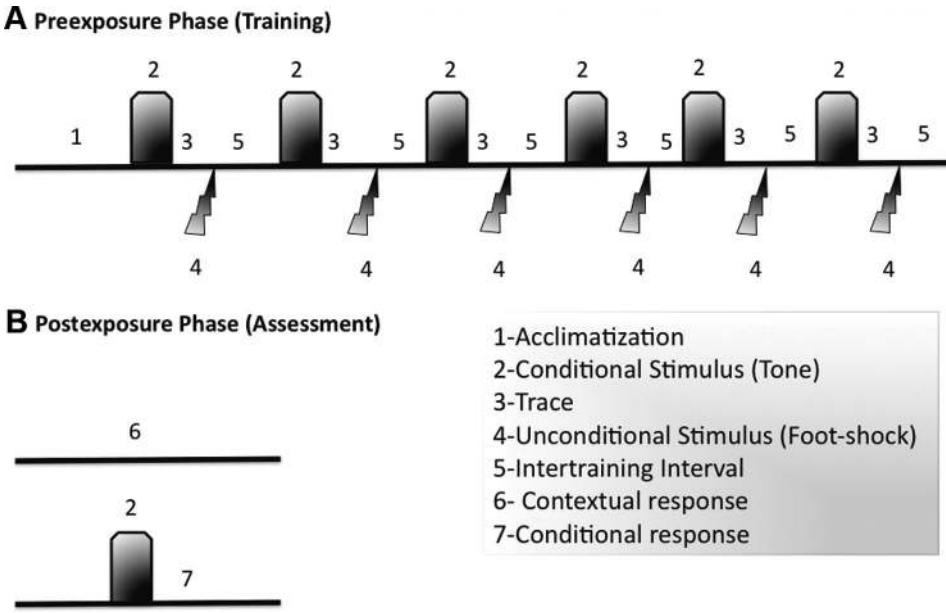

Fig. 1. Schematic representation of the fear-conditioning protocol. (*A*) Fear-conditioning training. (*B*) Contextual and conditional assessment after surgery and or anesthesia. Time is not to scale.

Although morphine is known to have effects on the immune response, buprenorphine does not alter the immune response at different concentrations up to 7 days after nociceptive stimuli;[31] therefore, it was chosen for this experiment. Eutectic mixture of local anesthetic was applied to the surgical site on completion of the surgical procedure, followed by daily application until day 3 postoperatively.

The surgical model was based on Harry *et al.*[32] In brief, after skin incision below the knee, soft tissue was reflected to expose bone, through which a 0.3-mm stainless steel rod was inserted into the medullary cavity. Once the tibia was internally fixated, the bone was fractured in the middiaphysis (tibial, midshaft) using surgical pliers. The wound was closed using a 2-0 Nylon suture (Ethicon, Inc., Somerville, NJ). Postintervention animals were left to recover on heated pads and then transferred to their own cages, where food and water were available.

Behavioral Tests

Fear Conditioning (FC). Room temperature was kept between 20° and 22°C and humidity between 35% and 55% during behavioral assessment. One day before surgery or anesthesia, animals were trained for FC to learn the task and establish long-term memory. Training and testing were performed in chambers with a video camera positioned at the top of the chamber to allow the subjects' behavior to be observed and recorded by an experimenter both on- and offline. The floor consisted of 32 stainless steel rods (1 mm in diameter) spaced 0.5 cm apart (center-to-center) and wired to a shock generator and scrambler to deliver a 0.70-mA foot shock.

The chambers were cleaned with a 5% sodium hydroxide solution scented with 0.2% mint extract, and pans containing a thin film of the same solution were placed underneath the grid floors. Background noise (60 dB) was provided by a fan positioned underneath the video camera.

The FC paradigm consists of a training phase before surgery and an evaluation phase after surgery when memory is assessed (fig. 1). A contextual interval of 25 s (trace), that is, the time between conditional and unconditional stimuli for assessment of hippocampal-dependent memory, was chosen as it was previously shown that less than 18 s is not enough for establishment of hippocampal memory, and above 30 s, other regions of the brain may be involved.[33] If the conditional stimulus (tone) is too weak (less than 50 dB), it does not increase connectivity within the learning centers, and if too strong, more than 75 dB, it acts as an unconditional stimulus; therefore, we chose a 70-dB tone. For the unconditional stimulus (shock) used to generate fear, too weak a stimulus may not trigger fear, whereas too strong may activate pain pathways, creating a confounding variable; therefore, we chose a 0.70-mA shock, which triggers maximum freezing behavior.[34]

Training was carried out 24 h before surgery. Mice were allowed to familiarize themselves with the surroundings (Context) for 120 s, followed by a 20-s, 70-dB tone (conditional stimulus) and then a delay of 25 s. This contextual interval was terminated by an unconditional stimulus, a 0.70-mA stimulus for 2-ms electrical foot shock. After six pairs of conditional-unconditional stimuli, the mice learned the association and established long-term memory. The pairs of conditional-unconditional stimuli were separated by random intervals from 45 to 60 s, the intertraining interval. The intertraining interval allowed the mice to disengage from the process of association before a new set of stimuli was introduced. After fear was established, the mice would freeze and

the percentage of time spent not moving was determined (% freezing time). Freezing time measured during exposure to the known context, or after a conditional stimulus in the known context, reflects hippocampal-dependent memory whereas assessment during delivery of the conditional stimulus (tone) assesses hippocampal-independent memory.

After surgery or anesthesia, we assessed hippocampal-dependent and -independent memory in the known context and in a novel context/environment 1, 3, and 7 days after surgery, exposure to anesthesia, or neither. Analysis was performed with freeze-frame/freeze-view modes. Threshold for freezing was chosen according to the individual animal's motion index histogram. An epoch of 0.75 s was used to count an episode of freezing. Percentage of total time spent freezing was used as an indicator of memory formation during training and retrieval of information after treatment.

Pain Threshold. Separate groups of C57BL/6-WT and Hsp72-Tg mice that were subjected to neither anesthesia nor surgery were assessed for sensory and motor activity to detect possible behavioral differences between C57BL/6-WT and Hsp72-Tg mice. Pain threshold was assessed using a hot plate at 55°C (Integrated Information Technology Center, Life Science, Inc., Woodland Hills, CA) to test acute thermal nociception. The latency to the nociceptive response was the time until the mouse started licking the hind paw or jumping. A cutoff time of 30 s was applied to protect animals from burn injury or distress.

Acoustic Startle Reflex. Acoustic function was tested in a mouse startle-reflex chamber made of Plexiglass vented with holes that was attached to a spring-loaded platform hinged to a support stand. A transducer assessed the displacement force of the animal chamber. The entire apparatus was housed in an acoustically insulated wooden box containing speakers (Med Associates, Inc., St. Albans, VT). The startle stimulus consisted of applying 110 dB of white noise lasting 10 s. Latency to startle response was used as a measure of sensorineural hearing (acoustic reflex). The Stanford Neuroscience Behavior Phenotyping and Pharmacology Behavior Core, directed by Dr. Mehrdad Shamloo, Ph.D., provided all behavioral equipment and training, except the use of the smart-home cage.

Assessment of Spontaneous Activity. Spontaneous locomotion was assessed for a period of 60 min, 72 h before surgery using the Smart-Home Cage System [Afasci, Inc., Redwood City, CA; loaned by Dr. Xinmin (Simon) Xie, M.D., Ph.D.] to compare C57BL/6-WT and Hsp72-Tg mice. Active count and travel distance were used as measures of motor activity detected as breaks of infrared sensors crossing the cage.

Immunohistochemistry and Morphometric Analysis

Animals were sacrificed 2 h after behavioral assessment on days 1 and 7. All animals received terminal anesthesia (isoflurane 5%), followed by transcardial bleeding and perfusion with 20 ml cold saline, followed by 4% paraformaldehyde. Brains were removed, postfixed, and then sections 50 μm thick were obtained using a vibratome (VT 1000 S; Leica Microsystems, Wetzlar, Germany). Immunohistochemistry was performed on free-floating sections under moderate shaking. All washes and incubations were done in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100. Sections were incubated for 1 h in blocking solution (0.1 M phosphate buffer, 0.3% Triton X-100, and 5% equine serum). After three washes in phosphate buffer, sections were incubated overnight at 4°C with a rat antimouse antibody for CD68 (diluted 1:200, MCA1957GA; Serotec, Raleigh, NC). Sections were then rinsed in phosphate buffer and incubated for 2 h at room temperature with an Alexa 488–conjugated goat antirat (diluted 1:200; Invitrogen, Carlsbad, CA) secondary antibody, washed, and mounted on glass slides using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Immunostaining was absent when the primary antibody was omitted.

For each animal, the number of CD68-immunoreactive cells in four subregions of the hippocampus, CA1, CA2, CA3, and hilus of dentate gyrus (−1.70 to −2.18 mm relative to bregma) were estimated from photomicrographs with a counting frame size of 0.4 mm^2. Sections immunostained for CD68 were photographed at 20× magnification using a digital camera attached to a Zeiss Axiovert 20 0M inverted epifluorescence microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). The total numbers of CD68-immunoreactive cells were counted in three counting frames per region (total, 12 frames per animal) using ImageJ software (National Institutes of Health). The numbers of cells in the three frames per regions were then averaged. The age range of animals studied was 6–12 months (C57BL/6-WT: 7.5 months ± 2.3; Hsp72-Tg: 8.2 months ± 2.6; mean ± SD; $P = 0.6$), not different between genotypes.

Statistical Analysis

The observer was unaware of the treatment received by mice at the time of assessment of behavior and assessment of cell counting for immunohistochemistry. Results were expressed as mean ± SEM. To study the treatment effect on memory and microglial activation, repeated two-way ANOVA and a *post hoc* Tukey multiple comparison test for genotype and condition was performed using a statistical package (GraphPad Prism 5; GraphPad, Inc., San Diego, CA; or SAS 9.1; SAS, Inc., Cary, NC). A P value of less than 0.05 was considered statistically significant.

Results

Development of the FC Paradigm

We developed a FC paradigm in which mice learned conditional reflexes to establish both hippocampal-dependent and -independent long-term memory for use to assess retrograde memory after surgery. In developing our paradigm, we

Table 1. Baseline Behavioral Variables

	C57BL6-WT	Hsp72-Tg
Acoustic reflex at 110 dB, mean ± SEM	46.0 ± 3.0	50.1 ± 5.9
Latency to startle (s) (95% CI)	(38.3–53.9)	(36.0–64.4)
—, n	6	8
Pain threshold, mean ± SEM	18.8 ± 1.6	19.09 ± 1.8
Latency to pain (s) (95% CI)	(15.4–22.3)	(15.1–23.0)
—, n	17	17
Motor activity, mean ± SEM	457.5 ± 39.8	416.2 ± 99.2
Travel distance (cm) (95% CI)	(355.0–560.1)	(160.9–671.4)
—, n	6	6
Long-term memory, mean ± SEM	75.6 ± 3.3	82.1 ± 2.9
Freezing time after training (%) (95% CI)	(69.0–82.3)	(76.23–88.1)
—, n	55	45

C57BL6-WT = C57 black 6–wild-type mice; Hsp72-Tg = heat shock protein 72 –transgenic mice.

standardized the time of day for training, the number of conditional stimuli, and the intensity of stimuli for establishment of long-term memory. We investigated the time of exposure to a familiar environment called context or contextual period before a subsequent conditional stimulus.

There was no difference in acquisition between training during light and dark times of the cycle; however, animals trained during daytime froze more than mice trained at night during the assessment phase, indicating that recollection was better during daytime. Therefore, animals were trained and assessed during the light cycle, consistent with previous results.[35]

We trained a cohort of C57BL/6-WT mice with a short (two pair of conditional-unconditional stimuli), medium-length (six pair of conditional-unconditional stimuli), and a long paradigm (eight pair of conditional-unconditional stimuli) to ascertain the numbers of conditional-unconditional pairs of stimuli required to develop and consolidate new information (acquisition). We found that two or eight pairs were less effective for establishment of long-term memory than six (data not shown). Therefore, six pairs were used for the subsequent study. The pairs of conditional-unconditional stimuli were separated by random intervals between 45 and 60 s each to prevent associative conditioning in between trials.

Finally, we assessed the time required to establish long-term memory. We identified 24 h as the minimum interval to establish long-term memory. This was assessed in a different group of C57BL/6-WT mice before this experiment. Animals were trained with a set of conditional stimuli and then assessed for memory formation in their training context and also in a novel context at different time points. Once we identified that memory was established at 24 h posttraining, we decided to perform surgery at 24 h posttraining (data not shown).

Behavioral Phenotype of Naïve Mice

There were no differences at baseline between C57BL/6-WT and Hsp72-Tg mice for acoustic reflex, pain threshold, or motor spontaneous activity (table 1). Acquisition of memory during training before surgery was similar between C57BL/6-WT and Hsp72-Tg. Asymptotic performance was achieved by the end of the sixth training session (fig. 2). There were no differences before surgery in acquisition during trace intervals, conditional, unconditional response, or intertraining intervals during training between the strains (data not shown).

Physiologic Variables

No statistical differences were detected among monitored physiologic variables between groups. Animal ages ranged from 3 to 14 months, with a median age of 6 months for the C57BL/6-WT group and 7 months (range, 3–14) for the Hsp72-Tg group. Animals weighed from 23 to 51 g, with a median weight of 37.2 g for the C57BL/6-WT group and 32.0 g (range, 21–49 g) for the Hsp72-Tg group. Rectal temperature during surgery and anesthesia was 35.9 ± 1.0°C in C57BL/6-WT mice and 36.1 ± 0.7°C in Hsp72-Tg.

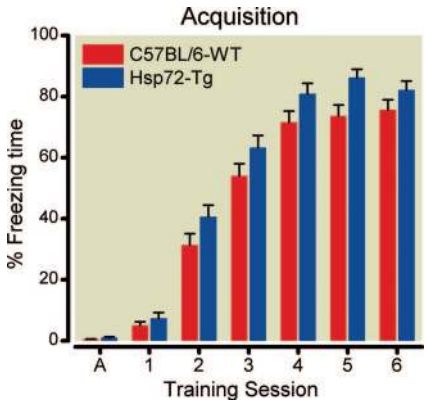

Fig. 2. Acquisition of memory during the training phase of the fear-conditioning protocol is similar in C57BL/6-WT (n = 55) and Hsp72-Tg mice (n = 45). Both mouse strains achieved maximal performance by the sixth training session. No differences in establishment of long-term memory were found between C57BL/6-WT and Hsp72-Tg mice. Values are mean ± SEM. Hsp72-Tg = heat shock protein 72 transgenic; WT = wild-type C57BL6.

Respiratory rate was between 20 and 30 breaths/min in both groups during maintenance of anesthesia with 1.4–1.6% isoflurane. Major bleeding was observed in three animals (1 C57BL/6-WT, 2 Hsp72-Tg), which were removed from the study and sacrificed immediately.

Effect of Surgery on Behavior

Contextual conditional response is a hippocampal-dependent memory task.

Treatment Effect. C57BL/6-WT mice showed reduced memory, based on treatment on days 1 (n = 14 for C, A; n = 12 for S) and 3 (n = 11 all groups) after anesthesia alone; mean ± SEM (95% confidence interval [CI]) values were as follows: 58.8 ± 5 (48–69.6) and 46.5 ± 4.8 (35.7–57.2), respectively, and after surgery also on days 1 and 3; 53.4 ± 5.8 (40.6–66.2) and 44.1 ± 6.9 (28.7–59.5), respectively ($P < 0.05$ and $P < 0.001$, respectively), compared with controls: 78.78 ± 4.6 (68.6–88.8) and 63.4 ± 6.2 (49.5–77.4), respectively, but not on day 7 (n = 11 all groups) (fig. 3). Hsp72-Tg mice showed no difference by treatment on any of the days (fig. 3). On day 3, the genotype had a strong effect on the contextual conditional response ($P < 0.0001$), whereas on day 7, no differences between groups were observed (fig. 3C). A subanalysis to identify a possible age-related difference on memory on days 1 and 3 after surgery and/or anesthesia was performed, and no age-related statistical differences were found (fig. 4). It should be noted, however, that the study was not designed or powered to assess the effect of age.

Hippocampal-independent Memory

Freezing behavior during the conditional stimulus assesses hippocampal-independent memory (*e.g.*, prefrontal cortex and amygdale). We assessed the dependence of the nonconditional response (tone) on either treatment or genotype (fig. 5).

Treatment Effect. We found that the response of Hsp72-Tg mice was not affected by treatment on any day, whereas C57BL/6-WT mice showed reduced memory after surgery on days 1 (n = 12) and 3 (n = 11); mean ± SEM (95% CI): 31.9 ± 5.2 (20.3–43.5) and 36.6 ± 6.0 (23.1–50.0), respectively, and day 3 after anesthesia 37.3 ± 3.5 (29.5–45.1) ($P < 0.05$), compared with the control group (n = 14); mean ± SEM (95% CI): 49.2 ± 5.04 (38.3–60.1) or anesthesia alone group (n = 14); mean ± SEM (95% CI): 46.1 ± 3.5 (38.5–53.8) on day 1, whereas those exposed to anesthesia alone differed from control only on day 3.

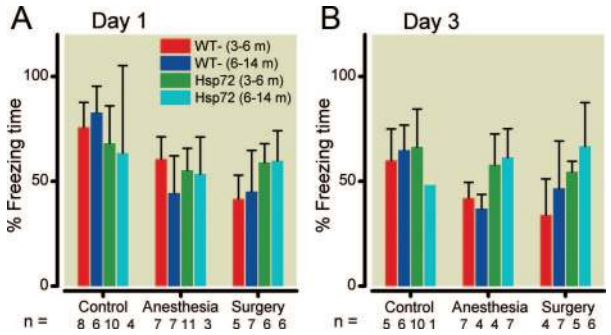

Fig. 4. Contextual conditional response was reanalyzed as percent freezing time after separating mice by age into two groups (young adult: 3–6 m; adult: 6.1–14 m) on days 1 and 3 after surgery. Groups are control, anesthesia (A), and surgery. Percent freezing time on day 1. Percent freezing time on day 3 (*B*). Values are mean ± SD. N for each group is shown under the bar. There were no statistically significant differences between the age groups for any condition. Hsp72-Tg = heat shock protein 72 transgenic; WT = wild-type C57BL6.

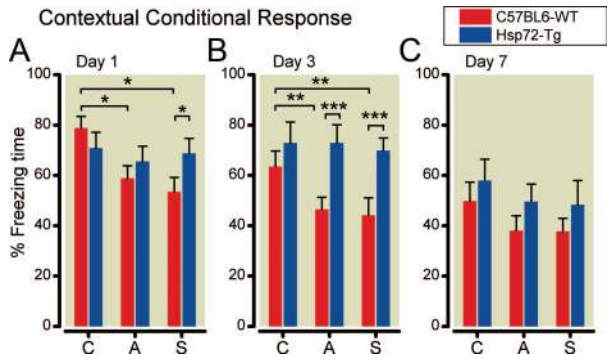

Fig. 3. Hippocampal-dependent memory is impaired after anesthesia or surgery. Contextual conditional response, a form of hippocampal-dependent memory, was assessed in separate groups of mice as % freezing time on days 1 (n = 14/group), 3 (n = 11), and 7 (n = 11) after surgery. Treatment groups are control (C), anesthesia (A), and surgery (S). Percent freezing time on day 1 (*A*). Percent freezing time on day 3 (*B*). Percent freezing time on day 7 (*C*). Values are mean ± SEM. Significant differences are expressed as follows: *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. Hsp72-Tg = heat shock protein 72 transgenic; WT = wild-type C57BL6.

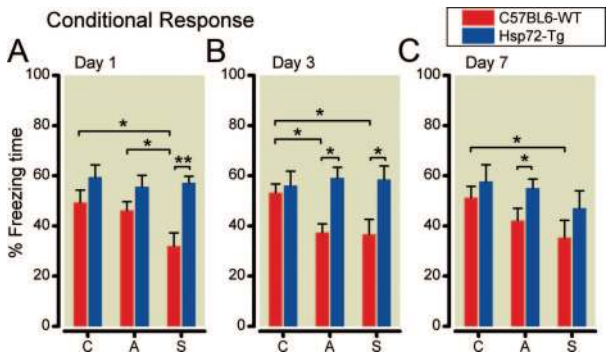

Fig. 5. Hippocampal-independent memory is impaired after anesthesia or surgery. Conditional response, a form of hippocampal-independent memory, was assessed as percent freezing time following the tone on days 1 (n = 14), 3 (n = 11), and 7 (n = 11) after treatment. Groups are control (C), anesthesia (A), and surgery (S). (*A*) Percent freezing time on day 1. (*B*) Percent freezing time on day 3. (*C*) Percent freezing time on day 7. Values are mean ± SEM. Significant differences are as follows: *$P < 0.05$. Hsp72-Tg = heat shock protein 72 transgenic; WT = wild-type C57BL6.

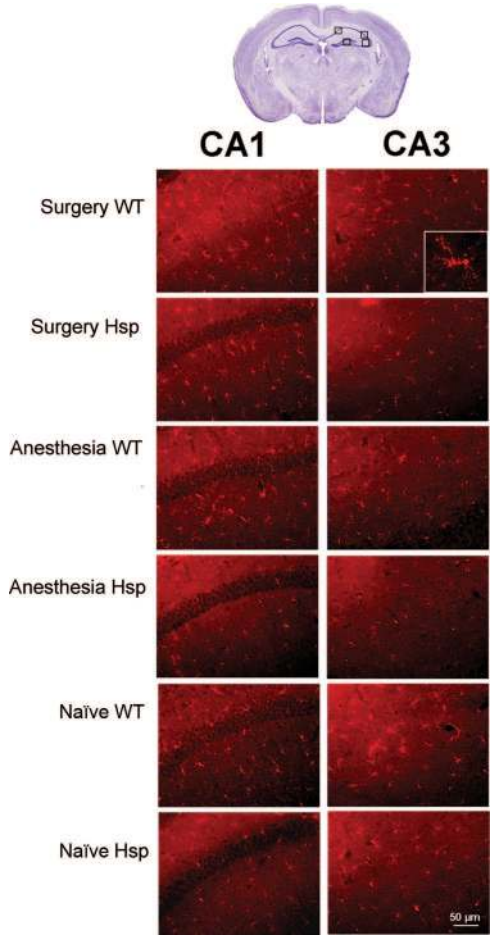

Fig. 6. Microglial activation was assessed by immunoreactivity for CD68 and cell counting. The subregions within the hippocampus assessed in this study are indicated by squares on the brain section at the top. Representative photomicrographs of the cornus ammonis (CA)1 and CA3 subregions of the hippocampus immunostained for CD68 on day 1 are shown for each of the conditions. Scale bar: 50 μm. In the insert, the scale bar represents 10 μm. Hsp = heat shock protein; WT = wild-type C57BL6.

Genotype Effect. We found that on day 1, the nonconditional response was strongly dependent on genotype after surgery ($P < 0.001$). On day 3, the genotype effect was significant after anesthesia alone and surgery ($P < 0.05$). Such dependence was still significant on day 7 for the surgery group by two-way ANOVA ($P < 0.05$), whereas the anesthesia alone C57BL/6-WT mice did not differ from control (fig. 5). No age-related differences on days 1, 3, and 7 after surgery and anesthesia were found (data not shown).

Microglial Activation

Activated microglia assessed by CD68 immunostaining were more prominent in animals that underwent surgery (fig. 6) and were observed throughout the brain, but there were also some region-specific differences between treatment groups. We analyzed numbers of CD68-positive cells in the CA1, CA2, CA3, and hilus of dentate gyrus, subsections of the hippocampal formation on days 1 and 7 after treatment. Two-way ANOVA for genotype, treatment, and their interaction were performed, and the genotype effect did not reach significance after Benjamini-Hochberg correction for multiple testing in any subsections (raw P values were 0.0638, 0.0405, 0.0694, and 0.0277 in CA1, CA2, CA3, and hilus of dentate gyrus, respectively, and adjusted P values were all 0.0694). In contrast, on day 7, there was a significant treatment effect with no genotype effect, with more activated microglia after surgery in all hippocampal regions and both genotypes (tables 2 and 3).

Discussion

This study establishes a new paradigm for postoperative cognitive dysfunction after anesthesia and tibial fracture surgery, in which FC training is performed before anesthesia and surgery, and memory deficits in C57BL/6-WT mice are detectable through 7 days postexposure. This model is sensitive to changes induced by exposure to anesthesia alone, as well as changes due to the combined effects of anesthesia and sur-

Table 2. Number of CD68 Immunopositive Cells in CA1 and CA3 on Days 1 and 7

Condition	CA1		CA3	
C - C57BL/6-WT (4)	24.2 ± 1.4	—	23.6 ± 1.3	—
C - Hsp72-Tg (7)	25.6 ± 1.0	—	22.9 ± 0.9	—
	Day 1	Day 7	Day 1	Day 7
A - C57BL/6-WT (3)	31.3 ± 2.5	25.2 ± 2.8	27.0 ± 1.3	26.5 ± 2.2
A - Hsp72-Tg (4)	24.1 ± 2.0	30.3 ± 0.9	21.1 ± 1.9	29.2 ± 0.7
S - C57BL/6-WT (4)	32.1 ± 2.2	34.8 ± 1.3	29.00 ± 1.6	32.7 ± 1.7
S - Hsp72-Tg (4)	26.6 ± 3.7	33.3 ± 0.8	25.9 ± 3.6	33.5 ± 1.1

Values are number of immunopositive cells within a 0.4-mm^2 counting frame, mean ± SEM. The number of animals in each experimental group is indicated in parentheses. Analysis by two-way ANOVA for treatment and genotype did not show any significant differences on day 1. On day 7, the model showed a very significant treatment effect (P values for cornus ammonis (CA)1 and CA3 were both <0.0001), but not a genotype or interaction effect. On day 7 in both CA1 and CA3, surgery (S) was significantly different from both anesthesia (A) and control (C) (P values for S *vs.* A and S *vs.* C were 0.0059 and <0.0001 in CA1, respectively, and are 0.0104 and <0.0001 in CA3, respectively) using the Tukey *post hoc* test. In CA3 on day 7, A and C were also significantly different ($P = 0.016$).

C57BL/6-WT = C57 black 6–wild-type mice; Hsp72-Tg = heat shock protein 72–transgenic mice.

Table 3. Number of CD68 Immunopositive Cells in CA2 and Hilus of Dentate Gyrus on Days 1 and 7

Condition	CA2		Hilus of Dentate Gyrus	
C: C57BL/6-WT (4)	24.7 ± 3.5	—	25.4 ± 4.6	—
C: Hsp72-Tg (7)	23.3 ± 1.1	—	19.9 ± 1.1	—
	Day 1	Day 7	Day 1	Day 7
A: C57BL/6-WT (3)	25.4 ± 8.5	27.2 ± 5.1	23.0 ± 7.1	25.2 ± 5.0
A: Hsp72-Tg (4)	23.2 ± 5.4	27.6 ± 4.6	21.50 ± 5.5	27.1 ± 3.4
S: C57BL/6-WT (4)	30.3 ± 3.8	35.1 ± 3.9	25.5 ± 4.4	31.4 ± 4.1
S: Hsp72-Tg (4)	24.7 ± 7.2	33.1 ± 3.9	21.7 ± 5.4	31.8 ± 2.8

Values are number of immunopositive cells within a 0.4-mm^2 counting frame, mean ± SEM. The number of animals in each experimental group is indicated in parentheses. Analysis by two-way ANOVA for treatment and genotype showed a significant genotype effect (*P* values for cornus ammonis (CA)2 and dentate gyrus (DG)3 were 0.0405 and 0.0277, respectively) on day 1. On day 7, both regions showed very significant treatment effects (*P* values for CA2 and DG <0.0001 and 0.0002, respectively), but not genotype effect. A Tukey *post hoc* test showed that on day 7, surgery (S) was significantly different from both anesthesia (A) and control (C) in both CA2 and DG (*P* values for S *vs*. A and S *vs*. C were 0.0051 and <0.0001 in CA2, respectively, and were 0.0154 and <0.0001 in DG, respectively). C57BL/6-WT = C57 black 6–wild-type mice; Hsp72-Tg = heat shock protein 72–transgenic mice.

gery. Mice overexpressing Hsp72 showed no cognitive impairment after the same treatments. This is consistent with a previous study showing heat shock pretreatment was associated with improved Morris water maze performance after traumatic brain injury.[36]

Whereas both hippocampal-dependent and -independent memory was impaired by both anesthesia and surgery, the effect of anesthesia alone was most notable on day 3. By day 7, no treatment showed a difference in hippocampal-dependent memory, whereas the WT surgical group still showed significant hippocampal-independent memory deficit at 7 days. Although we cannot rule out an effect of anesthetic on blood pressure or blood gases, the anesthetic was fairly brief and held constant for all groups, so any hemodynamic effect was unlikely to be different between genotypes. In addition, neither reduced blood pressure, oxygenation, nor depth of anesthesia were associated with POCD.[2,37] Further studies are warranted to examine the effect of depth of anesthesia on memory retrieval, as the current literature is unclear on this issue.

Isoflurane has been reported to suppress learning in a dose-dependent fashion.[38] Further, it has been demonstrated that isoflurane interferes with anterograde, but not retrograde, memory for Pavlovian fear conditioning.[39,40] In that study, FC training took place during delivery of anesthesia, whereas in this study, we trained animals before surgery or anesthesia. This allowed us to remove the influence of the acquisition phase on assessment of memory postoperatively. Whereas previous studies have demonstrated that ketamine, xylazine, and acepromazine anesthesia interfere with retrograde memory after FC training,[41] to the best of our knowledge, this is the first report showing impaired recall of FC after isoflurane-induced anesthesia. Further, this phenomenon is not observed in mice overexpressing the chaperone, Hsp72. However, a dose-response curve and exposure to different anesthetic agents are warranted to identify the effect of concentration and type of anesthesia on induction of Hsp72 in the future.

Work from several laboratories, including ours, suggests that an inflammatory component could contribute to impaired memory, and that inflammatory changes, particularly increased levels of IL-1β in the hippocampus, may be implicated in the setting of infection[18,39] or, as we previously reported, after splenectomy surgery[19] or traumatic brain injury.[42] It is likely that properly regulated levels of IL-1β are required for memory, as the lack of hippocampal IL-1β interferes with cognition and IL-1β is necessary for memory formation. Due to this previous work suggesting a link between inflammation in the hippocampus and memory impairment, we assessed the number of activated microglia in subregions of the hippocampus; however, we did not observe a significant correlation. This suggests either that the memory deficit is not correlated with numbers of activated microglia or that the relationship is complex, possibly different, early and later after injury.

Compared with previous reports linking inflammation and impaired memory, there are several likely factors that may explain why we did not observe a correlation with numbers of activated microglia. Our previous work,[19] and that of Maier *et al.*,[20,21] was performed in rats. There are known differences in immune response between rats and mice. Our previous article used a model of splenectomy, which directly affects the immune system, and a learning paradigm that was employed only after treatment, not, as here, a memory that was established before treatment and assessed after treatment. Microglial activation was not assessed in that report, only evidence of astrocyte activation. Thus, there are many differences between the two studies. Still, it is surprising that we did not find a stronger correlation between numbers of microglia and memory impairment. This could reflect the observation that microglia can play a positive role in plasticity, as well as contribute to memory impairment, in some settings. Conceivably, the early activation is deleterious, but the late activation may not be and, in contrast, may contribute to plasticity and the reestablishment of connectivity. Fur-

ther studies will be needed to test this hypothesis. In the current study, it is also possible that differences in peripheral inflammatory response could have influenced the response to injury, and this could have been affected by Hsp72 overexpression. Further studies would be needed to evaluate this possibility.

Hsp72 was previously shown to be lower in DBA mice that had learned a radial maze, but unchanged in C57BL6 mice after learning the maze,[29] whereas in contrast, heat shock cognate 70 was increased in rats learning the Morris water maze.[28] The exact relationship between plasticity and expression levels of Hsp72 is thus not yet clear. We found no difference in acquisition of FC between C57BL/6-WT and Hsp72-Tg mice that chronically overexpressed Hsp72 in all cell types, consistent with another report.[43] The differences observed after anesthesia/surgery may reflect differences in Hsp72 levels preceding exposure to anesthesia and/or surgery as well as responses to these exposures that varied with genotype and age. Whether the different genotypes had different changes in Hsp72 expression patterns after anesthesia and surgery is unknown. However, the pathways underlying memory acquisition, transition from short- to long-term memory, and memory retrieval are not fully understood, so it was not possible to evaluate possible differences in Hsp72 levels in the relevant pathways or how they may have changed after exposure to anesthesia and surgery. New investigations to explore the effect of age on memory retrieval, and to measure Hsp72 in different brain regions, may shed light on these factors. We are also not able to determine, at this point, whether effects of Hsp72 directly on memory processes is most important in the differences observed or to what extent the differences reflect a downstream effect *via* modulation of inflammation or other mediators that also affect memory.

Retrieval of information was fully protected in Hsp72-Tg mice up to day 7, although our sensitivity is reduced at this point, as all mice showed reduction in freezing time with time after training. These findings suggest that Hsp72 is important either to memory retrieval or protection from a process that degrades memory retrieval after anesthesia or surgery. Hsp72 is known to inhibit inducible nitric oxide synthase gene transcription *via* the inhibition of activation of its transcription factor, NFkB.[27,44] Hsp72 also stabilizes mitochondrial membrane potential and ameliorates reactive oxygen species production.[45] Whether the observed protection of memory involves reduced oxidative stress, such as from inhibition of induction of nitric oxide synthase, inhibition of cytokine production, or both, remains to be evaluated.

In summary, Hsp72 overexpression is associated with full maintenance of memory after isoflurane anesthesia and/or surgery. However, whether the mechanism of this improved cognitive performance, compared with WT, reflects direct effects on processes in the pathways serving memory establishment and retrieval and/or modulation of inflammation, which, in turn, affects memory, still remains to be elucidated.

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