Glial Expression of Small Heat Shock Proteins Following an Excitotoxic Lesion in the Immature Rat Brain

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ABSTRACT Heat shock proteins (HSPs) are chaperones induced under pathological conditions and involved in protein stabilization and cellular protection. In this study, we have evaluated the expression pattern of the glial cell–related HSP27, HSP32, and HSP47 following an excitotoxic lesion in the immature rat brain. Postnatal day 9 rats received an intracortical injection of N-methyl-D-aspartate and tissue was processed immunohistochemically for HSPs and double labeling using astroglial and microglial markers. HSP expression was quantified by image analysis. Excitotoxic damage caused primary cortical degeneration and secondary damage in the corresponding thalamus. In the injured cortex, reactive microglia/macrophages expressed HSP32 from 10 h until 14 days postlesion (PL), showing maximal levels at days 3–5. In parallel, most cortical reactive astrocytes showed expression of HSP47 from 10 h until 14 days PL and a population of them also displayed HSP27 labeling from 1 day PL. In addition, some cortical reactive astrocytes showed a temporary expression of HSP32 at day 1. In general, astroglial HSP expression in the cortex achieved maximal levels at days 3–5 PL. In the damaged thalamus, HSP32 was not significantly induced, but reactive astrocytes expressed HSP47 and some of them also HSP27. Thalamic astroglial HSP induction was transient, peaked at 5 days PL and reached basal levels by day 14. The injury-induced expression of HSP32, HSP27, and HSP47 in glial cells may contribute to glial cell protection and adaptation to damage, therefore playing an important role in the evolution of the glial response and the excitotoxic lesion outcome. HSP32 may provide antioxidant protective mechanisms to microglia/macrophages, whereas HSP47 could contribute to extracellular matrix remodeling and HSP27 may stabilize the astroglial cytoskeleton and participate in astroglial antioxidant mechanisms. *GLIA 38:1–14, 2002.*

INTRODUCTION

Heat shock proteins (HSPs) are molecular chaperones involved in protein stabilization during synthesis, folding, and assembling, participating in cellular protection by helping in the restoration of function of denaturing proteins (Samali and Orrenius, 1998; Sharp et al., 1999). Several families of HSPs, classified according to their molecular weights, have been identified in different organisms and play diverse functions within the cell, generally contributing to protective or adaptive responses against cellular stress (Samali and Orrenius, 1998; Sharp et al., 1999). Accordingly, events of the intracellular stress cascade such as free radical generation or accumulation of damaged proteins are

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the main factors triggering HSP synthesis, and the induction of HSPs seems to correlate with the ability of individual cells to survive stressful conditions (Bergeron et al., 1997).

In the brain, HSPs are often induced under several pathological conditions such as ischemia, excitotoxicity or neurodegenerative diseases (Sharp et al., 1999). In the last decade, extensive work has focused on the induction of HSP70 expression, mainly in neuronal cells, in response to ischemia, traumatic injury, epilepsy, hyperthermia, drug administration, and neurodegenerative diseases (Li and Mivechi, 1999; Sharp et al., 1999), where this HSP has been shown to provide neuroprotective mechanisms and neuronal rescue (Sharp et al., 1999). In contrast, fewer is known about the induction of HSPs in glial cells after neuropathological conditions. Several recent studies indicate that HSPs expressed in glial cells principally belong to the group of small-molecular-weight HSPs, including HSP32, HSP27, and HSP47 (Sharp et al., 1999), which could play an important role in the metabolic changes undergoing the glial response to neural damage.

HSP32 is the inducible form of the enzyme heme oxygenase (also called heme oxygenase-1, HO-1) and has been implied in the cellular protection against oxidative stress, metabolizing heme groups to antioxidant metabolites (Maines, 1997). HSP27 has been involved in the stabilization of cytoskeletal proteins and in the protective mechanisms against oxidative stress by abolishing the burst of intracellular reactive oxygen species (ROS) (Mehlen et al., 1996b). Finally, HSP47 is a collagen-specific molecular chaperone and its expression has been correlated with extracellular matrix stabilization (Tasab et al., 2000). Although the importance of these small HSP in the protection against oxidative stress and the mechanisms of cellular defense seems to be clearly established, studies after excitotoxic brain injury, where oxidative stress plays a major role, are scarce and exclusively focused on adult brain damage (Plumier et al., 1996; Kato et al., 1999; Nakaso et al., 1999; Gilberti and Trombetta, 2000). In this sense, in the last few years we have focused on the study of the processes undergoing neuronal excitotoxicity and the associated glial and inflammatory response in the immature rat cortex, describing the astroglial and microglial responses, including the expression of cytokines and inflammatory mediators (Acarin et al., 1999b, 1999c, 2000a, 2000b, 2001). Postnatal cortical brain damage is associated with enhanced plasticity that results in better lesion outcome and sparing of neuronal connections (Kolb, 1990; Kolb et al., 1998). In this sense, the injury-induced expression of HSP32, HSP27, and HSP47 in glial cells may contribute to glial cell protection and adaptation to damage, therefore playing an important role in the evolution of the glial response and the excitotoxic lesion outcome. Accordingly, the aim of this study was to evaluate the temporal and cellular pattern of expression of HSP32, HSP27, and HSP47 following an excitotoxic cortical lesion in the immature rat brain.

MATERIALS AND METHODS

Excitotoxic Lesions

Long-Evans black-hooded 9-day-old rats of both sexes (day of birth equals day 0; Harlan Sprague-Dawley) were used in this study. Under ether anesthesia, each pup was placed in a stereotaxic frame adapted for newborns (Kopf) and the skull was opened using a surgical blade. Thirty-seven nanomols of N-methyl-D-aspartate (NMDA; Sigma, M-32362) diluted in 0.15 μl of saline solution (0.9% NaCl) were injected into the right sensorimotor cortex at the level of coronal suture with a 0.5 μl Hamilton microsyringe. After suture, pups were placed on a thermal pad for 2 h to maintain normothermia before they were returned to their mothers. In control animals, the same procedure was followed except that they received an injection of 0.15 μl of saline solution. Four NMDA-injected animals and two saline-injected control animals were used for each of the six postinjection survival times. All efforts were made to minimize animal suffering. All these procedures received approval of the ethics committee of the Universitat Autònoma de Barcelona, in agreement with European Community directives.

Fixation and Histology

At 10 h, 1, 3, 5, 7, and 14 days after NMDA or saline injection, rats were anesthetized by ether inhalation and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were immediately removed, immersed in the same fixative for 4 h, and cryoprotected in a 30% sucrose solution before being quickly frozen with dry CO_2. Moreover, noninjected animals of 9, 10, 12, 14, and 16 days of age were included as additional controls in the corresponding groups and were processed together with injected animals. Frozen coronal sections (30 μm thick) were obtained using a Leitz cryostat. Alternate sections were mounted on gelatin-coated slides or stored free-floating in antifreeze buffer. Sections mounted on gelatin-coated slides were stained with toluidine blue for routine histological examination. Stored free-floating sections were processed with the immunohistochemical techniques.

HSP Immunohistochemistry

Parallel free-floating sections were processed immunohistochemically for the demonstration of HSP32, HSP27, and HSP47. After endogenous peroxidase blocking with 2% H_2O_2 in 70% methanol, sections were treated with buffer blocking (BB): 10% fetal calf serum (FCS) in Tris-buffered saline (TBS; pH 7.4) and 1% Triton X-100 for 30 min, and incubated at 4°C in the following primary antibodies: rabbit anti-HSP27 (StressGen, SPA-801; 1:2,500), mouse anti-HSP47 (StressGen, SPA-470; 1:1500), and rabbit anti–HO-1

(HSP32; StressGen, OSA-100; 1:100). After washing with TBS-1% Triton X-100, sections were incubated in the secondary antibody: either biotinylated antirabbit (Amersham, RPN-1004; 1:200) or biotinylated antimouse (Amersham, RPN-1001; 1:200). Sections were rinsed in TBS-1% Triton X-100 and incubated for 1 h at room temperature with streptavidin-peroxidase (Dako P-0364; 1:400). After rinsing again, the peroxidase reaction product was visualized incubating the sections in 100 ml of Tris buffer containing 50 mg 3,3′-diaminobenzidine and 33 μl of hydrogen peroxide. Finally, sections were mounted on gelatin-coated slides, dehydrated in alcohol, cleared in xylene, and coverslipped in DPX mounting medium. As negative controls, sections were incubated in media lacking primary antibodies.

Quantification of HSP Immunoreactivity

Immunohistochemically processed sections were digitized with a video camera mounted on a Leitz microscope and interfaced to a Macintosh computer. Immunohistochemical staining was quantified using National Institutes of Health image software (NIH 1.52) as previously described (Acarin et al., 1997, 1999b, 1999c). Briefly, the quantification measure, referred to as the reactivity grade, was defined as the ratio between values of a specific area in the lesioned hemisphere versus the same area in the contralateral control hemisphere. This ratio method compensates for any possible variability of staining between sections. The value of this ratio represents the intensity of immunolabeling by measuring the density of staining, and the density of labeled cells in that area, measured as the percentage of the delimited area above a staining threshold (threshold is defined as the density of staining in the contralateral control area). The formula used to calculate the reactivity grade is summarized as follows:

$$\text{Reactivity grade} = \underbrace{\frac{\text{Mean staining density}_{\text{Ipsilateral}}}{\text{Mean staining density}_{\text{Contralateral (threshold)}}}}_{\text{Factor considering a possible increase in marker binding}} \times \underbrace{\frac{\text{Area above threshold}_{\text{Ipsilateral}}}{\text{Area above threshold}_{\text{Contralateral}}}}_{\text{Factor considering possible hyperplasia and/or hypertrophia of labeled cells}}$$

The excitotoxic lesion induces de novo expression of HSP32 and HSP27 in the ipsilateral hemisphere but the contralateral hemisphere remained negative. Accordingly, for HSP32- and HSP27-processed sections, the factor considering the areas above threshold were omitted from the formula. A reactivity grade was obtained for each area of study (i.e., neocortex and thalamus), for each marker (HSP27, HSP32, and HSP47), and for each animal (mean of two sections). Measured areas included 0.38 mm^2 area of the neocortex at the level of the injection site, and 1.45 mm^2 area of the caudal thalamus, including the ventrobasal (VB) complex, reticular nuclei, and the internal capsule. Statistical analysis was performed using Statview 4.0 software. Analysis of variance (ANOVA) with Fisher's PLSD posthoc comparisons were used.

Double Immunohistochemistry for HSPs and Glial Markers

We used double-staining techniques for the simultaneous visualization of each HSP and microglial cells (by tomato lectin histochemistry) or astroglial cells (by glial fibrillary acidic protein, GFAP, immunohistochemistry). Free-floating sections were immunoreacted for HSP27, HSP32, and HSP47 as reported above, but using Cy3-conjugated antirabbit secondary antibody (Amersham, PA-4304) or Cy3-conjugated antimouse antibody (Amersham, PA-4302) at a dilution of 1:1,000. Sections were then further processed for GFAP immunocytochemistry as described elsewhere (Acarin et al., 1999c) by using the primary antibody rabbit anti-GFAP (Dakopatts, Z-0334, Denmark) diluted to 1:1,800 and a fluorescein-conjugated antirabbit secondary antibody (Amersham, N-1034) at 1:50. Sections for double staining with tomato lectin histochemistry were treated as described previously (Acarin et al., 1994). Briefly, sections were first incubated with the biotinylated lectin obtained from Lycopersicon esculentum (tomato; Sigma, L-9389) diluted to 6 μg/ml and then with Cy2-conjugated avidin (Amersham, PA-42000) at a dilution of 1:1,000. Double-stained sections were analyzed using a Leica TCS 4D confocal microscope.

RESULTS

As we have previously reported in detail (Acarin et al., 1999b, 1999c), the cortical NMDA injection caused neuronal death and glial reactivity in the sensorimotor cortex and a milder and transient glial response in the neuroanatomically connected ventrobasal complex of the thalamus and the internal capsule.

Briefly, in the neocortical injection site, at 10 h postlesion (PL), neuronal cell death was evident and glial cells showed the first signs of reactivity: astroglial cells began to overexpress GFAP and microglial cells changed to pseudopodic/ameboid forms. At 1–3 days PL, massive neuronal loss accompanied by serum protein extravasation and leukocyte infiltration was observed. Additionally, microglia/macrophages accumulated in the lesion site, showing maximal response at 3–5 days PL, and astroglial cells became strongly hypertrophied, presenting maximal response at day 7. Scarring of cortical tissue became apparent by 5 days

PL, showing highly hypertrophied astrocytes and macrophages until the last survival time examined. In the secondarily affected thalamus, no neuronal degeneration was observed; the microglial thalamic response was characterized by morphological changes to reactive ramified shapes, and the astroglial response was evident as a mild increase in GFAP expression and hypertrophy. This thalamic glial response was maximal at 5 days PL and decreased at longer survival times.

HSP32 (HO-1) Expression

Neocortical injection site

No expression of HSP32 was observed in normal control animals (Fig. 1A). In contrast, saline-injected controls showed mild HSP32 expression surrounding the needle track, where ramified HSP32-positive microglial cells were observed until 3 days postinjection.

In NMDA-injected animals, HSP32 expression was noticed from the first survival time analyzed, when mild HSP32 labeling was observed in scattered glial cells of the ipsilateral hemisphere. Those labeled cells were identified as microglial cells by their double staining with tomato lectin (Figs. 1B and 2A; note that tomato lectin also labels blood vessels), and most of them showed signs of activation: mainly pseudopodic/ameboid morphology. However, some HSP32-positive ramified microglial cells were also seen. From 1 day PL, HSP32-expressing microglia/macrophages were mostly ameboid/round-shaped (Figs. 1C and 2B), located in the degenerating area and the adjacent corpus callosum. At this time point, scattered reactive astroglial cells surrounding the neurodegenerative area also showed HSP32 labeling (Fig. 2C). Astroglial HSP32 expression was not observed at any other survival time (Fig. 2D). Maximal HSP32 expression was found at day 3 (Fig. 3A), when densely packed HSP32-positive microglia/macrophages accumulated in the degenerating area (Fig. 1D). At 5 days PL, when HSP32 labeling started to decrease, it was found in mildly stained microglia/macrophages of the forming glial scar and in reactive ramified microglial cells surrounding them (Fig. 1E). At longer survival times, labeling clearly decreased (Fig. 3A) and HSP32-positive cells were mainly reactive ramified microglial cells surrounding the glial scar (Fig. 1F).

Neuroanatomically connected thalamus

No expression of HSP32 was observed in the thalamus of either normal controls or saline-injected animals (Fig. 1G), whereas only a very mild expression of this heat shock protein was seen between days 1 and 3 PL (Fig. 1H) in the ipsilateral thalamus of NMDA-lesioned animals. However, image analysis quantitative study did not show significant differences (Fig. 3B).

HSP27 Expression

Neocortical injection site

In normal control animals, no expression of HSP27 was observed. However, saline-injected controls showed mild HSP27 expression in the ipsilateral cortex, where sparse HSP27-positive glial cells with the characteristic star-shaped and ramified morphology of astrocytes could be observed between days 1 to 3 postinjection (Fig. 4A).

After the NMDA injection, in addition to the scattered HSP27-positive astroglial cells also found in controls, de novo HSP27 expression was observed at 10 h PL in specific blood vessels in the lesioned cortical area (Fig. 4B). At 1 day PL, HSP27 expression became more evident and extended surrounding the degenerating area, concentrating on the edge of the lesion site, i.e., lateral margins, cortical layer VI, and adjacent corpus callosum, reaching the rostral hippocampus in some cases. The majority of HSP27-positive cells were identified as astroglial cells (Fig. 2E), although scattered endothelial cells were still seen (Fig. 2F). From 3 days PL, HSP27 expression was considerably enhanced (Figs. 4C and 5A) because the number of HSP27-labeled astrocytes increased substantially and cells became morphologically more reactive and hypertrophied (Figs. 2G and H and 4C and D), especially at 5 days PL. It should be noted that not all GFAP-positive astroglial cells showed HSP27 expression (Fig. 2G). Noteworthy is that astroglial HSP27 labeling was found in the cell body as well as in the thick and thin processes, even in GFAP-negative thinner ramifications (Fig. 2H). At longer survival times, labeling was restricted to the glial scar, formed by a dense network of HSP27-positive reactive astrocytes that was maintained until the last survival time analyzed.

Neuroanatomically connected thalamus

Both normal controls and saline-injected animals showed HSP27-positive astroglial cells in the internal capsule of both hemispheres but not in thalamic nuclei. In contrast, in NMDA-injected animals, de novo expression of HSP-27 in some astroglial cells located in the ipsilateral VB complex was observed at day 1 PL (Fig. 4G). Positive astroglial cells became more numerous at the following survival times, reaching maximal expression at days 5–7 PL (Figs. 4H and 5B) and returning to basal levels at longer times (Fig. 5B). Thalamic HSP27-positive astroglial cells were less hypertrophied and showed lower reactivity than positive astrocytes of the cortical lesion site (Fig. 4I).

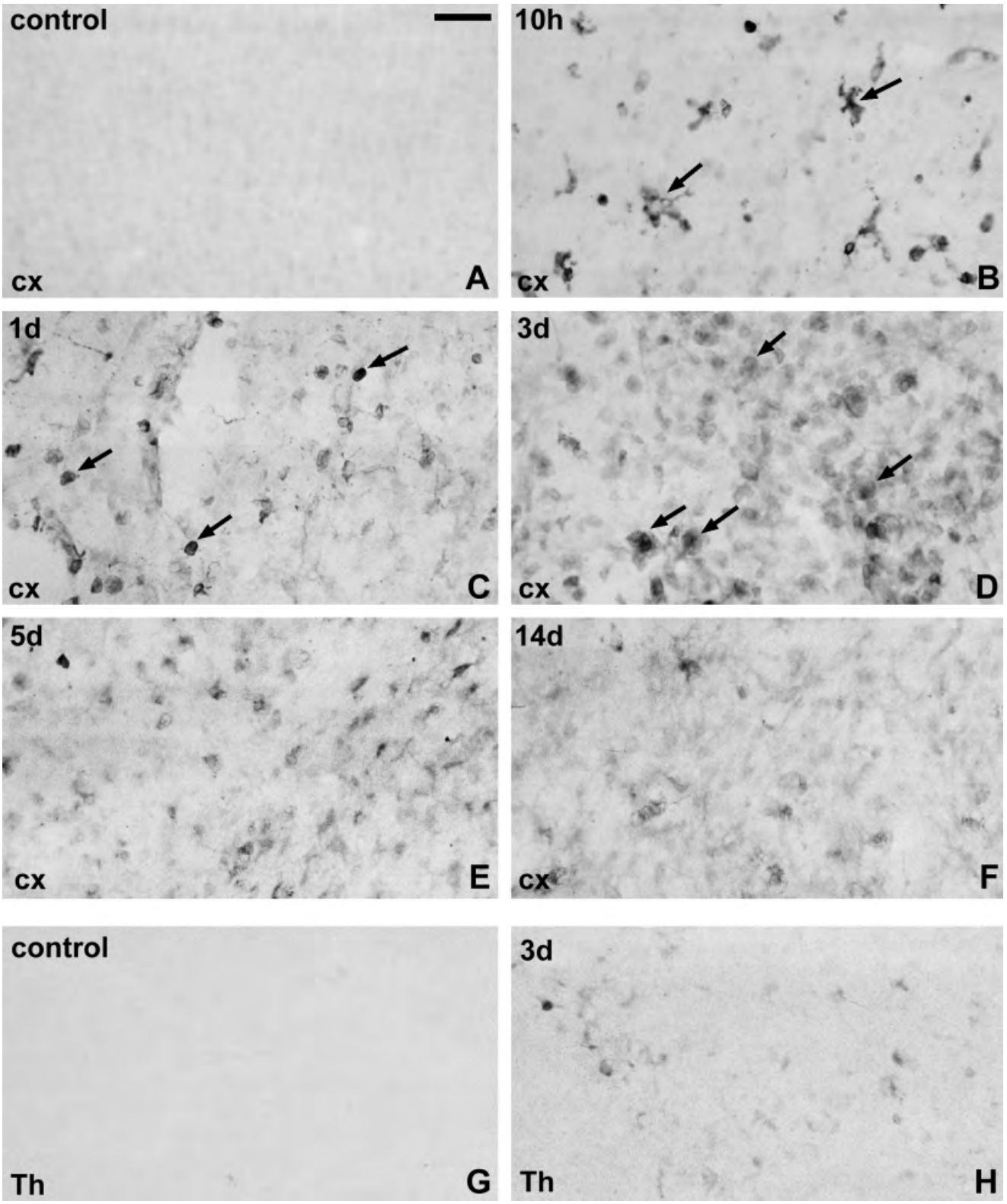
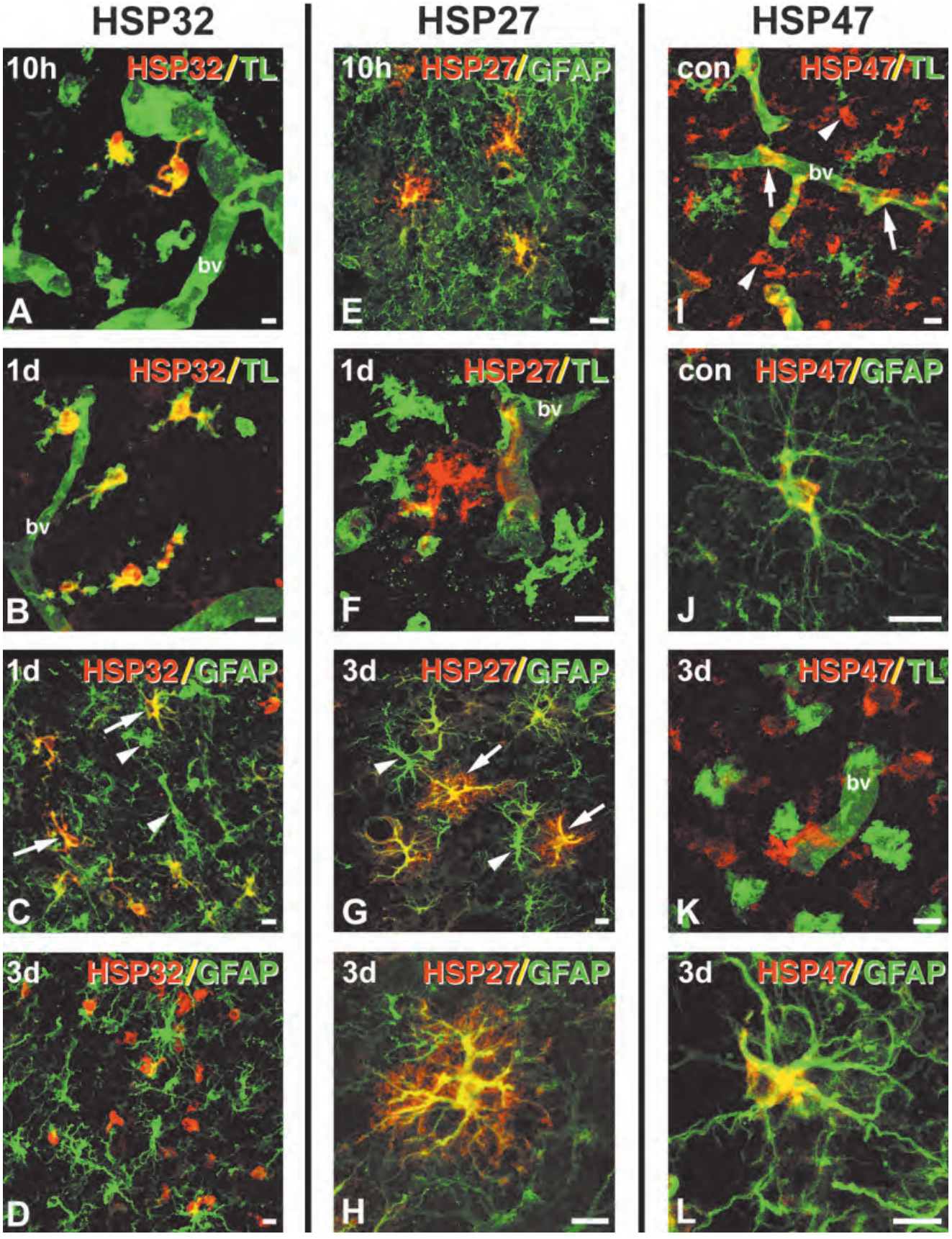


Fig. 1. HSP32 expression in saline-injected controls (**A**, **G**) and NMDA-injected animals (**B–F**, **H**), in the injected cortex (cx; A–F) and the corresponding ipsilateral thalamic nuclei (Th; G, H). HSP32 immunoreactivity is not observed in the cortex (A) and thalamus (G) of control animals. In the NMDA-injured cortex, HSP32 is already observed at 10 h PL (B), increases from day 1 PL (C) to day 3 PL (D), decreasing later on (E, F). As indicated by arrows, pseudopodic and round-shaped microglia/macrophages are immunolabeled. In contrast, only a faint immunoreactivity for HSP32 is seen in the thalamus (H). All micrographs are the same magnification. scale bar = 25 μm.


HSP32
HSP27
HSP47
10h HSP32/TL
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A
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Figure 2.

HSP47 Expression

Normal control animals showed basal expression of HSP47 on blood vessels throughout the brain and mild labeling in ramified astroglial cells (Figs. 3I and J and 6A), where HSP47 immunoreactivity was found in the cell body but was lacking in the GFAP-positive processes (Fig. 2J). HSP47-positive astrocytes were not homogeneously distributed, but delimited specific brain areas such as the cortical layers, the corpus callosum, hippocampal fields, and several thalamic nuclei. Interestingly, HSP47 staining of blood vessels and astroglial cells decreased with age, being almost absent in postnatal day 16 controls.

Neocortical injection site

In addition to the basal expression, saline injection caused an increase in astroglial HSP47 immunoreactivity, mainly surrounding the needle track, from the first survival time examined and until day 3 PL, when only scattered labeled cells remained.

In NMDA-injected animals, increased expression of HSP47 was already observed at 10 h PL in the lesioned hemisphere (Fig. 6B), increasing significantly at day 1 PL (Fig. 7A). At these early survival times, HSP47 expression increased in blood vessels within the lesioned hemisphere and in the perinuclear cytoplasm of astroglial cells located in the degenerating area, which showed signs of cell activation, with reorganization of cellular processes (Fig. 6B and C). Both the number of HSP47-positive cells and their immunoreactivity were increased at 3 days PL (Figs. 2K and L and 6D and E). From 5 days PL, blood vessel labeling diminished and astroglial HSP47 expression was further increased, correlating with astroglial hypertrophy and GFAP overexpression, showing maximal levels of HSP47 (Fig. 7A). At longer survival times, astroglial HSP47 expression was maintained, remaining in the positive glial scar.

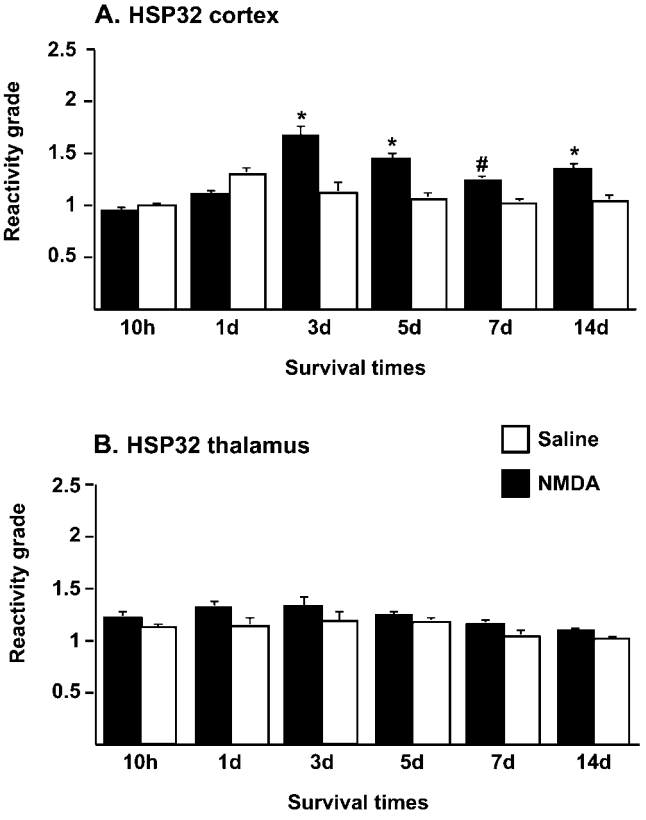

Fig. 3. Quantification of HSP32 expression in cortical (**A**) and thalamic (**B**) areas studied at several survival times after saline (white columns) or NMDA (black columns) injection. Data are presented as the mean values of reactivity grades ± SEM. Significative values are obtained comparing with saline-injected littermates of each survival time ($*P < 0.01$, $\#P < 0.06$). A, Significant increase of HSP32 immunolabeling is observed in the excitotoxically damaged cortex from 3 to 14 days PL, peaking at the day 3 PL. B, In contrast, HSP32 labeling in the thalamus is not significantly different from saline-injected controls.

Neuroanatomically connected thalamus

No thalamic overexpression of HSP47 was observed in saline-injected controls (Figs. 6H and 7B). In contrast, NMDA-injected animals showed increased HSP47 labeling in the ipsilateral thalamus, paralleling overexpression of this protein in the cortical lesion site (Fig. 7B). Thalamic HSP47 increase was first noticed in the VB nucleus at day 1 PL (Figs. 6I and 7B), when increased expression was found in blood vessels and astroglial cells. At days 3–5 PL, HSP47 was mainly observed in mildly hypertrophied astroglial cells (Fig. 6J and K), reaching maximal expression at 5 days PL and decreasing thereafter, achieving basal levels by day 14 PL (Fig. 7B).

Fig. 2. Identification of HSP32- (left column, **A**–**D**), HSP27- (middle column, **E**–**H**), and HSP47- (right column, **I**–**L**) positive cells in the postnatal control (con) brain (I, J) and at different times postinjury in excitotoxically lesioned cortex (A–H, K–L). HSP32, HSP27, and HSP47 labeling are shown in red, and GFAP (astroglial marker) and tomato lectin (TL; microglia/macrophage marker) in green. Note that tomato lectin also labels blood vessels (bv). Yellow-orange labeling shows colocalization of the markers specified in each micrograph. Right column: HSP32 is mainly expressed in microglia/macrophages (A, B), although a subpopulation of HSP32-positive astroglial cells is observed at 1 day postlesion (arrows in C). Note that at day 1, some astroglial cells do not colocalize with HSP32 (arrowheads in C) and that no HSP32-positive astrocytes are seen at day 3 (D). Middle column: HSP27 is found in some astrocytes as early as 10 h postlesion (E), whereas microglia/macrophage remain HSP27-negative (F). At day 3, HSP27 is strongly expressed in a population of hypertrophied reactive astrocytes (arrows in G), although some astrocytes remain HSP27-negative (arrowheads in G). HSP27 labeling shows a higher ramified astroglial morphology than GFAP immunostaining (H). Right column: HSP47 labeling shows ameboid shapes (arrowheads in I), which in control animals colocalize with TL-positive blood vessel structures (arrows in I) and with the soma of astroglial cells (J). After the lesion, neither lectin-positive blood vessels nor microglia/macrophages express HSP47 (K), whereas reactive astrocytes increase HSP47 cytoplasmic labeling (L). Scale bars = 10 μm.

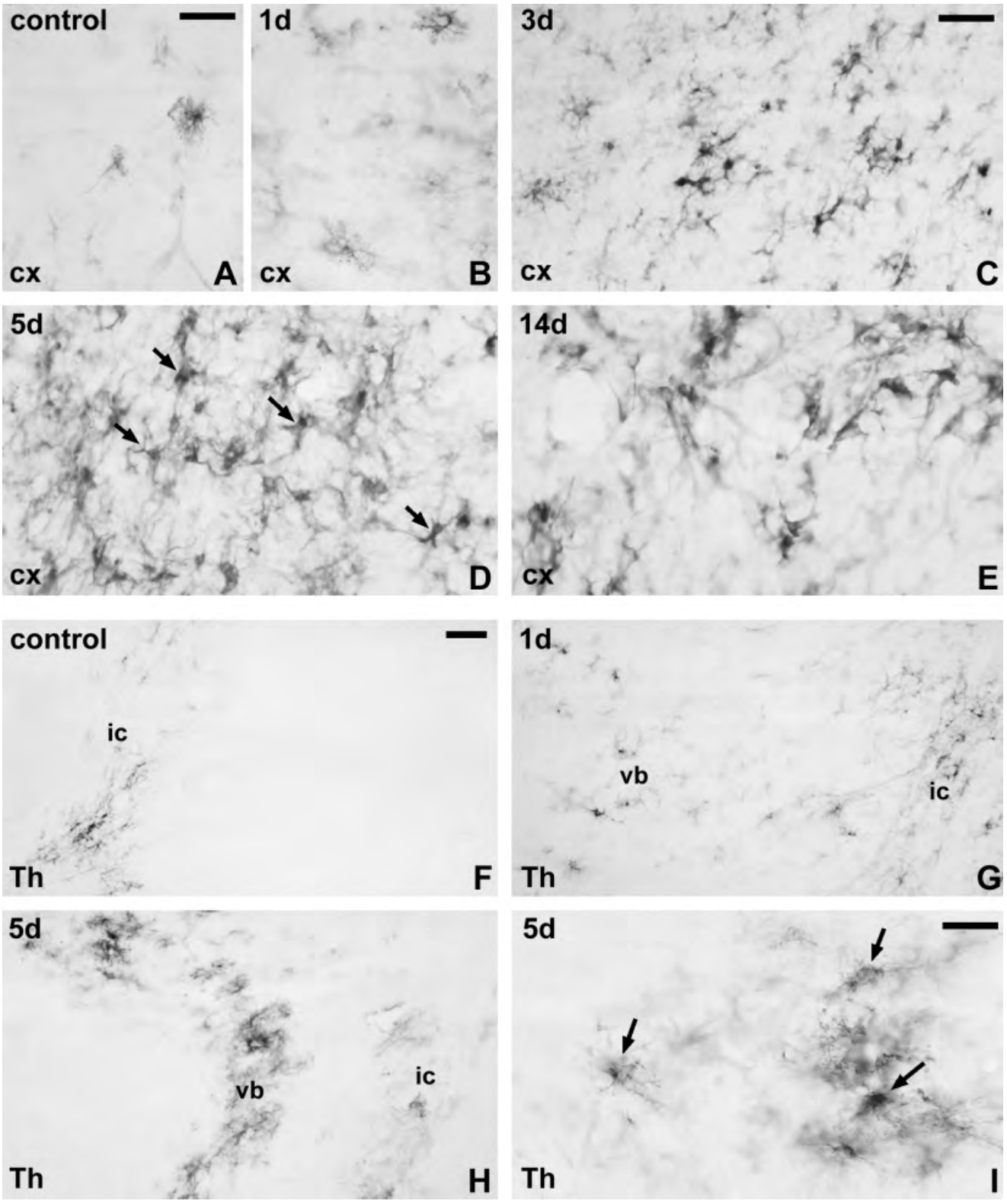

Fig. 4. HSP27 expression in saline-injected controls (**A**, **F**) and NMDA-injected animals (**B**–**E**, **G**–**I**), in the injected cortex (cx; A–E) and the corresponding ipsilateral thalamic nuclei (Th; F–I). Scattered astrocytes show HSP27 immunolabeling in the cortex (A) and internal capsule (ic in F) of saline-injected controls. In the excitotoxically damaged cortex, astroglial HSP27 immunoreactivity increases from day 1 PL (B). Maximal HSP27 labeling and astroglial hypertrophy is observed at 3 days PL (C) and 5 days PL (D). Glial scar astrocytes remain HSP27-positive (E). In the ipsilateral thalamus, increased HSP27 labeling is observed in the ventrobasal nucleus (vb) and the internal capsule (ic) from day 1 PL (G), achieving maximal immunoreactivity at 5 days PL (H). Thalamic HSP27-positive astrocytes (arrows in I) are not as hypertrophied as cortical reactive astrocytes (arrows in D). Scale bar, A, B = 50; D, E = 30; F–H = 60; I = 30 μm.

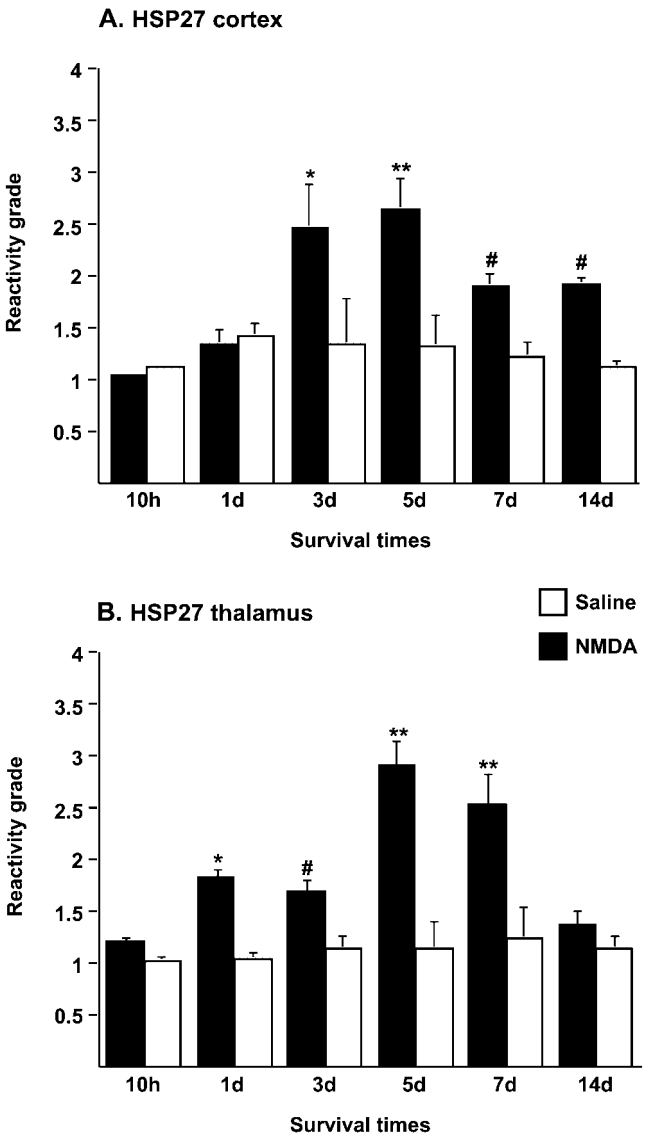

Fig. 5. Quantification of HSP27 expression in cortical (**A**) and thalamic (**B**) areas studied at several survival times after saline (white columns) or NMDA (black columns) injections. Data are presented as the mean values of reactivity grades ± SEM. Significative values are obtained comparing with saline-injected littermates of each survival time (**$P < 0.01$, *$P < 0.05$, #$P < 0.07$). A: Significant increase of HSP27 immunoreactivity is observed in the cortex from 3 to 14 days PL, showing peak values at days 3 and 5 PL. B: In the corresponding thalamus, HSP27 labeling is transitory, becomes maximal at 5–7 days PL, and diminishes later on.

DISCUSSION

This study demonstrates the astroglial and/or microglial expression of HSP32, HSP27, and HSP47 in the excitotoxically damaged postnatal cortex, as well as the expression of HSP27 and HSP47 in the secondarily affected thalamus. Glial expression of small HSPs parallels the glial and inflammatory response previously described in this lesion model (Acarin et al., 1999b, 1999c, 2000a, 2000b, 2001). The specific expression pattern and the possible contribution of each HSPs in the degenerating process will be discussed separately.

Cortical Microglia/Macrophage HSP32 (HO-1) Expression

After excitotoxic damage to the immature brain, we have observed HSP32 induction in microglia/macrophages located in the neurodegenerating area following a similar pattern to that previously observed after postnatal brain hypoxia-ischemia (Bergeron et al., 1997). Similarly, HSP32 expression is also induced under different neuropathological conditions in the adult brain, such as excitotoxicity (Nakaso et al., 1999; Gilberti and Trombetta, 2000), ischemia (Koistinaho et al., 1996; Turner et al., 1998; Beschorner et al., 2000), traumatic injury (Fukuda et al., 1996; Beschorner et al., 2000), endotoxin treatment (Kitamura et al., 1998b), photothrombosis (Bidmon et al., 2001), or experimental hemorrhage caused by intracerebral blood infusion (Matz et al., 1997; Turner et al., 1999). In most of these stressful conditions, both in the adult and postnatal brain, HSP32 has been demonstrated mainly in microglia/macrophages, although reactive astroglial cells have also been shown to express HSP32 in specific survival times and locations (Fukuda et al., 1996; Koistinaho et al., 1996; Kitamura et al., 1998b; Turner et al., 1998; Nakaso et al., 1999; Bidmon et al., 2001). Therefore, HSP32 expression seems to be a common characteristic of the microglial/macrophage response after different kinds of cerebral injury, although astroglial HSP32 expression also occurs at a lower extent.

Generally, the presence of extracellular heme, derived from extravasated hemoglobin or released from dying cells and interacting with the heme response site found in the HSP32 promoter, is thought to be the main activator of HSP32 following brain injury (Sharp et al., 1999). Furthermore, binding sites of well-known inflammatory transcription factors such as the nuclear factor kappa B (NF-κB) and the signal transducer and activator of transcription (STAT) families have also been recently identified in the HSP32 promoter (Brown and Sharp, 1999; Lee et al., 2000; Stuhlmeier, 2000). In our lesion model, HSP32 expression in microglia/macrophages correlates with the induction of major histocompatibility complex type II (MHC II), the time of maximal blood-brain barrier (BBB) disruption, and a pulse of activation of NF-κB and STAT3 in cortical microglial cells (Acarin et al., 1999b, 2000b), therefore indicating that both the presence of extravasated heme and the inflammatory process may contribute to HSP32 induction in the cortex. It should be noted that the thalamic microglial response occurs in the absence of BBB disruption, absence of MHC II expression, and absence of inflammatory transcription factor activation (Acarin et al., 1999b, 2000b), thus correlating with a lack of HSP32 expression in these thalamic nuclei.

As postulated before (Sharp et al., 1999; Turner et al., 1999), the breakdown of damaged heme proteins

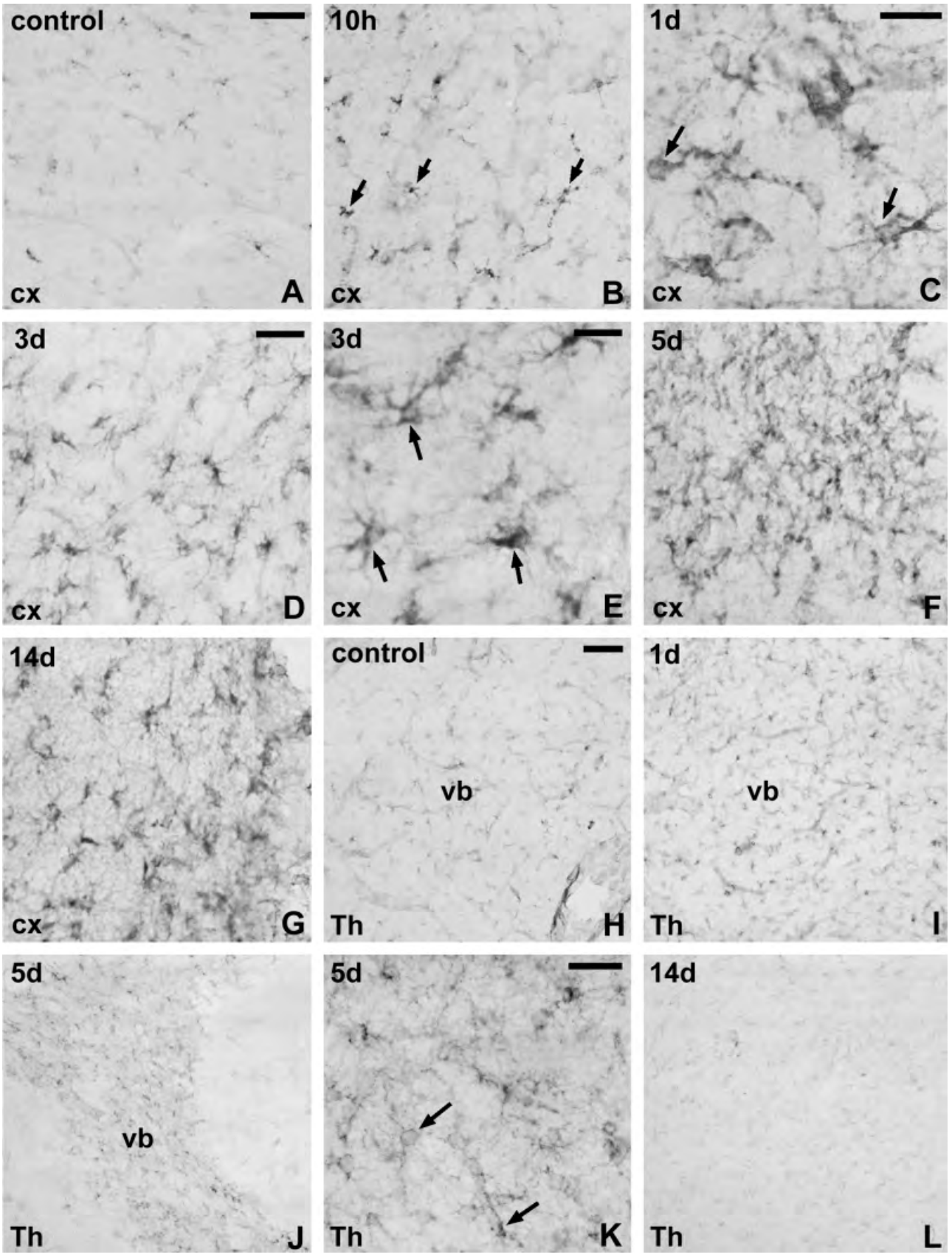
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Figure 6.

within the cell seems to be the main contribution of HSP32 to the recovery of disrupted cellular activity. HSP32 provides neuroprotection against oxidative stress by metabolizing heme to carbon monoxide, iron, and biliverdin, which is rapidly converted to bilirrubin, a potent antioxidant (Kitamura et al., 1998a; Turner et al., 1998; Doré et al., 1999). In this regard, HSP32 has been postulated to underlie the protective effects of hypoxic preconditioning prior to ischemia (Garnier et al., 2001) and to induce the expression of the potent antioxidant enzyme manganese superoxide dismutase (MnSOD) (Frankel et al., 2000). Finally, another protective role of this enzyme includes the anti-inflammatory effects of carbon monoxide, a product of HSP32 (Otterbein et al., 2000).

Cortical and Thalamic Astroglial HSP27 Expression

After the excitotoxic lesion, HSP27 expression is mainly found in astroglial cells, showing maximal expression at days 3–5 PL in the cortical lesion site, where expression is maintained in the long-term–formed glial scar. These results are in agreement with a previous study where we have reported astroglial HSP27 expression following a cortical aspiration lesion in the immature brain (Sanz et al., 2001), although this traumatic condition induces HSP27 increases more rapidly than the excitotoxic damage. To our knowledge, these are the only reports on HSP27 expression in the immature rat brain. However, in the adult brain, several studies have reported astroglial HSP27 induction after different pathological conditions, including ischemia (Kato et al., 1995; Wagstaff et al., 1996; Imura et al., 1999), photothrombotic injury (Plumier et al., 1997a), cortical application of potassium chloride (Plumier et al., 1997b), excitotoxicity (Plumier et al., 1996; Kato et al., 1999), axonal degeneration (Anguelova and Smirnova, 2000), and Alzheimer's disease (Renkawek et al., 1994). Therefore, astroglial HSP27 expression seems to be a common characteristic of very different types of brain injury, both during development and in adulthood.

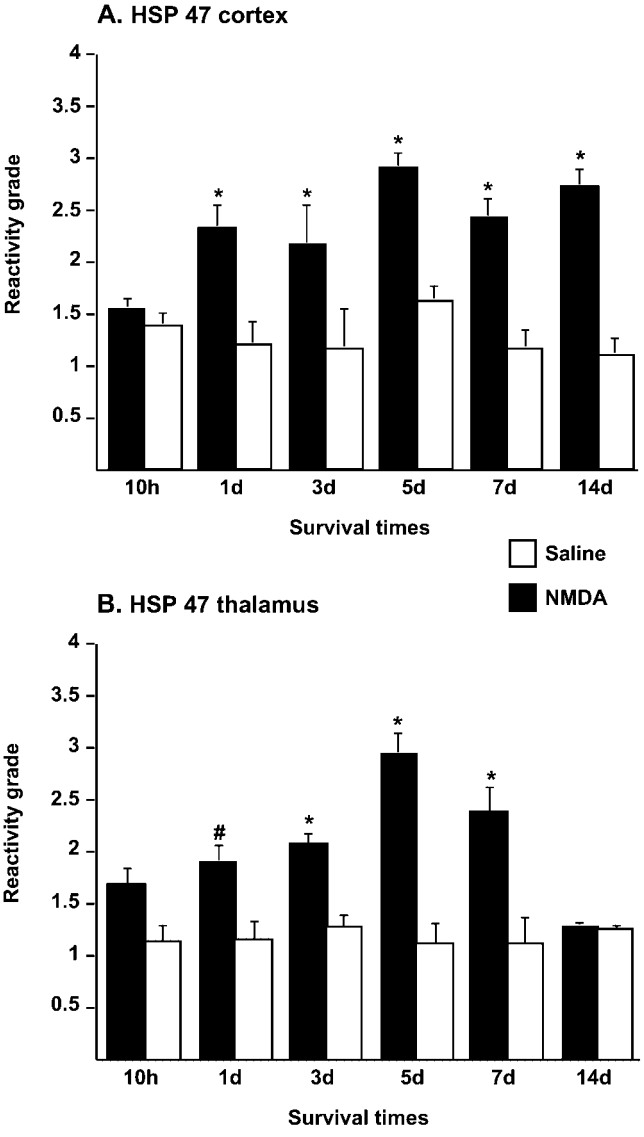

Fig. 7. Quantification of HSP47 expression in cortical (**A**) and thalamic (**B**) areas studied at several survival times after saline (white columns) or NMDA (black columns) injections. Data are presented as the mean values of reactivity grades ± SEM. Significative values are obtained comparing with saline-injected littermates of each survival time (*$P < 0.02$, #$P < 0.05$). A: Significant increase of HSP47 immunoreactivity is observed in the cortex from 1 to 14 days PL, showing peak values from 5 PL until the last survival time examined. B: In the corresponding thalamus, HSP47 labeling is transitory, becomes maximal at 5 days PL, and diminishes after 7 days PL.

Fig. 6. HSP47 expression in saline-injected controls (**A**, **H**) and NMDA-injected animals (**B**–**G**, **I**–**L**), in the injected cortex (cx; A–F) and the corresponding ipsilateral thalamic nuclei (Th; G-L). Mild HSP47 immunoreactivity is observed in blood vessels (arrows) and glial cells of saline-injected controls. In the excitotoxically damaged cortex, HSP47 immunoreactivity increases from 10 h PL (B, C) in ramified/pseudopodic glial cells (arrows in B, C). HSP47 labeling is further increased at 3 days PL (D, E) and 5 days PL (F), when HSP47-positive reactive astrocytes are observed (arrows in E). Glial scar astrocytes are still HSP47-positive (G). In the ipsilateral thalamus, increased HSP47 labeling is seen in the ventrobasal nucleus (vb) from day 1 PL (I), showing maximal immunoreactivity at 5 days PL (J, K) and decreasing later on (L). Thalamic HSP47-positive astrocytes (arrows in K) are not as HSP47-positive and clearly less hypertrophied than cortical reactive astrocytes (arrows in E). Scale bar, A, B, D, F, G = 25; C = 2; E = 2.5; H-J, L = 50; K = 30 μm.

Two different functions for HPS27 expression following brain damage have been suggested: first, it may serve as an antioxidant mechanism, and second, it could provide cytoskeletal stabilization. HSP27 has been shown to decrease intracellular reactive oxygen species, increasing levels of antioxidant glutathion, thus protecting against oxidative stress and cell death (Mehlen et al., 1996a, 1996b, 1997; Samali and Orrenius, 1998). It should be noted that several other antioxidant mechanisms have also been described in reac-

tive astrocytes (Makar et al., 1994; Peuchen et al., 1997), including the expression of the metal binding proteins metallothioneins, whose maximal expression levels in our excitotoxic lesion model coincide with maximal HSP27 immunoreactivity (Acarin et al., 1999a, 1999c). Therefore, astroglial HSP27 expression after injury may participate in the development of antioxidant mechanisms, contributing to the protective response to minimize damage caused by free radical production or increased presence of metal levels after the excitotoxic process.

Additionally, HSP27 is a molecular chaperone involved in the capping/decapping of actin filaments (Guay et al., 1997). In this sense, HSP27 expression has also been related to the dynamic regulation of actin filaments by phosphorylation/dephosphorylation mechanisms (Larsen et al., 1997; Costigan et al., 1998), thus stabilizing actin cytoskeleton (Guay et al., 1997) and protecting microfilaments from possible degradation and depolymerization following stressful conditions. In astroglial cells, in vitro studies have suggested that HSP27 may interact with the intermediate filaments GFAP and vimentin protecting them from stress-induced aggregation (Perng et al., 1999). Noteworthy is that in the excitotoxically damaged cortex, HSP27 labeling correlates with GFAP overexpression and de novo induction of vimentin, suggesting that HSP27 may act as a mechanism to protect GFAP and vimentin microfilaments, thus maintaining the integrity of astroglial cytoskeleton and promoting cell stabilization and survival.

Finally, it should be noted that HSP27 expression is also observed in the secondarily damaged thalamic nuclei, where mild astroglial hypertrophy and GFAP overexpression also occur. The dual function of HSP27 may imply that this protein could not only protect the cells where it is expressed (i.e., the astrocytes), but also influence the surrounding damaged tissue by inducing tissue remodeling and restoration. Actually, HSP27 expression in areas undergoing anatomical reorganization and axonal sprouting, both in the cortex and the secondarily affected thalamus, supports the idea proposed by some (Kato et al., 1999) that suggested a relationship between HSP27 expression and tissue remodeling.

Cortical and Thalamic Astroglial HSP47 Expression

Following excitotoxic damage to the postnatal cortex, HSP47 is expressed in astroglial cells within hours after the injury, being maintained in the glial scar. To our knowledge, this is the first study reporting HSP47 expression after immature brain damage, and only a few previous reports have focused on the expression of this heat shock protein after adult brain injury, including reports on focal cerebral ischemia and subarachnoid injection of lysed blood (Sharp et al., 1999; Turner et al., 1999). These studies in the adult brain have shown that both astrocytes and microglial cells are capable of HSP47 induction. In the postnatal brain, HSP47 immunoreactivity is mainly found in the perinuclear cytoplasm of astroglial cells, probably related to the endoplasmic reticulum, as has been suggested for other cell types (Tasab et al., 2000), giving an appearance of ameboid/pseudopodic cellular shapes (see Fig. 2) that at first resemble microglial cells but are GFAP-positive cells, unstained with microglial cell markers. This lack of microglial cell HSP47 expression in the injured immature brain may be attributed to the developmental stage.

As very few studies have focused on HSP47 brain expression, the possible function of this chaperone after neural damage is poorly understood. In peripheral organs, it is well known that HSP47 is a collagen-specific molecular chaperone located in the endoplasmic reticulum of synthesizing cells, playing a crucial role in the intracellular processing, folding, and secretion of collagen, thus participating in extracellular matrix formation and maintenance (Nagata, 1998; Turner et al., 1999; Tasab et al., 2000). Collagen is the main component of extracellular matrix in peripheral tissues and participates in the formation of fibrous scars, even in the brain, where it seems to participate in the axon growth inhibitory properties of scars (Stichel et al., 1999). In this regard, HSP47 expression may participate in tissue remodeling, a function attributed to glial scar astroglial cells that could imply the contribution of HSP47 to the stabilization of extracellular matrix proteins that underlie scar formation. Actually, glial basal expression of HSP47 in the central nervous system during early postnatal life may also support a possible role of this chaperone in the stabilization of other matrix components besides collagen, thus participating in the synthesis and maintenance of the extracellular matrix under physiological conditions during brain development. Nevertheless, in order to clarify its role, further studies analyzing HSP47 chaperone substrates and HSP47 expression in different neuropathological conditions would be needed.

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