Rapid, transient, and dose-dependent expression of Hsp70 messenger RNA in the rat brain after morphine treatment

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Abstract Induction of Hsp70 in the brain has been reported after intake of drugs of abuse like amphetamine and lysergic acid diethylamide. In this investigation, gene expression of Hsp70 and other heat shock genes in the rat brain was studied in response to morphine. Twenty milligrams per kilogram morphine intraperitoneally resulted in a marked induction of Hsp70 messenger RNA (mRNA) expression in the frontal cortex with a maximum increase of 13.2-fold after 2 hours. A moderate increase of Hsp27 mRNA expression (6.7-fold) could be observed after 4 hours, whereas mRNA expression of Hsp90 and of the constitutive Hsc70 did not exceed a mean factor of 1.8-fold during the 24 hours interval. The increase in Hsp70 mRNA was dose dependent, showing a significant elevation after doses ranging from 10 to 50 mg/kg morphine. In situ hybridization revealed enhanced Hsp70 mRNA expression mainly in cortical areas, in the hippocampus, in the paraventricular and supraoptic nuclei of the hypothalamus, in the locus coeruleus, as well in the pineal body. The double in situ hybridization technique revealed increased Hsp70 mRNA expression mainly in VGLUT1-positive neurons and to a lesser extent in olig1-positive oligodendroglia. Immunohistochemistry revealed a marked increase of Hsp70 protein in neuronal cells and blood vessels after 12 hours. In contrast to animal experiments, morphine did not increase Hsp70 mRNA expression in vitro in μ -opioid receptor (MOR1)-expressing human embryonic kidney 293 cells, suggesting no direct MOR1-mediated cellular effect. To exclude a body temperature-related morphine effect on Hsp70 mRNA expression, the temperature was recorded. Five to 20 mg/kg resulted in hyperthermia (maximum 40.6°), whereas a high dose (50 mg/kg) that produced the highest mRNA induction, showed a clear hypothermia (minimum 37.2°C). These findings argue against the possibility that Hsp70 induction by morphine is caused by its effect on body temperature. It may be speculated that increased expression of Hsp70 after morphine application protects brain structures against potentially hazardous effects of opiates.

INTRODUCTION

Hsp70 as well as Hsp27 are the major inducible heat shock proteins (Hsps) in the brain. Induction of Hsp70 messenger RNA (mRNA) or protein (or both) has been reported in response to various pharmacological stimuli such as convulsant drugs (Planas et al 1994; Krueger et al 1999) and drugs of abuse like amphetamine (Miller et al 1991), lysergic acid diethylamide (LSD) (Manzerra and

Brown 1990) and ethanol (Calabrese et al 2000). In addition, acute cocaine treatment has been reported to induce Hsp70 in murine liver (Salminen et al 1997). The *hsp70* gene is transcriptionally regulated by heat shock factors (HSFs), which bind on the promoter of the *hsp70* gene. Hsp90 binds to HSFs and suppresses transcription of the *hsp70* gene (Sharp et al 1999). Hsp90 is present constitutively in relatively high abundance in many cell types under unstressed conditions (Izumoto and Herbert 1993).

Using the DNA microarray technology we recently found that chronic morphine treatment (10 days treatment schedule using ascending morphine doses ranging from

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twice-daily 10 mg/kg to twice-daily 50 mg/kg), leading to morphine tolerance, resulted in an increased mRNA expression of several Hsps along with other genes in the brain of rats (Ammon et al 2003). In this investigation, we provide a detailed analysis of the dose- and time-dependent expression of Hsp70 mRNA and of related heat shock genes (Hsp27, Hsc70, and Hsp90) in response to acutely administered morphine by real-time polymerase chain reaction (PCR) or in situ hybridization (or both). In addition, expression of the corresponding Hsp70 protein was determined. To determine a potential μ -opioid receptor (MOR1)-mediated cellular response, MOR1-transfected human embryonic kidney (HEK) 293 cells were incubated with morphine and tested for Hsp70 mRNA expression. Because morphine is known to alter body temperature in rats, temperature measurements were performed to evaluate a possible relationship between morphine-induced temperature changes and gene expression.

MATERIALS AND METHODS

Animals

For all experiments, ethical approval was sought before the experiments, according to the requirements of the National Act on the Use of Experimental Animals (Germany). All possible steps were taken to avoid animals' suffering at each stage of the experiments. Eight-week-old male Wistar rats (Mol: Wist (shoe), Tierzucht Schönwalde, Germany) were used. The animals were housed under controlled laboratory conditions in a light (12 hours on–12 hours off), temperature ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and relative air humidity (55–60%) controlled environment with free access to food and water.

Drug application

1. Dose response: animals ($n = 4\text{--}5$ per group) received a single intraperitoneal (i.p.) injection of 5, 10, 20, or 50 mg/kg morphine or vehicle (saline). A separate group of animals receiving 50 mg/kg morphine was additionally treated with the opioid antagonist naloxone (10 mg/kg) 15 minutes before and 2 hours after morphine application. Brains were removed 4 hours after morphine injection.
2. Time course: animals ($n = 5$ per group) received a single morphine dose of 20 mg/kg i.p. Brains were removed after 1, 2, 4, 12, or 24 hours. Brains of control animals were taken without any treatment (naive) or 2 hours after saline application.
3. Animals for determination of cellular distribution of Hsp70 mRNA or Hsp70 protein: animals received a single morphine dose (50 mg/kg) or saline. Brains

were taken 2 hours later for in situ hybridization ($n = 3$ per group) or 12 hours later after transcardial perfusion for immunohistochemistry ($n = 2$ per group).

Tissue preparation, RNA isolation, and real-time PCR

After decapitation, frontal cortex was removed and immediately frozen at -70°C (Popov et al 1973). Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The integrity of RNA and the absence of larger amounts of contaminating genomic DNA were checked by gel electrophoresis.

As previously described by us (Mayer et al 2002), complementary DNA (cDNA) was created from RNA using TrueScript MMLV reverse transcriptase (Hybaid, Heidelberg, Germany) and dN_6 random primers. RNA ($0.2\text{ }\mu\text{g}$) was included in each reaction in a total volume of $20\text{ }\mu\text{L}$. The reaction was performed at 42°C for 2 hours. Thereafter, the mix was diluted 5-fold and $2\text{ }\mu\text{L}$ was added to the PCR reaction mix to yield a total volume of $20\text{ }\mu\text{L}$. The PCR reagents were from the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The reaction buffer contained 3.1 mM MgCl_2 . The amplification reaction consisted of 50 cycles of denaturation (95°C , 15 seconds), annealing (10 seconds), and elongation (72°C , 20 seconds).

Sequences and annealing temperatures for the respective upstream and downstream primers: Hsp70 (GenBank accession no. L16764): 5'-AAC TAC AAG GGC GAG AAC CGG TC, 5'-GAT GAT CCG CAG CAC GTT CAG A, 68°C , Hsp27 (GenBank accession no. M86389): 5'-ACT CAG CAG CGG TGT CTC AGA GAT CC, 5'-GGT GAA GCA CCG AGA GAT GTA GCC A, 71°C , Hsc70 (hspa8, GenBank accession no. NM_024351): 5'-CAA TGA CTC TCA GCG ACA GGC A, 5'-TGT CAA AGT CTT CTC CGC CCA A, 67°C , Hsp90 (GenBank accession no. S45392): 5'-CTC GTC AAG ATG CCT GAG GAA GTG C, 5'-CTC CAT GAA CGC CTT TGT ACC AGA CTT AG, 70°C .

Cycle numbers (crossing points, when amplification starts its exponential phase) were used for statistical analysis. The lower the cycle number, the higher the amount of initial template. Results are displayed as mean $\pm$ SD; statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttest. A 2-sided P value <0.05 was regarded as significant. In addition, fold inductions of mRNA expression of the different treatment groups compared with controls were calculated depending on amplification efficacy of the primer pairs used. Single values and means are graphically displayed on a log 2 scale.

In situ hybridization for hsp70 mRNA

After decapitation brains were immediately frozen at -70°C .

1. In situ hybridization for assessment of regional distribution and for mRNA quantification was performed as previously described (Erdtmann-Vourliotis et al 1999). In brief, 15- μm -thick frontal sections were fixed in 4% paraformaldehyde for 30 minutes. The oligonucleotide for Hsp70 (GenBank accession no. L16764): 5'-GGC CTC CTG CAC CAT GCG CTC GAT CTC CTC CTT GCT CA-3', complementary to base position 1743–1706, was radiolabeled with deoxyadenosine 5' [α - ^{32}P] thio] triphosphate (NEN Life Science Products, Cologne, Germany) using the terminal deoxynucleotidyl transferase reaction. The oligonucleotide was synthesized by IBA, Göttingen, Germany. To exclude unspecific hybridization, a sense probe of Hsp70 was used: 5'-ACT CGT TCC TCC TCT AGC TCG CGT ACC ACG TCC TCC GG-3' as a control.

Sections were incubated overnight at 42°C with 5×10^5 counts per minute of labeled probe in 50 μL of hybridization buffer. After washing twice in $1\times$ sodium chloride–sodium citrate (SSC) at 58°C and finally at room temperature, slides were dried and exposed to X-ray films (BIO-MAX film, Kodak, Germany) for 28 days. The anatomical atlas of Paxinos and Watson (1997) was used to identify the brain regions. Image editing software (Adobe Photoshop) was used to create montages that were printed on a digital image printer (FUJIX PICTOGRAPHY 3000). The signals were captured by a CCD camera for densitometric quantification. The quantification results are displayed as mean $\pm$ SEM; statistical analysis was performed by 1-way ANOVA followed by Tukey's multiple comparison posttest. A P value <0.05 was regarded as significant.

2. In situ hybridization for determination of cellular distribution of Hsp70 mRNA: the PCR product complementary to nucleotide 493–696 of the Hsp70 mRNA (GenBank accession no.: L16764) was amplified from rat brain cDNA and cloned into the pGEM-Teasy vector (Promega, Mannheim, Germany). The construct was verified by double-strand DNA sequencing. Riboprobes in antisense and sense orientation were generated from the linearized vector constructs by *in vitro* transcription using [^{32}P]-uridine triphosphate as label (1000 Ci/mmol; 15 μM concentration in the transcription reaction). The probes were subjected to mild alkaline hydrolysis (Angerer et al 1987) and purified using P-30 spin-columns (Bio-Rad). The probes were diluted in hybridization buffer ($3\times$ SSC), 50 mM NaPO_4 , 20 mM dithiothreitol, $1\times$ Denhardt's solution, 25-mg/

mL yeast transfer RNA, 10% dextran sulfate, and 50% formamide) to 50 000 dpm/ μL before hybridization for 14 hours at 60°C . In situ hybridization histochemistry was performed as described previously (Stumm et al 2001). Briefly, brain sections were fixed in phosphate-buffered 4% paraformaldehyde for 60 minutes, incubated for 10 minutes in 0.4% Triton X-100, and for 10 minutes in 0.1 M triethanolamine, pH 8.0 (Sigma, Deisenhofen, Germany) containing 0.25% v/v acetic anhydride (Sigma). After hybridization, the slides were washed in $2\times$ SSC and $1\times$ SSC before a 30-minute treatment with 1 unit/mL ribonuclease T1 and 20 $\mu\text{g}/\text{mL}$ RNase A (Roche Diagnostics) at 37°C in 10 mM Tris, pH 8.0, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid. Slides were extensively washed in $1\times$ and $0.2\times$ SSC at room temperature and subsequently in $0.2\times$ SSC for 60 minutes at 60°C . After washing in water, the tissue was dehydrated in 50% and 70% 2-propanol. The slides were exposed to X-ray films for 24 hours. Autoradiographic detection of ^{32}P was performed by coating the hybridized sections with NTB-2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) and by exposing them for 10 days. After processing with developer and fixer (Eastman Kodak), sections were counterstained with cresyl violet.

3. Double in situ hybridization histochemistry: two different mRNA transcripts in the same tissue section were detected by combining radioactive and nonradioactive in situ hybridization as described (Stumm et al 2002). Isotopically labeled riboprobes were transcribed for the mRNAs of Hsp70 (see above), the beta polypeptide of the complement component 1q (C1q), and glial fibrillary acidic protein (GFAP) (Stumm et al 2002). Digoxigenin (DIG)-labeled probes were transcribed for the mRNAs of Hsp70, the basic helix-loop-helix transcription factor olig1 (*SmaI-BamHI* fragment of 3'-untranslated region), and the vesicular glutamate transporter 1 (Stumm et al 2004) according to the manufacturers instructions (Roche Diagnostics). VGLUT1 is a selective marker for glutamatergic neurons (Takamori et al 2000; Fremeau et al 2001), GFAP identifies astrocytes, olig1 oligodendrocytes (Zhou et al 2000), and C1q microglial cells/macrophages (Schwaebler et al 1995; Schafer et al 2000). DIG-labeled probes were diluted in radioactive hybridization mix to a final concentration of 1 $\mu\text{g}/\text{mL}$, hybridized, and washed as above. Detection of DIG-labeled probes with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche Diagnostics) was performed as described (Stumm et al 2002). The ^{32}P -labeled probes were detected by K5 photoemulsion (Ilford, Dreieich, Germany). To estimate how many percent of cells in an identified population express Hsp70 mRNA, 50 marker-positive profiles were randomly selected for each com-

bination and analyzed for coexpression. Because of stereological issues, the results of this evaluation should be considered as semiquantitative estimation rather than as exact quantification.

Immunohistochemistry

Rats were deeply anaesthetized with chloral hydrate and transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were rapidly dissected and postfixed in the same fixative for 24 hours at room temperature. Tissue was cryoprotected by immersion in 30% sucrose for 48 hours at 4°C before sectioning using a freezing microtome. Free-floating sections (40 µm) were incubated with an antibody specific for Hsp70 (Stressgen, Victoria, BC, Canada SPA-810; Moon et al 2001) at a dilution of 1:1000. Characterization of the antibody has been reported previously (Milarski et al 1989). The primary antibody was detected using biotin-conjugated anti-mouse antibody, amplified by the tyramine amplification procedure and detected by streptavidine-Cy3 (Stumm et al 2002). For immunocytochemical controls, the primary antibody was omitted. Specimens were examined using a Leica TCS-NT laser scanning confocal microscope (Heidelberg, Germany) equipped with a krypton-argon laser. Cy3 was imaged with 568-nm excitation and 570–630 nm band pass emission filters.

Tissue culture experiments with MOR1 HEK 293 cells

Stable rat MOR1-transfected HEK 293 cells (Koch et al 1998) were used. These cells have been shown to exhibit MOR1 agonist coupling to cellular second messenger systems like adenylyl cyclase (Koch et al 1998). Cells were maintained in Dulbecco's Modified Eagle Medium (GIBCO Invitrogen Corporation, Karlsruhe, Germany), supplemented with 10% fetal calf serum and 1 µg/mL puromycin in a humidified atmosphere containing 10% CO₂ at 37°C. Approximately 3×10^6 cells per well were seeded in 60-mm dishes. Experiments were performed in triplicates. Cells were incubated for 2 hours at 37°C, 10% CO₂ with Opti-MEM I (GIBCO Invitrogen Corporation) containing either morphine HCl (10^{-5} M), naloxone HCl (10^{-6} M), naloxone HCl + morphine HCl (cells were preincubated with naloxone HCl, and morphine HCl was added after 15 minutes) or the respective volume of vehicle (phosphate-buffered saline). Additional cells underwent 2 hours incubation with Opti-MEM I at 42°C, 10% CO₂ to imitate heat stress. After incubation, cells were washed $1 \times$ with Opti-MEM I, placed on ice, and suspended in Qiagen Lysis Buffer (Qiagen). Extraction of RNA, reverse transcription, and real-time PCR experiments were performed as described above. Sequences and annealing

temperatures for the respective upstream and downstream primers: Hsp70 (accession number BC002453): 5'-CAA GGT GCA GGT GAG CTA CAA GG-3' and 5'-TTG ATG ATC CGC AGC ACG TTG AG-3', 69°C; beta actin (accession number X00351): 5'-CCA TGT ACG TTG CTA TCC AGG CTG T-3' and 5'-CTC CTT AAT GTC ACG CAC GAT TTC C-3', 69°C was used as a control house-keeping gene.

Body temperature after different morphine doses

In another panel of animals ($n = 5-6$ per group), changes in body temperature were assessed after injection of the different morphine doses as described above. Temperature was monitored rectally with a flexible digital thermometer (ama-digit ad 15th [Amarell Electronic, Kreuzertheim, Germany]) before as well as 30, 60, 90, 120, 180, 240, 300, and 360 minutes after morphine application. The stainless steel sensor was rectally introduced 7 cm deep. Statistical evaluation was performed using 2-way ANOVA, and a P value <0.05 was considered significant.

All drug applications and temperature measurements were carried out at a constant room temperature of 21°C.

RESULTS

1. mRNA expression of Hsp70, Hsp27, Hsc70, and Hsp90 in the frontal cortex after acute treatment: Figure 1 (A,B) shows the time course of the mRNA expression levels in the frontal cortex of rats in response to a 20-mg/kg dose of morphine. There was a rapid induction of Hsp70 mRNA levels with a mean maximum induction of 13.2-fold, 2 hours after drug application followed by a decline at 4 hours and return to control values at 24 hours. The increase in Hsp70 mRNA was significant for the 1-, 2-, and 4-hour time intervals. In another set of experiments, a dose-response relationship was tested 4 hours after morphine. Although the responsiveness of Hsp70 mRNA expression to morphine in these animals was less pronounced (3.5-fold instead of 7-fold induction after 4 hours after 20 mg/kg morphine), a clear dose dependency was seen with significant Hsp70 mRNA elevations after 10, 20, and 50 mg/kg as compared with control animals (Fig 1 C,D). Administration of the opioid antagonist naloxone reversed the morphine-induced increase of Hsp70 mRNA levels. In addition to Hsp70, Hsp27 mRNA was increased in response to a 20-mg/kg dose of morphine. A mean maximum increase of 6.7-fold was found 4 hours after morphine treatment (Fig 2A). In general, the mRNA of the constitutively expressed Hsc70 was more than 35-fold stronger expressed in the

Fig 1. (A,B) Time course of Hsp70 messenger RNA (mRNA) expression after 20 mg/kg morphine. (A) Crossing points in real-time polymerase chain reaction (PCR), when amplification starts its exponential phase: $n = 5$ per treatment group, mean $\pm$ SD; 1 hour vs control: $P < 0.001$, 2 hours vs control: $P < 0.001$, 4 hours vs control: $P < 0.001$, 1 hour vs 12 hours: $P < 0.001$, 2 hours vs 12 hours: $P < 0.001$, 4 hours vs 12 hours: $P < 0.001$, 1 hour vs 24 hours: $P < 0.001$, 2 hours vs 24 hours: $P < 0.001$, 4 hours vs 24 hours: $P < 0.001$. (B) Calculated—fold inductions compared with control: single values and means. (C,D) Hsp70 mRNA expression in response to different morphine doses (mg/kg), 4 hours. (C) Crossing points in real-time PCR, when amplification starts its exponential phase: $n = 4$ –5 per treatment group, mean $\pm$ SD; 10 mg/kg vs control: $P < 0.001$, 20 mg/kg vs control: $P < 0.001$, 50 mg/kg vs control: $P < 0.001$, 50 mg/kg vs 5 mg/kg: $P < 0.05$, 10 mg/kg vs 50 mg/kg + naloxone: $P < 0.01$, 20 mg/kg vs 50 mg/kg + naloxone: $P < 0.01$, 50 mg/kg vs 50 mg/kg + naloxone: $P < 0.001$. (D) Calculated—fold inductions compared with control: single values and means.

brain of control animals than that of Hsp70 or Hsp27. After application of 20 mg/kg morphine, minor but significant inductions of Hsc70 mRNA levels were found, which did not exceed a mean factor 1.7 at any of the given time intervals (Fig 2B).

Like Hsc70, Hsp90 mRNA was much stronger expressed in the brain of control animals than that of Hsp27 or Hsp70 (more than 25-fold). After application of 20 mg/kg morphine, small but statistically significant increases in the Hsp90 mRNA levels were observed, which did not exceed a mean factor 1.8 at any of the given time points (Fig 2C).

For the time course experiments, naive animals were used as controls. To exclude a possible role of injection stress or vehicle on gene expression, an additional control group that received saline and gene expression was analyzed after 2 hours, when maximal Hsp mRNA expression was assumed. There was no statistically significant difference in expression of the different Hsps between the 2 groups (data not shown).

2. Distribution of Hsp70 mRNA in brain measured by in situ hybridization after acute treatment: in contrast to saline-treated animals where Hsp70 mRNA was only marginally expressed, a dose of 50 mg/kg morphine induced a strong expression of Hsp70 mRNA within all cortical areas (Fig 3, column 3A–H). Furthermore, Hsp70 mRNA expression was observed in the bed nucleus of the stria terminalis (BSTM) (3C), the striohypothalamic nucleus (StHy) (3C), the medial preoptic (MPO) (3C), the supraoptic (SO) (3D) and paraventricular (PaV) (3D) hypothalamic nuclei, as well as in the habenula (Hb) (3E). In the hippocampus, Hsp70 mRNA was expressed in CA1-3/4 fields and in the dentate gyrus (3D–F). In addition, expression of Hsp70 mRNA was found in the red nuclei (RN) (3F), the pons (Pn) (3G), the pineal body (PB) (3H), the cerebellar cortex, as well as in the locus coeruleus (LC) (3I). Quantitatively similar effects albeit slightly less pronounced were observed in response to 20 mg/kg morphine (Fig 3, column 2). The morphine-induced Hsp70

Fig 2. (A) Time course of Hsp27 messenger RNA (mRNA) expression after application of 20 mg/kg morphine: calculated—fold inductions compared with control; $n = 5$ per treatment group single values and means. Statistical significance according to crossing points (not shown): 4 hours vs control: $0 < 0.01$, 4 hours vs 12 hours: $P < 0.01$, 4 hours vs 24 hours: $P < 0.01$. (B) Time course of Hsc70 mRNA expression after application of 20 mg/kg morphine: calculated—fold inductions compared with control; $n = 5$ per treatment group single values and means. Statistical significance according to crossing points (not shown): 1 hour vs control: $P < 0.05$, 2 hours vs control: $P < 0.01$, 4 hour vs control: $P < 0.001$, 24 hours vs control: $P < 0.05$. (C) Time course of Hsp90 mRNA expression after application of 20 mg/kg morphine: calculated—fold inductions compared with control; $n = 5$ per treatment group single values and means. Statistical significance according to crossing points (not shown): 1 hour vs control: $P < 0.001$, 2 hours vs control: $P < 0.001$, 4 hour vs control: $P < 0.001$, 24 hour vs control: $P < 0.01$.

expression was almost completely blocked by application of naloxone (Fig 3, column 4). Densitometric quantification of cortical areas at the level of caudate putamen revealed a significant increase in Hsp70 mRNA expression after 50 mg/kg morphine, which was blocked by naloxone (Fig 4A). At the level of the hippocampus, 50 mg/kg morphine resulted in a significant increase in Hsp70 expression in CA1, CA3/4 regions as well as in the dentate gyrus, which was decreased by cotreatment with naloxone (Fig 4 B–D). Unspecific hybridization could be excluded by hybridization with a sense probe (Fig 3, column 6B–I).

- Cell types expressing Hsp70 mRNA and the corresponding protein: low-power darkfield micrographs of hybridized coronal section at the level of the hippocampus revealed that morphine-induced Hsp70 mRNA is strongly expressed in white and gray matter throughout the brain and strongly upregulated in blood vessels, granule cells, and neurons in the hilar region (Fig 5C, C'). Interestingly, basal as well as morphine-induced Hsp70 mRNA expression is much higher in CA3 than in CA1. In addition, high-power images from brain sections of morphine-treated animals re-

vealed strong hybridization signals in blood vessels (Fig 6F). Using confocal images of coronal brain sections after immunohistochemical detection of the corresponding protein, intense Hsp70 immunoreactivity could be detected in granule cells and in blood vessels (Fig 6 D,E).

Additional in-detail analysis of cell types expressing Hsp70 mRNA after morphine treatment using semi-quantitative double labeling in situ hybridization experiments (Fig 7) revealed expression of Hsp70 mRNA in more than 90% of hippocampal VGLUT1-positive glutamatergic neurons, approximately 20% of olig1-positive oligodendrocytes in thalamus, and less than 5% of GFAP-positive astrocytes as well as Clq-positive microglial cells.

- Hsp70 mRNA expression in MOR1 HEK 293 cells after stimulation with morphine or naloxone (or both): as displayed in Figure 8, no significant differences in Hsp70 mRNA expression could be observed after stimulation with the MOR1 agonist or antagonist (or both). In contrast, a 2-hour incubation period at a high temperature (42°C) resulted in a significant elevation of Hsp70 mRNA expression. Neither drug treatment

Fig 3. Autoradiographic images displaying the overall distribution of Hsp70 messenger RNA (mRNA) after a single dose of morphine 20 mg/kg (column 2), 50 mg/kg (column 3), naloxone 10 mg/kg + morphine 50 mg/kg (column 4) compared with saline treatment (column 1) in coronal sections of the rat brain. Schematic drawings (column 5) are given according to the stereotactic coordinates of the rat brain atlas of Paxinos and Watson (1997). Column 6: Hsp70 sense probe after morphine 50 mg/kg. Line A: bregma 1.70 mm, line B: bregma 0.70 mm,

Fig 4. Quantification of Hsp70 messenger RNA (mRNA) expression, displayed as optical density of percentage of control after a single dose of morphine 20 mg/kg, 50 mg/kg, or 50 mg/kg + naloxone; mean, standard error of mean (SEM). (A) Cortical area: cingulate, frontal, parietal, and insular cortex at the level of caudate putamen (see line B, Fig 3). (B) CA1 of hippocampus (see line E, Fig 3). (C) CA3/4 of hippocampus (see line E, Fig 3). (D) Dentate gyrus (see line E, Fig 3). * $P < 0.05$, ** $P < 0.01$.

nor heat stress resulted in changes of the housekeeping gene beta actin.

- Measurement of rat body temperature in response to morphine doses: morphine doses of 5–20 mg/kg resulted in hyperthermia (maximum 40.3–40.6°C after 60 minutes), whereas the high dose of 50 mg/kg produced hypothermia (minimum 37.2°C after 240 minutes) (Fig 9 A,B). Two-way ANOVA revealed statistically significant differences according to dose and time ($P < 0.0001$, each). Naloxone given 15 minutes before and 120 minutes after morphine application prevented morphine-induced hypothermia but led to a temperature increase to 40.4°C after 120 minutes; this is most likely the result of residual morphine effects due to the short half-life of naloxone.

DISCUSSION

In a recent study, we could demonstrate that a 10-day treatment course with increasing morphine doses from twice-daily 10 mg/kg to twice-daily 50 mg/kg, leading to morphine tolerance, resulted in a significant increase of mRNA expression of Hsp70 and Hsp27 in the rat brain 4 hours after the last morphine dose (Ammon et al 2003). This study demonstrates that the same dosages applied as single doses from 10–50 mg/kg morphine also induce a marked increase of Hsp70 mRNA and the corresponding protein in the brain of rats. Gene expression was blocked by administration of the MOR1 antagonist naloxone. Morphine also induced a substantial increase in Hsp27 mRNA expression in the frontal cortex. As ex-

line C: -0.92 mm, line D: bregma -1.80 mm, line E: bregma -2.80 mm, line F: bregma -5.80, line G: bregma -6.80, line H: bregma -8.30, line I: bregma -9.80. BSTM, medial division of the bed nucleus of the stria terminalis; CA1, CA3/4, fields of hippocampus; cc, corpus callosum; Cg, cingulate cortex; Col, colliculi; CPu, caudate putamen; DG, dentate gyrus; Fr, frontal cortex; Hb, habenula; Hipp, hippocampus; In, insular cortex; LC, locus coeruleus; MPO, medial preoptic nucleus; Par, parietal cortex; PB, pineal body; Pir, piriform cortex; Pn, pons; RN, red nucleus; SO, supraoptic nucleus; StHy, striohypothalamic nucleus.

Fig 5. Cellular expression of Hsp70 messenger RNA (mRNA). (A–C) Low-power darkfield micrographs of representative hybridized coronal sections through the right brain hemisphere at the hippocampal level after hybridization with a riboprobe for Hsp70 mRNA (B,C) and after hybridization with the respective sense-probe (A). Animals that received morphine 50 mg/kg (A,C) or saline (B), respectively, are depicted. (B) After saline, moderate Hsp70 mRNA expression levels are detected in the retrosplenial cortex (RS), the CA3 subfield of the ammons horn (CA3), and the paraventricular nucleus (PVN). Very low Hsp70 mRNA levels are seen in the dentate gyrus (DG) and the CA1 subfield of the ammons horn (CA1). In other brain regions including the thalamus (Th), Hsp70 mRNA is not detected. (C) After morphine treatment, expression of Hsp70 mRNA is induced in the white and gray matter throughout the brain. (A'–C') High-power magnification of the dentate gyrus. (B') Note presence of very low Hsp70 mRNA levels in the granule cells (Gr). (C') After morphine, Hsp70 mRNA is strongly upregulated in blood vessels (arrowheads), granule cells, and neurons in the hilar region (Po). (A,A') Sparse nonspecific hybridization signals after sense-strand hybridization. Note that the white appearance of the white matter (cc, corpus callosum; fi, fimbria hippocampus; ic, internal capsule; opt, optic tract) is due to darkfield illumination and not a result of hybridization signals. Scale bars: (A–C) 2.5 mm; (A'–C') 0.4 mm. Exposure time (A–C) 10 days.

pected, there were only minor effects of morphine on mRNA expression of the constitutively expressed Hsc70 as well as on the expression of Hsp90. Regional distribution of Hsp70 mRNA revealed a marked morphine-induced expression in the neocortex, the hippocampus, the cerebellar cortex, the hypothalamic nuclei (supraoptic and paraventricular nuclei), the locus coeruleus, as well as in the pineal body. Examination of cell-specific expression revealed expression mainly in neurons and to a lesser extent in glia (oligodendroglia) as well as in blood vessels.

The regional distribution of Hsp70 mRNA after morphine application in this study is only partially consistent with the known distribution of MOR1s: Although MOR1 localization has been described in cortex, hippocampus, locus coeruleus, and habenula, areas that show increases in Hsp70 expression in response to morphine, a very high expression of MOR1s has been found in thalamic nuclei in which the expression of Hsp70 was not particularly

increased in response to morphine. In addition, in the caudate putamen and nucleus accumbens, a patchy distribution of MOR1s has been found, which is not reflected by a similar pattern of Hsp70 expression (Mansour et al 1987). Furthermore, morphine did not influence Hsp70 mRNA expression in vitro in a MOR1-transfected cell line in this study. Therefore, it is not likely that morphine-induced Hsp70 mRNA expression is exclusively mediated by a direct activation of MOR1-containing neurons. The fact that prior administration of the opiate receptor antagonist naloxone in vivo prevented morphine-induced Hsp70 mRNA expression in this study and that naloxone-precipitated morphine withdrawal abolished Hsp70 mRNA induction mediated by chronic morphine application (Ammon et al 2003) would rather imply a MOR1-related indirect mechanism, which still has to be determined.

The question arises whether the observed morphine-induced Hsp70 mRNA expression in a variety of brain

Fig 6. Expression of Hsp70 in neuronal cells and blood vessels (protein: 12 hours, messenger RNA (mRNA): 2 hours) after morphine treatment. (A–E), Confocal images of coronal brain sections taken from saline-treated (A,B), and morphine-treated animals (D,E) after immunohistochemical detection of Hsp70. (A) After saline treatment, faint Hsp70-immunoreactivity was present in the granule cell layer, and Hsp70-immunoreactivity was absent from hippocampal blood-vessels (arrowheads). (D) In the morphine-treated animal, intense Hsp70-immunoreactivity is detected in the granule cell layer and in blood vessels (arrowheads). (B,E) High-power images of blood vessels in the brain parenchyma. Note the very intense Hsp70-immunostaining in blood vessels after morphine (E) and absence of HSP70-immunoreactivity from blood vessels after saline. (C,F) High-power images of brain sections from a saline-treated (C) and a morphine-treated (F) animal after hybridization for Hsp70 mRNA. (C) Note that in the saline-treated animal, hybridization signals for Hsp70 mRNA are largely absent from the depicted blood vessel (arrowheads) and other brain cells. (F) After morphine, the depicted blood vessel shows strong hybridization signals (arrowheads), and other brain cells are moderately labeled. Scale bars: (A,B,D,E) 80 μ m; (C,F) 75 μ m. Exposure time: (C,F) 10 days.

regions is due to a general stress-like situation after morphine administration such as respiratory depression, to a counterregulatory action against potential hazardous cellular effects of morphine, or to a drug-specific activation of Hsp70 transcription ie, like activation by geldanamycin. The latter, a benzoquinone ansamycin, has been shown to bind Hsp90 in vitro, resulting in dissociation of HSF-1 and subsequent binding of HSF-1 on the promoter region of the *hsp70* gene initiating transcription. Interest-

ingly, rats pretreated with geldanamycin have been shown to be more protected against subsequent focal cerebral ischemia showing reduced infarct volumes (Lu et al 2002).

In the rat, acute morphine administration is known to stimulate the hypothalamic pituitary adrenal (HPA) axis, as reflected by elevated adrenocorticotropin and corticosterone levels in peripheral blood. Morphine has been reported to stimulate the HPA axis after administration of

Fig 7. Cell types expressing Hsp70 messenger RNA (mRNA) after morphine treatment. Double in situ hybridization with a probe for Hsp70 mRNA (A–D) and probes for VGLUT1 (A), olig1 (B), C1q (C), and GFAP (D), respectively. Isotopically labeled probes (^{35}S) are detected as *grains* and digoxigenin (DIG)-labeled probes as *gray* reaction product. (A,B) Hsp70 mRNA expression is present in VGLUT1-positive glutamatergic neurons in the hippocampal CA3 field (A, arrows) and olig1-positive oligodendrocytes in the thalamus (B, arrows). (A) Note also VGLUT1-negative cell expressing Hsp70 mRNA (arrowhead). (C) Hsp70 mRNA expression (arrows) is not detected in C1q mRNA-positive microglial cells in the thalamus (arrowhead). (D) Several GFAP mRNA-expressing astrocytes that are Hsp70 mRNA negative (arrowheads) surround a large Hsp70 mRNA-expressing blood vessel (arrows). Scale bar, 60 μm .

Fig 8. Hsp70 messenger RNA (mRNA) (A) expression in μ -opioid receptor (MOR1) HEK 293 cells after 2 hours exposure to morphine 10^{-5} M, naloxone 10^{-6} M, naloxone + morphine or after a 2 hours exposure to 42°C (drug free): calculated—fold inductions compared with controls; $n = 3$ per group; single values and means. Statistical significance according to crossing points (not shown): 42°C heat vs controls: $P < 0.001$, 42°C heat vs morphine: $P < 0.001$, 42°C heat vs naloxone: $P < 0.001$, 42°C heat vs naloxone + morphine: $P < 0.001$. Beta actin mRNA expression (B): no statistical significant differences between groups.

relatively high doses (≥ 20 mg/kg), and it has been argued that this effect might be due to a stress effect ie, respiratory depression. The actions of opioids on the HPA axis are believed to be mediated, directly or indirectly, by the release of corticotropin-releasing factor and the ability to stimulate the HPA axis can be blocked by opioid antagonists (Pechnick 1993). Interestingly, metyrapone, an inhibitor of corticosterone synthesis, has been shown to

block the kainate-induced expression of Hsp70 (Czyrak et al 2000). It could be likely, that the upregulation of the Hsp70 expression in the paraventricular nucleus of the hypothalamus in this study may reflect some effects of morphine on the endocrine system. On the other hand, naloxone-induced opiate withdrawal in morphine tolerant rats, which also acts as a strong stimulus for the activation of the HPA axis, abolished morphine-induced Hsp70 expression in our former study (Ammon et al 2003). Therefore, additional pathways must be taken into account.

It is likely that toxic cellular effects of high morphine doses lead to compensatory protective mechanisms like activating the blood brain barrier. A slight increase of the drug efflux pump P-glycoprotein in the brain of morphine-tolerant rats has been reported (Aquilante et al 2000). A combination of Hsp70 and P-glycoprotein expressed in blood vessels may play a role in neuroprotection. The toxic effects of morphine and heroin have been demonstrated in vitro (Oliveira et al 2002). In addition, opioids have been shown to disrupt Ca^{2+} homeostasis and induce carbonyl oxyradical production in mouse astrocytes (Hauser et al 1998).

Hsps may protect cells by mechanisms unrelated to their chaperone function as well. Several articles have now established that Hsp70 also can interfere with apoptosis in various systems and that Hsp70 probably acts at multiple sites to confer protection in models of apoptosis (Yenari 2002). Interestingly, in the model of permanent middle cerebral artery occlusion, it has been shown that ischemic cells capable of translating Hsp70 protein generally do not undergo DNA fragmentation, indicating a reversible injury (States et al 1996). One might speculate therefore that increased Hsp70 expression after morphine application might contribute to protect cells against potentially hazardous effects of opiates. However, the reported effects of morphine on apoptosis on cells of the central nervous system are contradictory, possibly depending on experimental conditions (Boronat et al 2001; Hu et al 2002; Iglesias et al 2003).

A neuroprotective effect of Hsp70 in vivo is well established. Induction of Hsp70 by previous moderate heat shock or ischemia has been shown to reduce vulnerability of neuronal cells when exposed to subsequent thermal stress or ischemia (Kelty et al 2002). Furthermore it has been reported that transgenic mice overexpressing Hsp70 are more protected against ischemic neuronal and cardiac distress (Plumier et al 1995; Rajdev et al 2000), and it has been demonstrated that *hsp70.1* gene- or *hsp70.3* gene-disrupted mice displayed reduced thermotolerance and increased cellular apoptosis after thermal stress (Huang et al 2001).

In this investigation, mainly neuronal cells as well as oligodendroglia showed an enhanced morphine-mediat-

Fig 9. Time course of rat body temperature in response to the different doses (mg/kg) of morphine. (A) Low morphine doses. (B) High morphine dose with or without naloxone; mean, standard error of SEM.

A) low morphine doses

B) high morphine dose, with or without naloxone

ed Hsp70 mRNA expression in different brain areas. In addition, a strong Hsp70 expression has been observed in blood vessels, which suggests also an endothelial expression. Interestingly, a differential cell-specific expression of Hsp70 mRNA and the corresponding protein according to different stressors has been discussed recently (Krueger et al 1999). In line with the phenomenon of hypoxic preconditioning, whereby a mild hypoxemia induces Hsps and protects organs against subsequent more severe episodes, a single morphine application may contribute through Hsp70 expression in different cell types to a better tolerance to the next subsequent dose and may contribute to the phenomenon of development of morphine tolerance.

Because morphine is known to alter body temperature in rats, one could have speculated that upregulation of Hsp70 might be caused by the hyperthermic action of morphine. Other drugs of abuse such as LSD and amphetamine are known to produce hyperthermia. LSD-induced Hsp70 mRNA expression is not present when hy-

perthermia is blocked (Manzerra and Brown 1990). In contrast, amphetamine treatment seems to result in a higher Hsp70 mRNA expression in heat shocked rats, leading to the hypothesis of a direct toxic effect of amphetamine or of a catecholamine-mediated potentiation of the heat shock response in certain cell types (Miller et al 1991).

In this study, treatment of rats with doses between 5–20 mg/kg morphine resulted in marked elevations in the body temperature, which peaked 60 minutes after drug administration. In contrast, a high dose of 50 mg/kg morphine decreased body temperature. It has long been recognized that opiates like morphine exhibit complex effects on body temperature: the effects of morphine are known to be species specific, showing a dose-dependent dual response (low-dose hyperthermia and high-dose hypothermia) in rats and mice at thermoneutral ambient temperature (Rosow et al 1980; Geller et al 1983). Interestingly, environmental temperature can profoundly affect body temperature responses to morphine. At cool

ambient temperatures, dose-related hypothermia has been described, whereas warm ambient temperatures have been reported to result in dose-related hyperthermia (Paolino and Bertrand 1968; Rosow et al 1980). To avoid these confounding factors, we chose a thermoneutral ambient temperature (21°C) for the experiments.

The exact mechanisms involved in the body temperature changes induced by morphine are still not known with certainty, and several potential mechanisms involved in the dual temperature response after morphine administration have been discussed: The primary site of thermoregulatory control in mammals is the preoptic anterior hypothalamus (POAH), and it has been assumed that low-dose morphine-induced hyperthermia is due to an upward setting of the hypothalamic set point and to a consequent vasoconstriction that decreases heat loss and that high-dose morphine-induced hypothermia is primarily attributed to decreased oxygen consumption (Adler et al 1988).

In the POAH, there is convincing evidence for a differential involvement of the respective opioid-receptor subtypes, indicating that MOR1s mediate hyperthermia, whereas kappa opioid receptors mediate hypothermia (Chen et al 1996; Xin et al 1997). The finding that morphine might produce hypothermia only at relatively high and nonselective doses may be important. On the other hand, in the study of Baker et al (2002), no effect on morphine-mediated hypothermia was evident using the specific kappa receptor antagonist nor-binaltorphimine.

Morphine is known to alter locomotor activity and other behavioral parameters. Low doses (5 mg/kg and less) have been associated with increased locomotor activity (Bartoletti et al 1983; Jorenby et al 1988). One could therefore speculate that heat generated by increased muscular activity would elevate body temperature. Nevertheless, because morphine-induced hyperthermia frequently accompanies a cataleptic state in rats and because hypothermia may even accompany "running fits" in mice, heat production due to activity seems to be an insufficient explanation for changes in temperature (according to Adler et al 1988).

In this study, a dose-dependent catalepsy with increasing sedation and plastic rigidity of posture, most pronounced at doses of 20 and 50 mg/kg, was observed.

Our finding that a high dose of morphine induces Hsp70 mRNA expression despite decreased body temperature argues against the fact that Hsp70 mRNA induction by morphine is solely due to morphine-induced hyperthermia. Phencyclidine, a drug of abuse that causes hypothermia has also been reported to induce Hsp70 mRNA in multiple brain regions, possibly by blockade of NMDA-receptors of GABA-ergic interneurons, leading to disinhibition of pyramidal neurons. In addition, phencyclidine depolarizes neurons and produces high, poten-

tially damaging intracellular calcium levels (Sharp et al 1994). Alcohol, which causes hypothermia in rats at normal ambient temperatures, induces Hsp70 expression in various brain areas, an effect that was attributed to the cellular oxidative stress caused by alcohol (Calabrese et al 2000). Thus, it appears to be likely that increased Hsp70 expression after several drugs of abuse is not exclusively mediated by their effects on body temperature.

In summary, this study demonstrates a rapid and transient induction of Hsp70 by morphine in several brain regions in neuronal cells, glia (mainly oligodendroglia), and blood vessels. It might be speculated that Hsp70 protects the brain against potentially harmful effects of morphine. However, the exact mechanism of action still has to be determined. According to our results, a direct MOR1-mediated cellular effect on Hsp70 expression as well as an induction solely caused by morphine-mediated changes in body temperature appear to be unlikely.

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