Cell Stress and Chaperones
DOI 10.1007/s12192-008-0013-9

ORIGINAL PAPER

05-3037-SCR

Changes in the regulation of heat shock gene expression in neuronal cell differentiation

Jay Oza · Jingxian Yang · Kuang Yu Chen · Alice Y.-C. Liu

Received: 17 May 2007 / Revised: 1 August 2007 / Accepted: 9 August 2007

Abstract Neuronal differentiation of the NG108-15 neuroblastoma–glioma hybrid cells is accompanied by a marked attenuation in the heat shock induction of the Hsp70-firefly luciferase reporter gene activity. Analysis of the amount and activation of heat shock factor 1, induction of mRNA^{hsp}, and the synthesis and accumulation of heat shock proteins (HSPs) in the undifferentiated and differentiated cells suggest a transcriptional mechanism for this attenuation. Concomitant with a decreased induction of the 72-kDa Hsp70 protein in the differentiated cells, there is an increased abundance of the constitutive 73-kDa Hsc70, a protein known to function in vesicle trafficking. Assessment of sensitivity of the undifferentiated and differentiated cells against stress-induced cell death reveals a significantly greater vulnerability of the differentiated cells toward the cytotoxic effects of arsenite and glutamate/glycine. This study shows that changes in regulation of the HSP and HSC proteins are components of the neuronal cell differentiation program and that the attenuated induction of HSPs likely contributes to neuronal vulnerability whereas the increased expression of Hsc70 likely has a role in neural-specific functions.

Q1 **Keywords** Heat shock gene expression · Neuronal cell differentiation · Heat shock protein

J. Oza · J. Yang · A. Y.-C. Liu (✉)
Department of Cell Biology and Neuroscience,
Division of Life Sciences, Rutgers State University of New Jersey,
604 Allison Road,
Piscataway, NJ 08854-8082, USA
e-mail: liu@biology.rutgers.edu

K. Y. Chen
Department of Chemistry and Chemical Biology,
Rutgers State University of New Jersey,
Piscataway, NJ, USA

Introduction

Induction of the heat shock response (HSR; a.k.a. stress response) is a primary and evolutionarily conserved genetic response to diverse stressors, mediated by activation of the heat shock transcription factor HSF1, culminating in the induction of a family of heat shock proteins (HSPs) that function as chaperones to help in the folding/refolding of nonnative protein, proteases to help in the degradation of irreversibly damaged proteins, and other proteins essential for the protection and recovery from cell damages associated with perturbation of protein homeostasis (Lis and Wu 1993; Morimoto 1993, 1998; Morimoto et al. 1994; Voellmy 1994; Hendrick and Hartl 1995; Feige et al. 1996).

Evidence in the literature suggests that induction of the HSR and ability to upregulate expression of the HSP chaperones—mechanisms that provide important defense against the dire consequences of protein mis-folding and aberrant protein interactions—are decreased in various brain and spinal cord neurons in vivo and in vitro (Manzerra and Brown 1996; Marcuccilli et al. 1996; Nishimura and Dwyer 1996; Guzhova et al. 2001; Batulan et al. 2003; Chen and Brown 2007); in general, neurons, in comparison with glial and ependymal cells, have a higher threshold for induction of the HSR, requiring a greater intensity or duration of stress for a diminished response. Given the importance of protein mis-folding and aggregation in the pathogenesis of various neurodegenerative diseases—including Alzheimer's, Huntington's, Parkinson's, Lou Gehrig's, and prion diseases—it is clear that changes in expression of the HSP chaperones in neurons would have significant implications (Welch and Gambetti 1998; Sharp et al. 1999; Sherman and Goldberg 2001; Bonini 2002; Muchowski 2002; Benn and Brown 2004; Landsbury

2004; Westerheide and Morimoto 2005; Morimoto 2006; Muchowski and Wacker 2005).

We commenced this study to determine if neural differentiation may be accompanied by changes in regulation of heat shock gene expression. Using the NG108-15 tumor neural progenitor cells as our model, we show in this study that their differentiation into neuron-like cells is accompanied by a decreased induction of the heat-inducible HSPs and an increased expression of the constitutive Hsc70 protein.

Materials and methods

Cell culture and induction of neural differentiation Cells of the NG108-15 mouse neuroblastoma–glioma hybrid lineage (Nelson et al. 1976; Nirenberg et al. 1983, 1984) were grown in Dulbecco's modified Eagle's medium (Mediatech Inc.) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc.), 50 μg/ml streptomycin, and 50 U/ml of penicillin. Cells were subcultured at or near confluency by minimal trypsinization (0.25% trypsin; Mediatech Inc.) and dispersion into single cell suspension in new growth medium and plating onto new growing surfaces.

Differentiation of the NG108-15 cells was induced by the subculturing of cells (1:4 split ratio) into a low serum-containing medium (2%, as opposed to the normal 10%, fetal bovine serum) supplemented with 1-mM dibutyryl cAMP (Meyer et al. 1988). Differentiation, scored by % of neurite-positive cells (neurite defined as processes>2× soma diameter), was visible within hours, and >80% of the cells was neurite-positive 2 days after induction with dibutyryl cAMP, as compared to <10% of neurite-positive cells in the undifferentiated culture. Two other parameters used to confirm the neural differentiation phenotype were (1) immunocytochemical staining for neural specific tubulin βIII and neurofilament and (2) voltage clamp recording to validate the presence of voltage-gated sodium channels in the differentiated cells but not the undifferentiated cells (data not shown). In previous studies, it was shown that the differentiated NG108-15 cells form functional synapse with muscle cells at relatively high frequency (Nelson et al. 1976; Nirenberg et al. 1983, 1984).

Primary hippocampal neuron culture was obtained from embryonic day 16 rat embryos according to methods described (Magby et al. 2006). Briefly, hippocampi were dissected from surrounding brain tissue, and meninges were removed. Hippocampi were dissociated by trypsinization, followed by trituration through fire-polished Pasteur pipettes. Neurons were plated in poly-D-lysine-coated plates and maintained in serum-free medium composed of a 1:1 mixture of Ham's F12 and Eagle's MEM supplemented with 25 mg/ml insulin, 100 mg/ml transferring, 60 mM putrescine, 20 nM progesterone, 30 nM selenium, and 6 mg/ml glucose. Cells were plated at a density of 4×10^5 cells/35 mm plate. Experiments were done on cells after 12–15 days in culture, a time when the cells formed an extensive and elaborate neuritic network.

Unless indicated otherwise, the condition for heat shock was at 42°C for a specified time period. Cells were either harvested immediately for analysis of HSF1 or mRNA^{hsp} or allowed to recover at 37°C for a specified time period for analysis of Hsp70-firefly luciferase reporter gene expression and induction of HSP synthesis and accumulation.

Assay of Hsp70 promoter-driven firefly luciferase reporter The Hsp70 promoter-driven firefly luciferase reporter was constructed by ligating a 1,036 bp *Kpn*I and *Nco*I restriction enzyme fragment of the mouse Hsp70 promoter-luciferase reporter, pLHSEU4 (Yanagida et al. 2000), to the *Kpn*I/*Nco*I digested pGL3E (5,006 bp; Promega Inc.). For screening of the effects of heat shock on the Hsp70-luciferase reporter gene activity, undifferentiated and differentiated cells in either 35- or 60-mm plates were transfected with the Hsp70-firefly luciferase reporter along with the internal control of phRLSV40 (synthetic humanized Renilla luciferase DNA; Promega Inc. E6261). Unless indicated otherwise, the amount of each DNA used was 0.5 μg/35-mm plate or 1.5 μg/60-mm plate, and the amount of Lipofectamine 2000 used (in microliters) was three times that of the total amount of DNA (in micrograms). Six hours after DNA transfection, cells were plated into individual wells of a 96 Stripwell™ plate (Corning/Costar 9102); these identically transfected cells allowed for testing of the effects of different times and temperature of heat shock on reporter gene expression.

To evaluate heat shock induction of the Hsp70-luciferase reporter gene, strips of eight wells or designated wells of cells were placed in a 42°C incubator for 2 h followed by recovery at 37°C for 4 h before harvesting. Undifferentiated and differentiated cells were processed in parallel to minimize experimental noise due to variation in incubator temperature, quality/amount of the luciferase assay reagent, and decay of the luciferase luminescence signal. The Dual-Glo luciferase assay reagent system from Promega Inc. (E2920) was used to assay for first the firefly then the Renilla luciferase activity according to manufacturer's instructions. We have also used the Bright-Glo luciferase assay reagent (E2610) from Promega Inc.; qualitatively similar results were obtained, although the Bright-Glo reagent gave a stronger signal with a shorter half-life. Luciferase activity was measured using the Perkin Elmer Victor 2 multiplate reader equipped with dual injectors. Result of the Hsp70-firefly luciferase activity was normalized against that of the Renilla luciferase, and, to facilitate comparison across experiments for statistical analysis, this ratio was set at 1 for the undifferentiated control. By normalizing the Hsp70-firefly luciferase activity against that of the Renilla luciferase internal control, we effectively minimized variations in experimental result due to possible differences in transfection efficiency and cell viability as well as nonselective and toxic effects of the treatment conditions/reagents on gene expression.

Analysis of HSF1 by Western blotting and electrophoretic mobility shift assay Whole cell extract was prepared as previously described (Huang et al. 1994). Immuno-Western blot probing for HSF1 was done using a 1:5,000–1:10,000 dilution of a rabbit polyclonal antibody, RTG88, we generated against a recombinant histidine-tagged human HSF1 protein. For assessment of the activation of HSF1 DNA-binding activity, electrophoretic mobility shift assay was done according to methods described using 20 μg of whole cell extract protein, 0.5 μg of poly(dI–dC).poly(dI–dC), and [^{32}P]labeled HSE in a total reaction volume of 10 μl (Huang et al. 1994). After 20 min of incubation at room temperature, 2-μl aliquot of a five times loading buffer was added and samples analyzed by electrophoresis in 4% acrylamide gel.

Northern blot quantitation of HSP mRNAs RNA was isolated from undifferentiated and differentiated cells incubated under control (37°C) and heat shocked (42°C, 2 h) conditions after the Trizol reagent protocol for RNA isolation from Invitrogen Inc. Concentration of the RNA was determined spectrophotometrically. For Northern blotting, 20 μg of the RNA sample was used. The RNA membrane was pre-hybridized at 60°C for 1 h in a pre-hybridization solution of 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 1 M NaCl, and 100 μg/ml of sheared salmon sperm DNA. Probing of the mRNA$^{hsp89\alpha}$, mRNA^{Hsp70}, and RNA^{hsp25} were done, respectively, by hybridization with [^{32}P]-labeled pHS801 (for Hsp89α), pH2.3 (Hsp 70), and pHS208 (Hsp25) DNA at 60C overnight in a hybridization oven (Hickey et al. 1986). After extensive washing, the membrane was exposed to X-ray film for signal detection.

Assessment of the synthesis of HSPs by 35S]methionine incorporation Confluent cultures in 35-mm plates were refurbished with serum-free medium. The condition for heat shock was 42°C. To assess the induction of HSP synthesis at various times of heat shock, cells were pulse labeled with 50–100 μCi/ml of [^{35}S]methionine/cysteine (Amersham Pro-Mix, a 70:30% mixture of [^{35}S]methionine and [^{35}S] cysteine) for the last an hour immediately before harvesting. For example, for cells that were heat shocked for 6 h, [^{35}S] methionine was added at *t*=5 h, and cells were harvested at *t*=6 h. Cells were harvested by first removing the [^{35}S]-containing medium, rinsed twice with ice cold phosphate-buffered saline (PBS), and scraped into 0.2 ml of a buffer of 10 mM Tris, pH 7.4 containing 1 mM ethylenediamine-tetraacetic acid and 50 μg/ml of phenylmethylsulfonyl fluoride. Cell homogenate was prepared by freezing and thawing the cell suspension once and passing it through a 25G needle. A 5-μl aliquot of the cell homogenate was used to determine the amount of radioactivity incorporated into total cellular protein (trichloroacetic acid-insoluble). Aliquots of the cell extracts containing an equal amount of radioactivity (50–100 K cpm) were subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Immuno-Western blot detection of the heat inducible Hsp70 and constitutive Hsc70 Immuno-Western blot detection and quantitation of the heat-inducible Hsp70 and the constitutive Hsc70 were done using (1) the RTG76 rabbit polyclonal antibody (1:5,000–1:10,000 dilution) that we generated against a histidine-tagged human Hsp70-recombinant protein and that recognizes both the HSP and Hsc70 proteins and (2) a rabbit polyclonal antibody from Stressgen (SPA816) that specifically recognizes the 73-kDa Hsc70 protein. Membrane was incubated with the primary antibody at 4°C overnight followed by horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The antibodies were diluted in Tris-buffered saline with 0.1% Tween 20 and 3% nonfat dry milk, and the immunoblot was probed using Amersham ECL-plus or Millipore Immobilon Western blot reagent.

Immunochemical staining for Hsc70 Undifferentiated and differentiated cells in 60-mm plates were fixed with 4% paraformaldehyde for 30 min at 4°C, permeabilized with 0.1% TritonX100 in PBS for 30 min at 4°C, and washed three times with cold PBS. Wax pen circled areas (~1 cm in diameter) of the fixed and permeabilized cells were overlaid with the Hsc70-specific antibody (Stressgen SPA816 at 1:50 dilution) and incubated at 4°C for 1 h. After washing off the primary antibody, cells were overlaid with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G and incubated at 4°C for 1 h. Cells were viewed using a Nikon Diaphot 300 microscope and phase and fluorescent images captured with a SPOT camera system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Assay for cell viability and activation of caspase 3/7 Cells in 96-well plates were used. To test for vulnerability of oxidative stress-induced cell death, sodium arsenite was added to individual wells to final concentrations as indicated and incubated for time periods specified (12–24 h). The ability of glutamate to elicit excitotoxic cell death was evaluated in the presence of 0-, 10-, and 50-μM glycine and incubation at 37°C for time periods indicated

(12–24 h). Cell viability was determined using the CellTiter-Glo luminescent cell viability assay reagent from Promega Inc., and results were normalized against that of the untreated control (100%). Caspase 3 and 7 activity was determined using the Caspase-Glo™ 3/7 assay reagent from Promega Inc., and the readouts were normalized against signal from cell viability assay.

Results

Neural differentiation is associated with an attenuated induction of the Hsp70-luciferase reporter gene

We used the Hsp70 promoter-firefly luciferase reporter gene to assess induction of the HSR in the undifferentiated versus the differentiated NG108-15 cells. Figure 1 presents the average ± standard deviation of Hsp70-luciferase reporter gene activity of the control- and heat shocked- (42°C for 0.5, 1, 2 h) undifferentiated and differentiated NG108-15 cells. Our results showed that heat shock elicited a time-dependent increase in reporter gene activity. Furthermore, induction of the Hsp70-luciferase reporter gene activity was significantly lower in the differentiated cells when compared to that of the undifferentiated cells. The fold of induction of the Hsp70-luciferase reporter by a 2-h heat shock at 42°C of the undifferentiated cells ranged from 16–41 times over that of the control, and, for the differentiated cells, the induction ranged from 4–10 times over that of the differentiated control. Such quantitative difference in induction of the Hsp70-luciferase reporter gene activity of the undifferentiated versus the differentiated cells was observed regardless of the time and temperature of heat shock; the result was very reproducible over the course of a 2-year study. An alternative approach we took to affirm this observation was to transfect undifferentiated NG108-15 cells and divided the transfected cells into two halves: induce half of the cells to differentiate with dibutyryl cAMP (48 h) with the other half serving as the undifferentiated control. Result similar to that presented in Fig. 1 was obtained.

To validate that the attenuated induction of the Hsp70-luciferase reporter gene is indeed a feature associated with neural differentiation, we carried out two studies: (1) a comparison of the control and heat-induced reporter gene activity of the undifferentiated and differentiated NG108-15 cells with that of E16 (embryonic day 16) rat hippocampal neurons. As shown in Fig. 2a, the control and heat-induced Hsp70-luciferase reporter for the undifferentiated, differentiated NG108-15 cells, and the E16 hippocampal neurons were 1 and 37, 0.9 and 7, and 0.2 and 1.5, respectively. (2) The attenuated induction of the Hsp70-luciferase

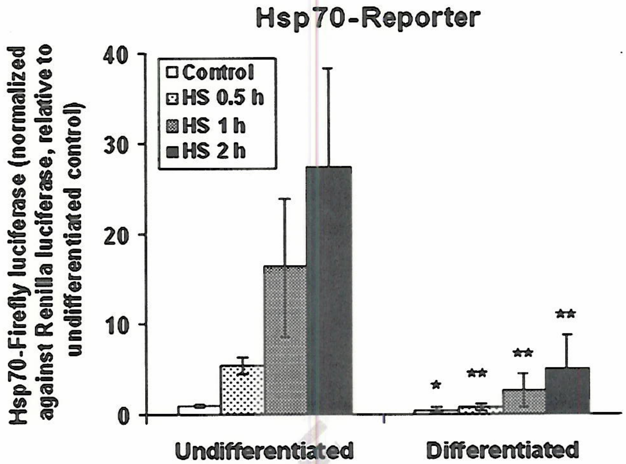

Fig. 1 Neural differentiation of the NG108-15 cells is associated with an attenuated heat shock induction of the Hsp70-firefly luciferase reporter gene. NG108-15 neuroblastoma–glioma hybrid cells were induced to differentiate by subculturing of the cells into a Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 1-mM dibutyryl cAMP for 2 days. Undifferentiated and differentiated cells in 35-mm plates were transfected with the Hsp70-firefly luciferase reporter DNA together with the Renilla luciferase DNA as an internal control, and the transfected cells were plated into wells of a 96 Stripwell plate. Cells were heat shocked at 42°C for time periods as indicated (0.5, 1, and 2 h) followed by recovery at 37°C; all cells were harvested at 6 h. The relative luminescence unit of the firefly luciferase readout was normalized against that of the Renilla luciferase. To facilitate comparison across experiments, this ratio was set at 1 for the undifferentiated control. The result presented represents the average ± standard deviation, $N=8$ (four separate experiments, each with two independent determinations). Result on Student's t-tests of probability of difference (probability <0.01, **highly significant; probability between 0.01 and 0.05, *significant) in the Hsp70-luciferase reporter gene activity between paired samples of the undifferentiated and differentiated cells is as illustrated

reporter is not a direct effect of dibutyryl cAMP. In Fig. 2b, we show that the treatment of a near confluent culture of the undifferentiated NG108-15 cells with 1-mM dibutyryl cAMP for 2 days—when cells were mostly recalcitrant to the neural inductive effect of dibutyryl cAMP (induced undifferentiated)—failed to elicit a comparable decrease in the heat-induced Hsp70-luciferase reporter. (Note: This "recalcitrance" may be due to the need of cells to undergo a round of quantal mitosis to commit to the differentiation process [Macieira-Coelho 1995] and/or cell crowding that block neurite extension. Our effort to determine the % of neurite positive cells in the induced-undifferentiated culture gave estimates between 25–35%.)

A transcriptional mechanism for the attenuated HSR in neural differentiation

Induction of the HSR is initiated by the activation of HSF1—a process that converts HSF1 from a cytosolic, latent monomer to a nuclear localized, hyperphosphorylated,

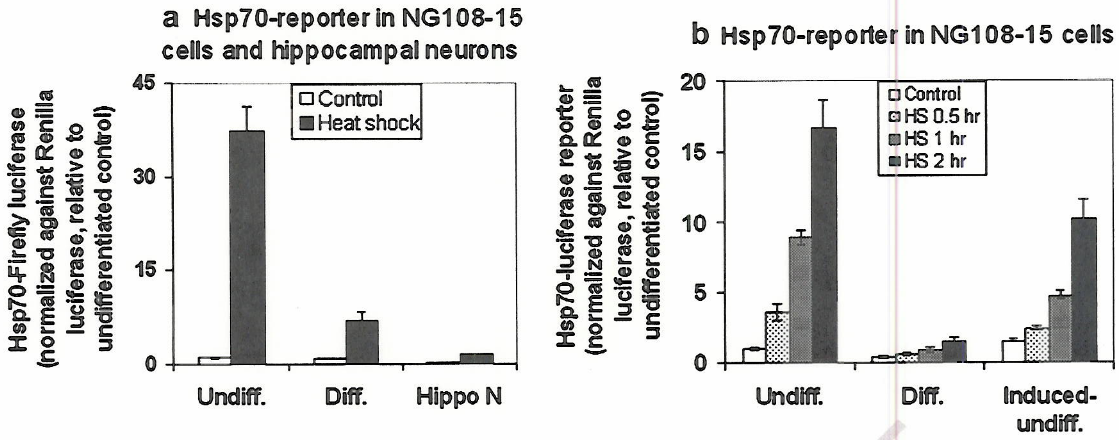

Fig. 2 **a** Comparison of the control and heat shock-induced Hsp70-luciferase reporter activity in the undifferentiated and differentiated NG108-15 cells and of E16 hippocampal neurons. The culturing and differentiation condition of the NG108-15 cells were as described in the text. Sprague–Dawley rat hippocampal neuron from E16 fetus at 14 days of culture was obtained as previously described (Magby et al. 2006). Cells were transfected with the Hsp70-firefly luciferase DNA together with the Renilla luciferase internal control. Results of the Hsp70-firefly luciferase activity (relative luminescence unit) were normalized against that of the Renilla luciferase (relative luminescence unit), and the ratio for the undifferentiated control was set at 1. The results for the control and heat shocked cells were—undifferentiated: 1 and 37; differentiated: 0.9 and 7; hippocampal neuron (Hippo N): 0.2 and 1.5. Result represents the average ± standard deviation, *N*=4. **b** Hsp70-luciferase reporter activity in the undifferentiated, differentiated, and induced-undifferentiated NG108-15 cells. The culturing and differentiation condition of the NG108-15 cells were as described in the text. To test if the attenuated induction of the Hsp70-reporter is a direct effect of dibutyryl cAMP independent of neural differentiation, we treated a plate of near confluent undifferentiated NG108-15 cells with 1-mM dibutyryl cAMP for 48 h before DNA transfection, and this was designated as "induced-undifferentiated." The % of neurite-positive cells in the undifferentiated, differentiated, and induced-undifferentiated cultures were <10, >80, and ~30%, respectively. Result of the Hsp70-firefly luciferase activity was normalized against that of the Renilla luciferase, and this ratio was set as 1 for the undifferentiated control. Result represents the average ± standard deviation, *N*=4

DNA-binding trimer—and culminates in increased steady-state level of the HSP proteins. In experiments presented in Fig. 3, we determined the amount and activation of HSF1 and the mRNA level of Hsp89α, Hsp70, and Hsp25 in the undifferentiated versus the differentiated NG108-15 cells. We show that, while there was little/no difference in the abundance of HSF1 protein in extracts of the undifferentiated and differentiated NG108-15 cells (Fig. 3a), the DNA-binding activity of HSF1 in the differentiated cells was resistant to stress-induced activation. Electrophoretic mobility shift assay of the DNA-binding activity of HSF1 in Fig. 3b showed a much more robust activation in the undifferentiated than the differentiated cells. Analysis by Northern blot of the steady-state level of mRNA of HSPs in Fig. 3c showed that heat shock induction of the mRNA of Hsp89α, Hsp70, and Hsp25 was greater in the undifferentiated than the differentiated cells.

We also determined the induction of HSP synthesis in the undifferentiated and differentiated NG108-15 cells by the incorporation of [^{35}S]methionine into newly synthesized proteins. The result in Fig. 4 on the profile of new protein synthesis showed a heat shock time-dependent increase in the synthesis of a number of proteins, marked as Hsp98, Hsp89, Hsp72, Hsp50, and Hsp25. In particular, we note that induction of the three major HSPs, Hsp98, 89, and 72, starts at 2 h of heat shock, peaks at 6 h, and decreases at 8 in the undifferentiated cells. The magnitude of induction of the HSPs—as indicated by intensity of the bands—was greater in the undifferentiated than in the differentiated cells. Together, these results support a transcriptional mechanism of the attenuated induction of HSPs in the differentiated NG108-15 cells.

Increased expression of Hsc70 in neural differentiation

In Fig. 5, we used immuno-Western blot technique to affirm the specificity and to evaluate changes of the Hsp70 versus Hsc70 protein in neural differentiation. The experiment shown in Fig. 5a was probed using the RTG76 antibody that recognizes the inducible and constitutive Hsp70 proteins. We show that, while the heat shock induction of the 72-kDa Hsp70 protein is markedly attenuated in the differentiated NG108-15 cells, expression of the 73-kDa Hsc70 protein was clearly upregulated in the differentiated neural cells. Neuronal specificity of these changes in expression of the Hsp70 versus Hsc70 protein in the differentiated NG108-15 cells was further evaluated using extracts from normal and Hsp70 knockout murine embryo fibroblasts (Hsp70−/− MEF). The identity of the 72-kDa protein as the heat-inducible Hsp70 was validated by (1) its induction by heat shock in NG108-15 (compare lanes 1 and 2) and wild-type MEF (lane 5 and 6) and (2) its absence in extracts of the Hsp70−/− MEF (lanes 9–12). That the attenuated induction of the 72-kDa Hsp70 protein was specific to the

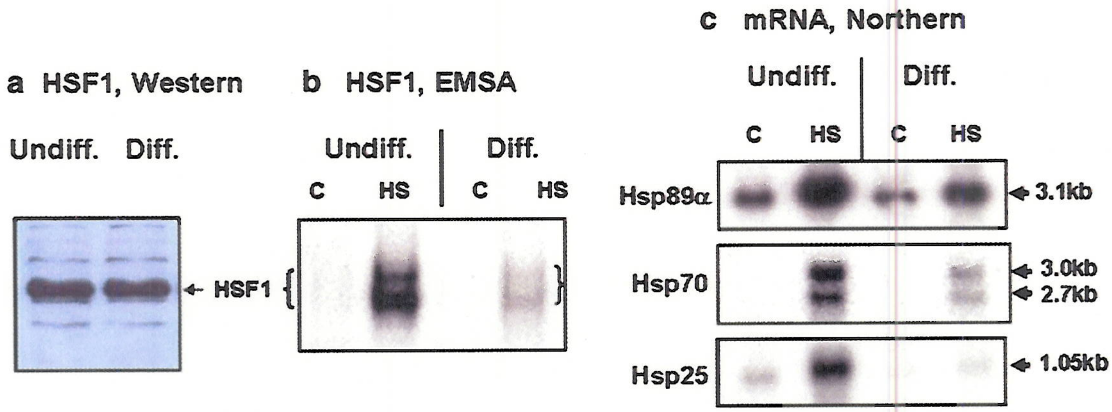

Fig. 3 Determination of the amount and activation of HSF1, and induction of mRNA of HSPs in the undifferentiated and differentiated NG108-15 cells. Cells in 100-mm plates were used. Condition for heat shock was 2 h at 42°C. **a** Immuno-Western blot probing for HSF1 of undifferentiated and differentiated NG108-15 cells. Ten-microgram aliquots of whole cell extracts were loaded onto an 8% SDS-acrylamide gel for analysis. **b** DNA-binding activity of HSF1 in extracts from control and heat shocked (42°C, 1 h) cells was determined by electrophoretic mobility shift assay. The relative DNA-binding activity in the four samples (*left to right*) were 1, 40, 0.5, and 12, respectively. **c** Northern blot quantitation of mRNA of Hsp89α, Hsp70, and Hsp25 in the undifferentiated and differentiated NG108-15 cells. Cells were heat shocked at 42°C for 2 h, and RNA was isolated according to methods described in the text. Probing of the mRNA of Hsp89α, Hsp70, and Hsp25 were done by hybridization with [^{32}P]labeled Hsp89α cDNA (pHS801), Hsp70 DNA (pH2.3), and Hsp25 cDNA (pHS208). The size of the transcripts are as indicated (in kb). The relative abundance of the mRNA, quantitated by densitometric scanning were Hsp89α (*left to right*): 6, 21, 4.3, 9; Hsp70: not determined, 9.6, not determined, 2.8; Hsp25: 1.3, 6.8, 0.4, 1

differentiated neural cells (compare lanes 2 and 4 of Fig. 5a), as opposed to effects of dibutyryl cAMP independent of neural differentiation, was supported by the observation that treatment of MEF with 1-mM dibutyryl cAMP for 2 days failed to produce the same effect; rather, dibutyryl cAMP boosted the heat shock induction of the 72-kDa Hsp70 protein in MEF (compare lanes 6 and 8, Fig. 5a). In a previous study, we reported on effects of cAMP and cAMP-dependent protein kinase in promoting Hsp70 gene expression (Choi et al. 1991). Neural specificity of the upregulation of Hsc70 expression was supported by the increase in 73-kDa Hsc70 protein in the differentiated NG108-15 cells (lanes 3 and 4

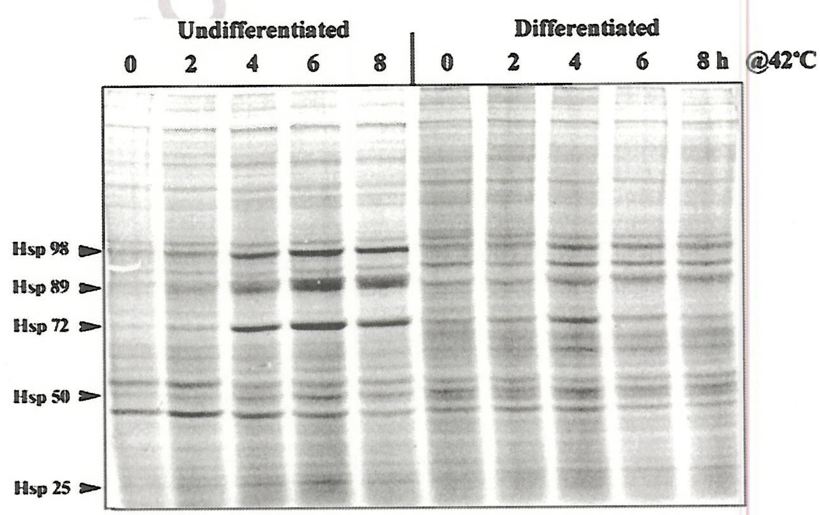

Fig. 4 Synthesis of heat shock proteins in the undifferentiated and differentiated NG108-15 cells. Undifferentiated and differentiated NG108-15 cells in 35-mm plates were used. Cells were heat shocked at 42°C for time periods of 2, 4, 6, and 8 h. To monitor the induction of HSP synthesis, [^{35}S]methionine/cysteine (50 μCi/ml) was added to the medium for the last hour before harvesting of the cells. The amount of radioactivity incorporated into newly synthesized proteins was determined by precipitation of proteins with trichloroacetic acid followed by liquid scintillation counting. Aliquots of the cell homogenate containing an identical amount radioactively labeled protein (60,000 cpm of trichloroacetic acid-insoluble material) were analyzed by SDS-PAGE and autoradiography. The positions of the major HSPs, Hsp98, Hsp89, Hsp72, Hsp50, and Hsp25, are indicated by *arrowheads*

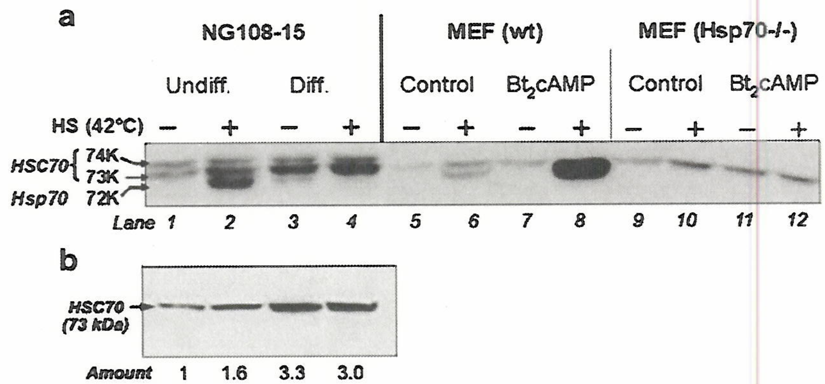

Fig. 5 Attenuated induction of the Hsp70 protein and increased expression of the constitutive Hsc70 protein in the differentiated NG108-15 cells. **a** Immuno-Western blot probing for Hsp70 and Hsc70. Extracts from control- and heat shocked- (42°C, 2 h, followed by recovery at 37°C for 6 h) undifferentiated and differentiated NG108 cells were probed using the RTG76 antibody that detects the 72-kDa Hsp70 and the 74- and 73-kDa Hsc70 proteins. To validate the identity of the protein bands and to assess the specificity of effects of dibutyryl cAMP, we included in this experiment extracts from the wild type and the Hsp70−/− MEF. Where indicated, MEF were treated with 1-mM dibutyryl cAMP for 48 h. The condition of the heat shock was 2 h at 42°C followed by recovery incubation at 37°C for 6 h. Aliquots of whole cell lysate containing 10-μg protein were subjected to SDS-PAGE (8%) after the transfer of proteins onto polyvinylidene fluoride membrane and antibody probing. The positions of the 74- and 73-kDa Hsc70 and the 72-kDa Hsp70 are as indicated. **b** Immuno-Western blot probing for Hsc70. To unequivocally determine the increase in Hsc70 expression in neural differentiation, extracts of the control- and heat shocked-undifferentiated and differentiated NG108-15 cells, as shown in *lanes 1 through 4* of (**a**), were probed using an antibody specific for the constitutive Hsc70 protein (Stressgen, SPA-816). The relative abundance in the different samples determined by densitometry is shown at the *bottom of the figure*

versus 1 and 2) but not in the dibutyryl cAMP-treated MEF (wild-type lanes 5–8; Hsp70−/−, lanes 9–12).

To validate the increased expression of Hsc70 in the differentiated NG108-15 cells, we used a commercially available Hsc70-specific antibody (Stressgen, SPA816) to probe for Hsc70 by both immuno-Western blot and immunocytochemistry. Result in Fig. 5b shows that this antibody specifically recognized the 73-kDa Hsc70 protein. Heat shock (42°C, 2 h followed by recovery at 37°C for 6 h) had a variable but insignificant effect on the expression of Hsc70 (the relative abundance of the Hsc70 protein of Fig. 5b as determined by densitometry is indicated at the bottom of the figure). The average ± standard deviation of Hsc70 from five determinations of two separate experiments for undifferentiated-control and undifferentiated-heat shocked cells and differentiated-control and differentiated-heat shocked cells were 1, 1.1±0.3, 3.52±0.42, and 3.48±0.5, respectively.

In Fig. 6, we used immunocytochemical techniques to probe for the abundance and localization of the Hsc70 protein using the Stressgen Hsc70-specific antibody. In general, the differentiated cells showed significantly stronger staining for Hsc70 than the undifferentiated cells, and heat shock at 42°C for 2 h followed by recovery at 37°C for 6 h had no obvious effect either on the staining intensity or the localization of Hsc70. The staining pattern revealed that Hsc70 is located in the cytoplasm and the neuritic processes. In the differentiated cells, we noticed structures resembling neuronal varicosities (indicated by arrow heads in the figure) at the terminus of or along the neuritic shafts staining strongly for Hsc70. Furthermore, there appears to be a correlation between morphological differentiation (number and length of neurite) and the Hsc70 staining intensity at the individual cell level. As shown in panels f and g, the highly differentiated cells stained brightly for Hsc70, whereas the less differentiated cells—less so (e.g., the three cells in the upper left hand corner of panel g and cells in the lower left hand corner in panel f). Together, the results in Figs. 5b and 6 demonstrate unequivocally an increase expression of the constitutive Hsc70 protein in neuronal cell differentiation.

Vulnerability of the differentiated NG108-15 cells to stress-induced cell death

Induction of the HSPs provides a buffering capacity against the toxic effects of mis-folded proteins; their activation under conditions of stress is a powerful cyto-protective mechanism for survival (Amin et al. 1996; Yenari et al. 1998, 1999; Akbar et al. 2003). These considerations suggest that the attenuated induction of HSPs in the differentiated may be associated with vulnerability to stress-induced cell death.

To evaluate this possibility, we determined the effects of increasing concentrations of arsenite (Fig. 7) and glutamate/glucine (Fig. 8) on cell viability and activation of caspase 3/7. Arsenite was chosen for its ability to elicit oxidative stress, and, indeed, the cytotoxic effects of arsenite were

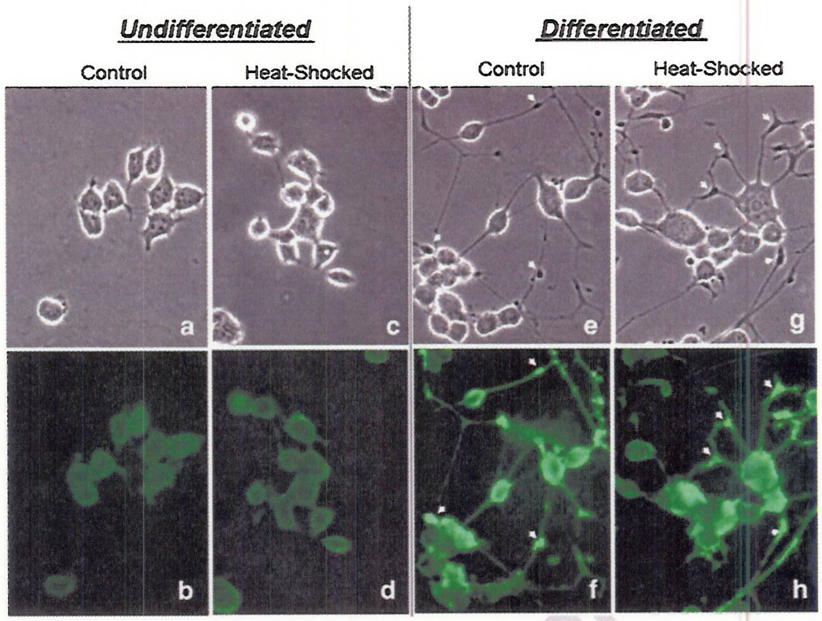

Fig. 6 Phase contrast and Hsc70 immuno-fluorescence photomicrographs of the control- and heat shocked-undifferentiated and differentiated NG108-15 cells. Undifferentiated and differentiated (1-mM dibutyryl cAMP in a 2% fetal bovine serum supplemented medium for 3 days at 37°C) NG108-15 cells were incubated under control and heat shocked conditions (42°C for 2 h followed by recovery at 37°C for 6 h) and processed for immunocytochemical staining for Hsc70 according to methods described in the text. The phase contrast (**a**, **c**, **e**, and **g**) and FITC fluorescence (**b**, **d**, **f**, and **h**) views of these cells are illustrated. The *arrowheads* in **e**, **f**, **g**, and **h** point to examples of varicosity-like structures at the terminus (**h**) of or along (**f** and **h**) the neuritic shaft of the differentiated NG108-15 cells

negated by the transfection and expression of superoxide dismutase 1 (data not shown). Glutamate/glycine was chosen for its ability to bind to and activate the *N*-methyl-D-aspartate receptor (NMDAR) protein and, at appropriate concentrations and time of incubation, elicit excitotoxic cell death in NMDAR-positive neurons (Michaelis 1998; Schubert and Piasecki 2001). We show in Fig. 7 that the differentiated NG108-15 cells exhibited exquisite sensitivity toward the cytotoxic effects of arsenite. In the differentiated cells, arsenite caused a significant and dose-dependent loss of cell viability beginning at 10 μM and, at 50 μM, <15% of cells were viable (Fig. 7a). Under the same condition, the undifferentiated NG108-15 cells were more resistant against the cytotoxic effects of arsenite with >90% of cells viable up to 50-μM arsenite, followed by a steep decline in cell viability in the presence of 70- and 100-μM arsenite. The cause of cell death is likely due to apoptosis, as there was a significant and arsenite dose-

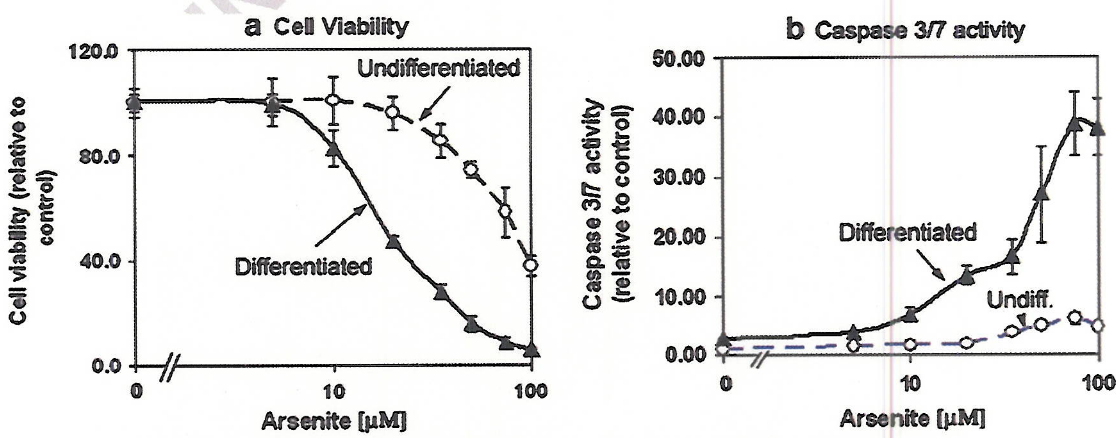

Fig. 7 Differentiated NG108-15 cells exhibited greater sensitivity toward oxidative stress-induced cell death and activation of caspase 3/7 activity. Undifferentiated and differentiated NG108-15 cells in 96 Stripwell™ plate were used. To induce oxidative stress, sodium arsenite was added to designated wells to final concentrations of 1, 5, 10, 20, 35, 50, 75, and 100 μM and incubated at 37°C for 16 h. **a** Cell viability, relative to that of the untreated (i.e., without arsenite) control of 100, is presented. Results represent average ± standard deviation, *N*=4. **b** Caspase 3/7 activity (relative luminescence unit, normalized against cell viability signal) was assayed using the Caspase3/7 Glo reagent from Promega Inc. Results represent average ± standard deviation, *N*=4

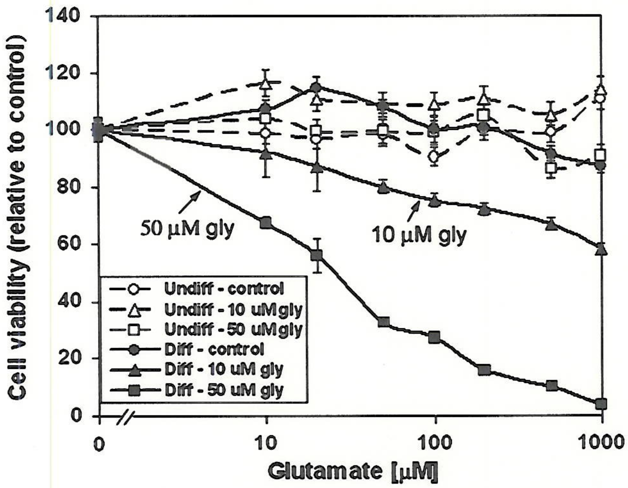

Fig. 8 Susceptibility of the differentiated but not the undifferentiated NG108-15 cells to the excitotoxic effects of glutamate/glycine. Undifferentiated and differentiated NG108-15 cells in 96 Stripwell™ plate were used. To test for the effects of glutamate and glycine, cells were refurbished with Dulbecco's phosphate-buffered saline without added amino acids. Glutamate was added to individual wells to final concentrations of 0, 10, 20, 50, 100, 200, and 500 µM and 1 mM either without (*circle symbol*) or with 10 (*triangle symbol*) and 50 µM (*square symbol*) glycine (*gly*). Cells were incubated at 37°C overnight (16 h). Cell viability was assayed using the CellTiter Glo luminescence reagent from Promega Inc. Results presented are relative to that of the untreated (i.e., without glutamate or glycine) control of 100. Results represent average ± standard deviation, *N*=4

dependent activation of caspase 3/7 activity particularly in the differentiated cells (Fig. 7b). A maximal activation of caspase 3/7 was observed after 16-h incubation at 37°C with 50-µM sodium arsenite, and this activation was approximately five times greater in the differentiated cells than in the undifferentiated cells.

The excitotoxic effects of increasing concentrations of glutamate and glycine (Fig. 8) appeared also to be selective for the differentiated cells. Glutamate, without glycine, had little or no effect on viability of the differentiated NG108-15 cells; the addition of 10- and 50-µM glycine, however, gave a glutamate dose-dependent decrease in viability of the differentiated cells. Viability of the undifferentiated cells was not statistically affected by the concentration and combination of glutamate and glycine used.

Discussion

In our present study of the regulation of heat shock gene expression in neural differentiation, we observed that differentiation of the NG108-15 tumor neural progenitor cells into neuron-like cells is associated with an attenuated HSR. Our result is consistent with previous observations of a reduced induction of Hsp70 during neuronal differentiation of the PC12 cells (Dwyer et al. 1996; Hatayama et al. 1997) and of differences in induction of the heat shock genes in regions of the mammalian brain—a robust response in glial and ependymal cells as compared to a null, delayed, or diminished response in neurons (Manzerra and Brown 1996; Marcuccilli et al. 1996; Nishimura and Dwyer 1996; Tytell et al. 1996). Studies in various neuronal systems noted a high threshold for induction for the stress response, a defect attributed to the lack of activation of the heat shock transcription factor, HSF1 (Marcuccilli et al. 1996; Nishimura and Dwyer 1996; Batulan et al. 2003). Together, these observations strongly suggest that an attenuated HSR may be a common feature of the differentiated neuronal cell. This limited ability of neurons to mount the protective HSR is likely to have dire consequences, as protein mis-folding and aberrant protein interactions are known to have fundamentally important roles in the pathogenesis of various neurodegenerative conditions (Welch and Gambetti 1998; Sharp et al. 1999; Sherman and Goldberg 2001; Bonini 2002; Muchowski 2002; Benn and Brown 2004; Landsbury 2004; Westerheide and Morimoto 2005; Morimoto 2006; Muchowski and Wacker 2005).

The molecular mechanism of this attenuated HSR in differentiated neurons is not entirely clear. We showed that, while the amount of HSF1 in the differentiated cells is not significantly different from that of the undifferentiated cells, the HSF1 of the differentiated cells was nonetheless recalcitrant to heat-induced activation. In a previous study on PC12 cells, neural differentiation was associated with a marked increase in the HSF1 DNA-binding activity, although induction of the HSP mRNA and protein was markedly reduced (Hatayama et al. 1997). The cause of this difference in regulation of HSF1 DNA-binding activity in the PC12 versus NG108-15 cells is not entirely clear. Studies on embryonic motor neurons showed that, while the attenuated HSR in neurons cannot be rectified by the transfection and expression of a wild-type HSF1, the transfection and expression of a constitutively active form of HSF1 were effective in reinstating the HSR (Batulan et al. 2003). Together, these results suggest changes in the sensing and/or signaling mechanism leading to the activation of HSF1 in the differentiated neuron.

The increased expression of Hsc70 protein in the differentiated NG108-15 cells is of interest and, perhaps, of significance. Hsc70 can, by interacting with various co-chaperone proteins, guide the sequential restructuring of stable or transient protein complexes to promote a temporal and spatial regulation of the endo-and exocytotic machinery and to ensure a vectorial passage through the vesicle cycle (Zinsmaier and Bronk 2001; Young et al. 2003). In other words, localized co-chaperones can harness the adenosine triphosphate-dependent mechanisms of Hsc70 for conformational work in vesicle secretion and recycling, protein transport, and the regulated assembly and/or disassembly of protein complexes. Our observation that the differentiated NG108-15 cells—notably, varicosity-like structures on neuritic shafts—staining strongly for Hsc70, is consistent with this suggested function of Hsc70. In neurons, varicosities are known structures filled with synaptic vesicles and release neurotransmitter by synaptic vesicle exocytosis (Mandell et al. 1993; Cooper et al. 1995; Chiti and Teschemacher 2007). In previous studies on PC12 cells, differentiation of these cells was not associated with observable changes in expression of the constitutive Hsc70 protein, although there was a significant decrease in induction of Hsp70 (Dwyer et al. 1996; Hatayama et al. 1997). The reason(s) for such difference in regulation of HSC 70 expression upon differentiation of the PC12 versus NG108-15 cells is not clear. Possibilities may include differences in the cell model used or stages of differentiation attained in the different studies. To better understand the mechanism and the functional significance of the changes in heat shock gene expression in neural differentiation, we plan to evaluate if changes in expression of Hsc70, by using sense and anti-sense vectors of Hsc70 DNA, may modulate induction of the HSPs and/or differentiation of the NG108-15 cells.

Unlike the stress-induced Hsp70, however, Hsc70 may not afford significant protection against stress-induced pathologies. We show in Fig. 7 that the differentiated NG108-15 cells are exquisitely sensitive to the cytotoxic effect of arsenite. Given that arsenite is both an inducer of the HSR and an elicitor of oxidative stress (Khalil et al. 2006), we inferred that the limited induction of HSPs in the differentiated cells coupled with their increased sensitivity to oxidative stress-induced pathologies likely contributed to the demise of the differentiated cells in the presence of arsenite.

The selective sensitivity of the differentiated NG108-15 cells to glutamate and glycine is of interest. The possibility that this selective cytotoxic effect of glutamate and glycine in the differentiated NG108-15 cells is due to activation of the NMDAR protein is supported by our observation that, whereas glutamate plus glycine gave dose-dependent cytotoxic effects, glutamate alone was without effect. Previous studies showed that NMDARs are heteromeric composed of NR1 subunits, which binds glycine, and NR2 subunit, which binds glutamate; both NR1 and NR2 subunits are required to create a functional receptor (Waxman and Lynch 2005). Importantly, expression and function of the NMDAR protein appeared to be modulated in neural differentiation: (1) Neurogenesis is correlated with the expression of various NMDAR subunits (Varju et al. 2001; Pizzi et al. 2002), and (2) differentiation of the NG108-15 cells is associated with an increase in the NMDAR mRNA level (Beczkowska et al. 1996, 1997). Therefore, it is most likely that the selective vulnerability of the differentiated NG108-15 cells toward glutamate plus glycine, shown in Fig. 8, is due, at least in part, to the increased expression and function of NMDAR as part of the neural differentiation program. The possibility that expression of the HSP chaperones may afford protection against the cytotoxic effects of glutamate and glycine is supported by a previous observation that conditioning heat shock and increased synthesis of HSPs protect cortical neurons from glutamate toxicity (Rordorf et al. 1991). HSPs can suppress stress-induced apoptosis by many and varied mechanisms including blocking cytochrome c release from mitochondria, preventing apoptosome formation, and inhibiting the activation of caspase 3 and downstream events (Mosser et al. 2000; Gabai and Sherman 2002).

In summary, our study provides evidence that changes in expression of the HSP and HSC proteins are components of the neural differentiation program. It seems likely that the attenuated induction of HSPs contributes to neuronal vulnerability to stress-induced pathologies and death, whereas the increased expression of Hsc70 may support various neural-specific functions such as vesicle trafficking in the differentiated cells.

Dibutyryl cAMP	N^6,2′-*O*-dibutyryl adenosine 3′:5′-cyclic mono-phosphate
HSF1	heat shock factor 1
HSR	heat shock response
HSP	heat shock protein
Hsp70	the 72-kDa heat shock protein
Hsc70	the 74- and 73-kDa constitutively expressed heat shock cognates
Hsp70−/−	Hsp70 knockout
MEF	murine embryo fibroblasts
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA receptor
PBS	phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4)

Acknowledgement We are grateful to Dr. Mark Plummer of the Department of Cell Biology and Neuroscience for providing us with the rat embryonic hippocampal neuron culture (Magby et al. 2006). We thank Dr. Gutian Xiao for the Hsp70 knockout MEF. This work was supported in part by grants from the NSF (MCB0240009) and NJ Commission on Spinal Cord Research (05-3037-SCR-E-0).

References

Akbar MT, Lundberg AMC, Liu K et al (2003) The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death. J Biol Chem 278:19956–19965

Amin V, Cumming DVE, Latchman DS (1996) Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies. Neuro Lett 206:45–48

Batulan Z, Shinder GA, Minotti, S, He BP, Doroudchi MM, Nalbantoglu J, Strong, MJ, Durham HD (2003) High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. J Neuroscience 23: 5789–5798

Beczkowska IW, Buck J, Inturrisi CE (1996) Retinoic acid-induced increase in delta-opioid receptor and *N*-methyl-D-aspartate receptor mRNA levels in neuroblastoma × glioma (NG108-15) cells. Brain Res Bull 39:193–199

Beczkowska IW, Gracy KN, Pickel VM, Inturrisi CE (1997) Detection of delta opiod receptor and *N*-methyl-D-aspartate receptor like immunoreactivity in retinoic acid-differentiated neuroblastoma × glioma (NG108-15) cells. J Neurosci Res 47:83–89

Benn SC, Brown RH (2004) Putting the heat on ALS. Nat Med 10:345–347

Bonini NM (2002) Chaperoning brain degeneration. Proc Natl Acad Sci U S A 99:16407–16411

Chen S, Brown IR (2007) Neuronal expression of constitutive heat shock proteins: implications for neurodegenerative diseases. Cell Stress Chaperones 12:51–58

Chiti Z, Teschemacher AG (2007) Exocytosis of norepinephrine at axon varicosities and neuronal cell bodies in the rat brain. FASEB J 21:1–11

Choi HS, Li B, Lin Z, Huang LE, Liu AY-C (1991) cAMP- and cAMP-dependent protein kinase regulate the human heat shock protein 70 gene promoter activity. J Biol Chem 266:11858–11865

Cooper RL, Marin L, Atwood HL (1995) Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. J Neurosci 15:4209–4222

Dwyer DS, Liu Y, Miao S, Bradley RJ (1996) Neuronal differentiation in PC12 cells is accompanied by diminished inducibility of Hsp70 and HPS 60 in response to heat and ethanol. Neurochemical Res 21:659–666

Feige U, Morimoto RI, Yahara I, Polla BS (eds) (1996) Stress-inducible cellular responses. Birkhauser, Basel

Gabai VL, Sherman MY (2002) Interplay between molecular chaperones and signaling pathways in survival of heat shock. J Appl Physiol 92:1742–1748

Guzhova I, Kislyakova K, Moskaliova O, Friedlanskaya I, Tytell M, Cheetham M, Margulis V (2001) In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res 914:66–73

Hatayama T, Takahashi H, Yamagishi N (1997) Reduced induction of Hsp70 in PC12 cells during neuronal differentiation. J Biochem 122:904–910

Hendrick JP, Hartl FU (1995) The role of molecular chaperones in protein folding. FEBS J 9:1559–1569

Hickey E, Brandon SE, Potter R, Stein G, Stein J, Weber LA (1986) Sequence and organization of genes encoding the human 27 kDa heat shock protein. Nucleic Acids Res 14:4127–4145

Huang LE, Zhang H, Bae SW, Liu AY-C (1994) Thiol reducing reagents inhibit the heat shock response: involvement of a redox mechanism in the heat shock signal transduction pathway. J Biol Chem 269:30718–30725

Khalil S, Luciano J, Chen W, Liu, AYC (2006) Dynamic regulation and involvement of the heat shock transcriptional response in arsenic carcinogenesis. J Cell Physiol 207:562–569

Landsbury PT (2004) Back to the future: the 'old-fashioned' way to new medications for neurodegeneration. Nat Rev Neurosci 5: S51–S57

Lis J, Wu C (1993) Protein traffic on the heat shock promoter: parking, stalling, and trucking along. Cell 74:1–4

Macieira-Coelho A (1995) The last mitoses of the human fibroblast proliferative life span, physiopathologic implications. Mech Ageing Dev 82:91–104

Magby JP, Bi C, Chen ZY, Lee FS, Plummer MR (2006) Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. J Neuroscience 26:13531–13536

Mandell JW, MacLeish PR, Townes-Anderson E (1993) Process outgrowth and synaptic varicosity formation by adult photoreceptors in vitro. J Neuroscience 13:3533–3548

Manzerra P, Brown IR (1996) The neuronal stress response: nuclear translocation of heat shock proteins as an indicator of hyperthermic stress. Exp Cell Res 229:35–47

Marcuccilli CJ, Mathur SK, Morimoto RI, Miller RJ (1996) Regulatory differences in the stress response of hippocampal neurons and glial cells after heat shock. J Neuroscience 16:478–485

Meyer SA, Lin A, Liu AYC (1988) Neurite extension and increased expression of R_1 cyclic AMP-binding protein in ouabain-resistant neuroblastoma mutants. J Neurochem 51:950–959

Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol 54:369–415

Morimoto RI (1993) Cells in stress: transcriptional activation of heat shock genes. Science 259:1409–1410

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:378–3796

Morimoto RI (2006) Stress, aging, and neurodegenerative disease. N Eng J Med 355:2254–2255

Morimoto RI, Tissieres A, Georgopoulos C (eds) (1994) The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, New York

Mosser DD, Carbon AE, Bourget L, Merlin AB, Sherman MY, Morimoto RI, Massie B (2000) The chaperone function of Hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20:7146–7159

Muchowski PJ (2002) Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? Neuron 35:9–12

Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11–22

Nelson P, Christian C, Nirenberg M (1976) Synapse formation between clonal neuroblastoma × glioma hybrid cells and striated muscle cells. Proc Natl Acad Sci U S A 73:123–127

Nirenberg M, Wilson S, Higashida H et al (1983) Synapse formation by neuroblastoma hybrid cells. Cold Spring Harbor Symp Quant Biol 48:707–715

Nirenberg M, Wilson S, Higashida H et al (1984) Modulation of synapse formation by cyclic adenosine monophsophate. Science 222:794–799

Nishimura RN, Dwyer BE (1996) Evidence for different mechanisms of induction of Hsp70i: a comparison of cultured rat cortical neurons with astrocytes. Mol Brain Res 36:227–239

Pizzi M, Boroni F, Bianschetti A et al (2002) Expression of functional NR1/NR2B-type NMDA receptors in neuronally differentiated SK–N–SH human cell line. Euro J Neuro Sci 16: 2342–2350

Rordorf G, Koroshetz WJ, Bonventre JV (1991) Heat shock protects cultured neurons from glutamate toxicity. Neuron 7:1043–1051

Schubert D, Piasecki D (2001) Oxidative glutamate toxicity can be a component of the excitotoxicity cascade. J Neuroscience 21:7455–7462

Sharp FR, Massa SM, Swanson RA (1999) Heat shock protein protection. Trends in Neuroscience 22:97–99

Sherman MY, Goldberg AL (2001) Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. Neuron 29:15–32

Tytell M, Greenberg SG, Lasek RJ (1996) Heat shock-like protein is transferred from glia to axon. Brain Res 363:161–164

cell damages caused by the perturbation of protein conformation (Feige et al., 1996; Hendrick and Hartl, 1995; Morimoto, 1998; Morimoto et al., 1994).

Two lines of observation suggest that problems of protein folding and of the supportive role of HSPs in preventing the buildup of mis-folded or non-native proteins have fundamentally important roles in the genesis and pathology of neurodegenerative diseases. The first is that an abnormality/defect in protein folding is at the crux of Alzheimer's, Huntington's, Parkinson's, amyotrophic lateral sclerosis, and prion diseases. The diseases are characterized by changes, due to genetic or epigenetic factors, in the folding of specific proteins to conformations prone to aggregation resulting in the accumulation of toxic protein fibrils and aggregates that likely contribute to neuron pathology and death (Forman et al., 2004; Morimoto, 2006; Muchowski and Wacker, 2005; Sherman and Goldberg, 2001). The second line of observation is that induction of the HSR and the ability to up-regulate expression of the HSP chaperones – mechanisms that normally provide important defense against the dire consequences of protein mis-folding and aberrant protein interactions – are decreased in various brain and spinal cord neurons *in vivo* and *in vitro* (Batulan et al., 2003; Brown and Rush, 1999; Foster and Brown, 1997; Manzerra and Brown, 1996; Manzerra et al., 1997; Marcuccilli et al., 1996; Nishimura and Dwyer, 1996). In general, neurons, in comparison with glial and ependymal cells, have a higher threshold for induction of the HSR, requiring a greater intensity or duration of stress for a diminished response.

We initiated this study to determine if induction of the heat shock transcriptional response (HSR) may be regulated in the course of neuronal cell differentiation. To facilitate this analysis, we developed a semi-high throughput hsp70–firefly luciferase reporter gene screening assay to assess possible changes in regulation of the HSR. Using tumor neural progenitor cells and primary embryonic neurons as model systems, we show in this study that differentiation of the neural progenitor cells from a state resembling stem cells into a state resembling neurons is accompanied by a decreased induction of the HSR and an increased vulnerability to stress induced cell death.

2. Results

We routinely scored neural differentiation by measuring the % of neurite-positive cells in the population, this being <10% and >80% for the undifferentiated and differentiated cultures,

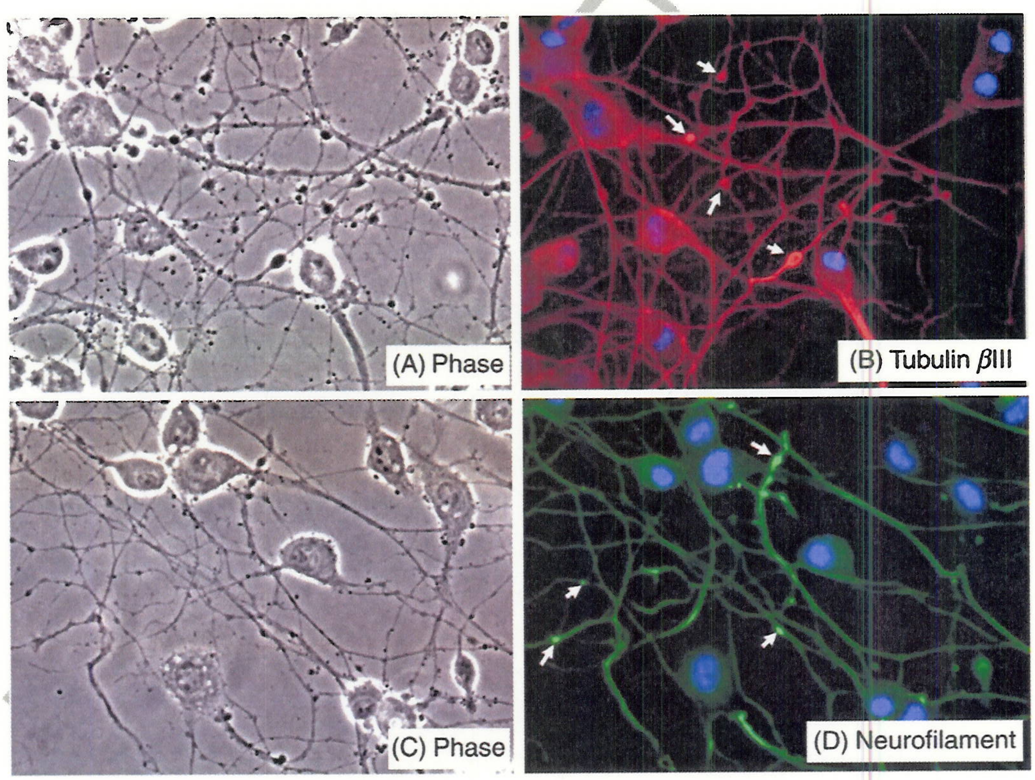

Fig. 1 – Immunocytochemical staining of differentiated NG108-15 cells for tubulin βIII and neurofilament. Dibutyryl cAMP-induced (1 mM, 3 days) differentiated NG108-15 cells in 35 mm plates were fixed and stained according to methods described in the text. The secondary antibody used for the staining of tubulin βIII was conjugated to Texas red, and for neurofilament was conjugated to FITC. Nuclei were counter stained with 10 μM Hoechst 33342. (A) and (B): Phase contrast and rhodamine fluorescence views, respectively, of the tubulin βIII stained differentiated NG108-15 cells. (C) and (D): Phase contrast and FITC fluorescence views, respectively, of the neurofilament stained cells. Arrow heads identify "varicosity-like" structures along the neuritic shafts.

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

respectively. To ascertain the neural differentiation phenotype, differentiated NG108-15 cells were stained for neural specific tubulin βIII and neurofilament. The result in Fig. 1 showed positive staining of both the cell body and neurites of the differentiated NG108-15 cells; furthermore, we noted strongly stained structures – indicated by arrowheads in Fig. 1B and D – resembling varicosities along the neuritic shafts. Voltage clamp recording of the (A) undifferentiated and (B) differentiated NG108-15 cells in Fig. 2 demonstrated the presence of voltage-gated sodium channels in the differentiated cells but not the undifferentiated cells. The voltage-dependent, constant amplitude inward sodium current (−pA on the Y-axis) – the basis of the depolarizing upstroke in action potential – was observed when the differentiated cell was clamped at voltage≥−40 mV, and the latency of this inward sodium current decreased with an increasingly positive voltage clamp. The unique presence of the voltage-gated sodium channel is to be contrasted with the ubiquitous outward (+pA) potassium current observed in both the undifferentiated and the differentiated cells. In previous studies, it was shown that the differentiated NG108-15 cells form a functional synapse with muscle cells at relatively high frequencies (Nelson et al., 1976; Nirenberg et al., 1983, 1984). All of these features underscore the neuronal phenotype of the differentiated NG108-15 cells, a prototype of the tumor neuroprogenitor cell lines used in this study.

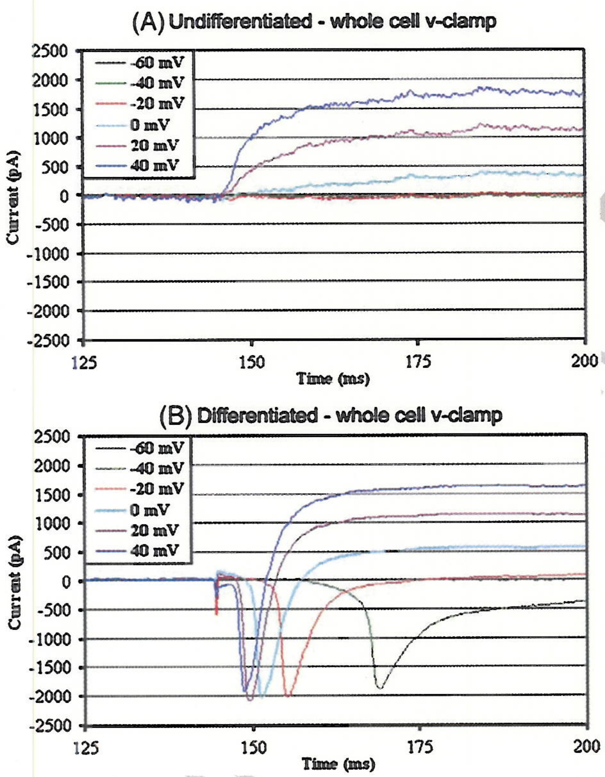

Fig. 2 – Whole cell voltage clamp recordings of the (A) undifferentiated and (B) differentiated NG108-15 cells. Undifferentiated and differentiated NG108-15 cells in 60 mm plates were used. For voltage clamp recording, cells were clamped at voltages as indicated. Signals were recorded with an Axopath 200A amplifier. The voltage-gated inward sodium current is indicated by a negative deflection (−pA) whereas the outward potassium current is indicated by a positive deflection (+pA). Result is representative of 4 different recordings from two separate experiments.

In Fig. 3 we used the hsp70 promoter–luciferase reporter gene to assess possible changes in induction of the heat shock response upon neuronal differentiation of the tumor cells and in primary embryonic neurons of the hippocampus, cortex, and spinal cord. The result in Fig. 3A, B, and C represents, respectively, the raw hsp70–luciferase activity in relative luminescence unit (RLU), fold of increase in reporter gene activity under heat shock condition over that of the control (HS/control), and after normalization against that of the co-transfected *Renilla* luciferase activity. We show that induction of the hsp70–reporter gene activity was highest in the undifferentiated NG108 cells. Differentiation of the NG108-15 cells resulted in a significant drop in reporter gene expression, and reporter gene activity of the primary embryonic neurons (hippocampal, cortical and spinal cord neuron culture) was lower than that of the differentiated NG108-15 cells. This observation would suggest that induction of the hsp70–reporter, and hence the heat shock response (HSR) is attenuated in the course of neural differentiation: from the undifferentiated progenitor to the early differentiated neural cells, and then the mature differentiated neuron.

To evaluate if the attenuated induction of the hsp70–reporter gene is indeed a common feature of neural differentiation, we screened for reporter gene expression in the undifferentiated and differentiated N18 and NS20 mouse neuroblastoma cells (differentiation induced by the addition of 1 mM dibutyryl cAMP), the PC12 pheochromocytoma cells (differentiation induced by the addition of 50 ng/ml of nerve growth factor), and the C17.2 surrogate stem cells (differentiation induced by serum removal). Morphological differentiation was validated by neurite extension (data not shown). As shown in Fig. 3C, heat shock induction of the hsp70–reporter gene is attenuated in the differentiated cells when compared to that of the undifferentiated cells. Experiments done using other neuroblastoma cells including the NB15, N2a, and NIE-115 cell lines further supported our contention that neural differentiation is associated with an attenuated induction of the hsp70–reporter; in each case, morphological differentiation is correlated with decreased induction of the hsp70–reporter gene (data not shown).

Figs. 4 and 5 are experiments aimed to validate specificity of the attenuated induction of hsp70–reporter gene in the differentiated NG108-15 cells. In Fig. 4A various treatment and cell culture conditions were used to modulate morphological differentiation: NG108-15 cells were induced to differentiate by treatment with dibutyryl cAMP (1 mM, 48 h — standard differentiation protocol), forskolin (10 μM, 48 h), or retinoic acid (10 μM, 48 h). Our result showed that regardless of the agent used, differentiation of the NG108-15 cells was associated with a decreased hsp70–reporter gene expression. Further, treatment of a near confluent culture of the NG108-15 cells with dibutyryl cAMP (undiff + cAMP) – a condition not permissive for neural differentiation (cell crowding blocked neurite extension) – failed to elicit a comparable decrease in hsp70–reporter gene expression. Fig. 4B presents the

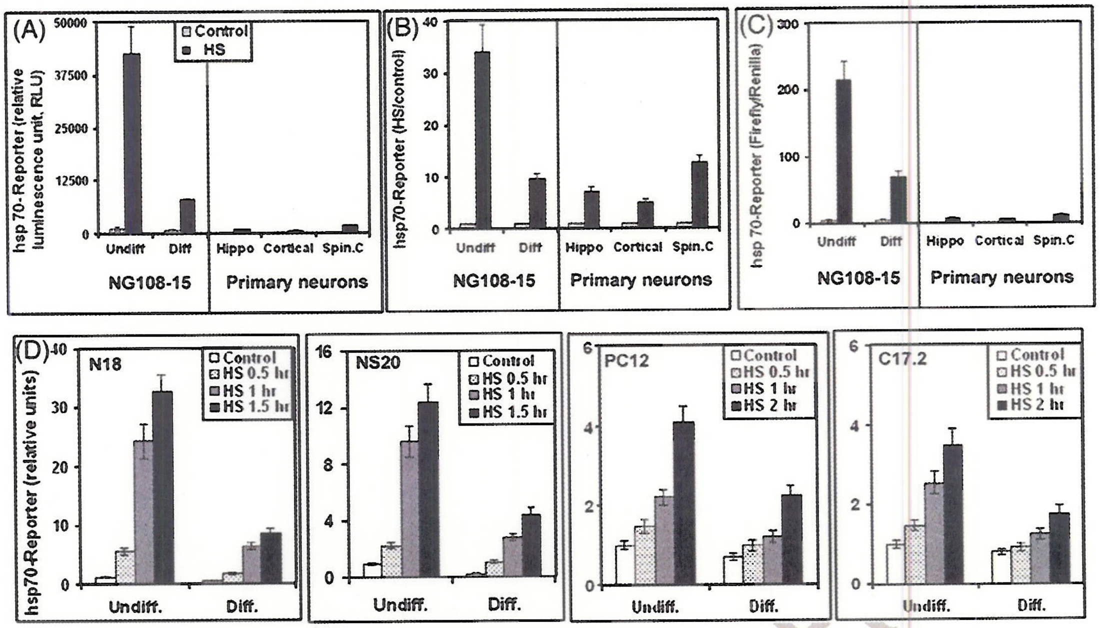

Fig. 3 – An attenuated induction of the hsp70–firefly luciferase reporter is a feature of the differentiated neuron. (A) A comparison of the basal (37 °C) and heat shock-induced (42 °C) hsp–firefly luciferase activity (in relative luminescence unit, RLU) in the undifferentiated versus differentiated NG108-15 cells and primary embryonic neurons from rat E15 hippocampus, cortex, and spinal cord. The result presented represents the average ± standard deviation, N = 4. (B) Fold of induction of the hsp70–firefly luciferase activity by heat shock over that of the control (HS/control). (C) Hsp70–firefly luciferase activity normalized against that of the co-transfected *Renilla* luciferase activity. The result presented represents the average ± standard deviation, N = 4. (D) Induction of hsp70–firefly luciferase in the undifferentiated and differentiated N18, NS20, PC12 and C17.2 neuroprogenitor cells. Cells were induced to differentiate according to methods described. Cells were transfected with the hsp70–firefly luciferase reporter DNA along with the *Renilla* luciferase DNA. Cells were heat shocked at 42 °C for time periods as indicated (0.5, 1, and 2 h) followed by recovery at 37 °C; all cells were harvested at 6 h. To facilitate comparison across experiments, the firefly/*Renilla* luciferase ratio was set at 1 for the undifferentiated control. The result presented represents the average ± standard deviation, N = 8.

time course of change in hsp70–reporter gene expression in the control and dibutyryl cAMP-induced differentiating cells. Result showed a quantal decrease in reporter gene expression at 48 h, but not at 24 h after the induction of differentiation. We further assessed the correlation of neurite extension and induction of the hsp70–reporter gene by culturing NG108-15 cells at varying plating densities and in the presence of different concentrations of serum and dibutyryl cAMP to effect various degrees of morphological differentiation. A plot in Fig. 5A of the heat shock-induced hsp70–reporter gene activity against neurite extension (neurite defined as a process with a length >2× the diameter of the cell body; the unit length × number of neurites were counted and divided by the number of cell bodies in a microscopic field to get a "neurite extension" score) showed a robust negative correlation. Representative photomicrographs of NG108-15 cells with neurite extension scores of 0.2 and 8.3 are shown in Fig. 5B along with photomicrograph of a representative hippocampal neuron culture with a neurite extension score of ~38. Together the results in Figs. 3–5 provide strong support for the contention that neural differentiation is associated with an attenuated induction of the hsp70–reporter gene.

To better understand the mechanism of this change in hsp70–reporter gene expression, we analyzed activation of the HSF1 DNA-binding activity, induction of the mRNA of hsp70 and synthesis and accumulation of the HSP70 protein. The result in Fig. 6A shows that while heat shock at 42 °C activated the HSF1 DNA-binding activity in both the undifferentiated and differentiated cells, the magnitude of the increase in HSF1 DNA-binding activity was greater in the undifferentiated than in the differentiated cells. Further, we show in Fig. 6B and C that heat shock induction of the mRNA of hsp70 and the 72 kDa HSP70 protein were significantly reduced in the differentiated cells when compared to that of the undifferentiated cells. The decreased induction of the HSP70 protein was further validated by immunocytochemical staining. We show in Fig. 7 that heat shock at 42 °C for 2 h followed by recovery at 37 °C for 6 h greatly increased the HSP70 staining intensity of the undifferentiated NG108-15 cells and weakly in the differentiated cells. HSP70 appear to be primarily a cytoplasmic protein — as opposed to a nuclear or neuritic localization.

Induction of the HSP protein in general and of the HSP70 protein in particular has been demonstrated to confer cytoprotection. The attenuated HSR in the differentiated neural cells would suggest a vulnerability of these cells when stressed or challenged, a vulnerability that should be rectified – at least in part – by conditioning heat shock to pre-induce HSPs or by the forced expression of HSP70 using gene transfer technology. In Fig. 8, we examined the dose–response effect of a non-

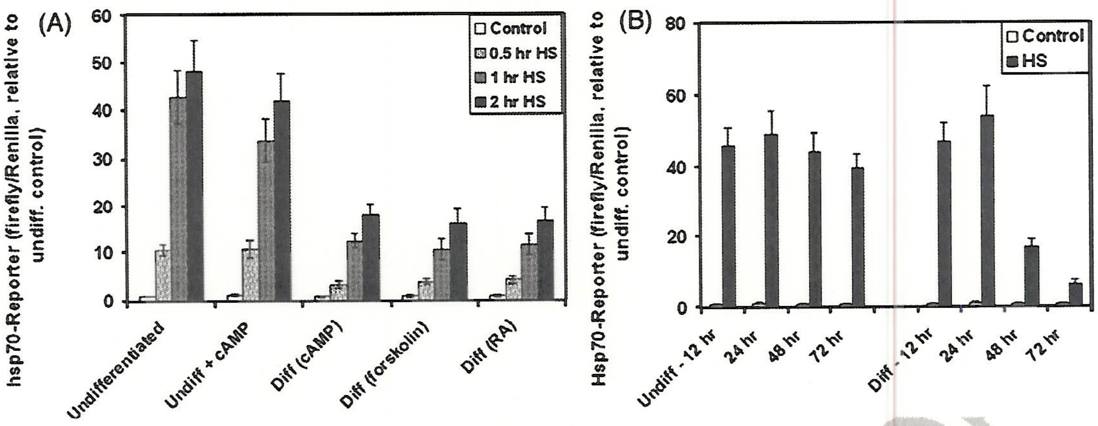

Fig. 4 – Specificity and time course of change in hsp70–reporter gene expression in neural differentiation.
(A) Specificity of the attenuated induction of hsp70–reporter gene. Plates of NG108-15 cells were induced to differentiate by the addition of dibutyryl cAMP [Diff (cAMP); 1 mM, 48 h], forskolin [Diff (forskolin); 10 μM 48 h] or retinoic acid [Diff (RA); 10 μM 48 h]. To validate that the decreased expression of hsp70–reporter is not a direct effect of the treatment of cells with dibutyryl cAMP, a plate of near confluent undifferentiated NG108-15 cells was treated with dibutyryl cAMP under conditions not permissive for neurite extension (Undiff+cAMP). These five groups of cells were transfected with the hsp70–firefly luciferase reporter DNA together with the *Renilla* luciferase DNA as an internal control. Hsp70–reporter gene activity, calculated as the firefly/*Renilla* luciferase ratio and relative to that of the undifferentiated control, of the control and heat shocked cells (0.5, 1, and 2 h at 42 °C, followed by recovery at 37 °C for a total of 6 h) is shown. (B) Time course of change in the basal and heat shock-induced hsp70–reporter. A 100 mm plate of near confluent undifferentiated NG108-15 cells were transfected with the hsp70–firefly luciferase reporter DNA along with the *Renilla* luciferase DNA as an internal control. At the end of this DNA transfection procedure (t=0), the cells were divided and plated into a 96 well plate under "undifferentiated" (standard medium) and "differentiated" (DMEM supplemented with 2% FBS and 1 mM dibutyryl cAMP) conditions. At various times thereafter (12, 24, 48, and 72 h), cells were heat shocked at 42 °C for 2 h followed by recovery at 37 °C for 4 h prior to harvesting for reporter gene assay. The result on the firefly/*Renilla* luciferase ratio, relative to that of the undifferentiated control, is presented. The result represents the average±standard deviation, N=4.

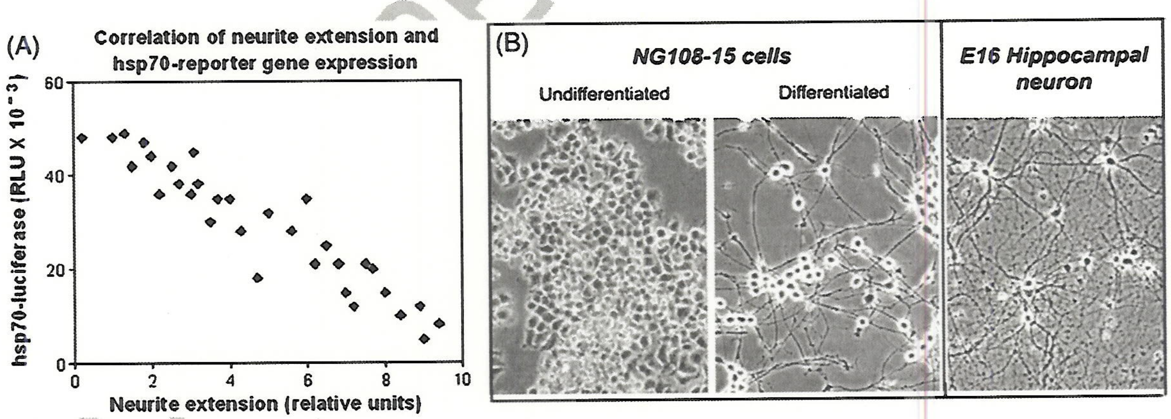

Fig. 5 – (A) Induction of the hsp70–reporter is negatively correlated with morphological differentiation. NG108-15 neuroblastoma cells were transfected with the hsp70–firefly luciferase DNA. 6 h after DNA transfection cells were subcultured and plated in 24 well plates at varying plating density in DMEM supplemented with different concentrations of serum (1, 2, 4, 6, 8 and 10%) and dibutyryl cAMP (0.2, 0.4, 0.6, 0.6 and 1 mM) to effect varying degrees of morphological differentiation. After 48 h of culture a 37 °C, cells were scored for neurites (neurite defined as a process >2× the diameter of the cell body; the length×number of neurites were counted and divided by the number of cell bodies in the field to get a "neurite extension" score). Cells were heat shocked at 42 °C for 2 h followed by recovery at 37 °C for 4 h to determine induction of the hsp70–reporter gene. (B) Representative phase contrast photomicrographs of the undifferentiated and differentiated NG108-15 cells and primary embryonic hippocampal neurons in culture, with neurite extension scores of 0.2, 8.3 and 38, respectively.

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

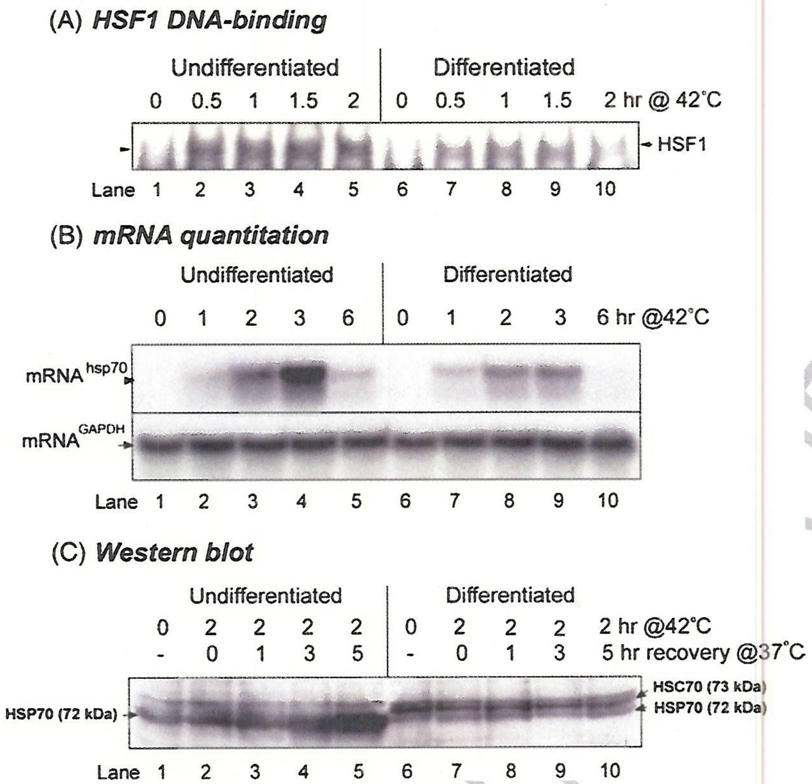

Fig. 6 – Activation of the HSF1 DNA-binding activity, and induction of hsp70 mRNA and protein in the undifferentiated and differentiated NG108-15 cells. (A) Heat shock-dependent activation of the HSF1 DNA-binding activity. Electrophoretic mobility shift assay was used to assess the HSF1 DNA-binding activity in the control- and heat shocked- (42 °C, 0.5, 1 and 2 h) undifferentiated (lanes 1–5) and differentiated (lane 5–10) NG108-15 cells. The position on the gel of the HSF1–HSE complex is as indicated. (B) Heat shock induction of the mRNA^{hsp70}. Cells were heat shocked at 42 °C for time periods of 0, 1, 2, 3 and 6 h. RNA was prepared and probed according to methods described. Abundance of mRNA of the house keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) served as the internal control. (C) Induction of the 72 kDa HSP70 protein. Undifferentiated (lanes 1–5) and differentiated (lanes 6–10) NG108-15 cells were heat shocked at 42 °C for 2 h followed by recovery at 37 °C for 0, 1, 3, and 5 h prior to harvesting. Aliquots of whole cell lysate containing 10 μg protein were subjected to SDS-PAGE (8%) followed by the transfer of proteins onto PVDF membrane and probing by the Stressgen anti-HSP70 polyclonal antibody (SPA812). The position on the gel of the 72 kDa HSP70 and the 73 kDa HSC70 protein are as indicated.

selective oxidizer, arsenite, on (A) viability and (B) caspase 3/7 activation in the differentiated NG108-15 cells; results on the undifferentiated NG108-15 cells were included for comparison. We show that arsenite caused a dose-dependent decrease in viability of both the undifferentiated and the differentiated NG108-15 cells, with the differentiated cells being much more sensitive to the cytotoxic effects of arsenite. Conditioning heat shock (42 °C, 2 h; pre-HS) and expression of HSP70 by gene transfer at 24 h prior to the arsenite challenge of the differentiated cells significantly blunted the cytotoxic effects, increasing cell viability from 10% to, respectively, 80 and 70% in the presence of 200 μM arsenite. The cause of cell death likely involves apoptosis as arsenite caused a dose-dependent activation of caspase 3/7 activity, and this activation was blunted by conditioning heat shock and increased expression of HSP70. Treatment of the undifferentiated NG108-15 cells also caused a dose-dependent increase in caspase 3/7 activity, however the magnitude of the increase was muted when compared to that of the differentiated cells.

We also tested the effects of activation of the NMDA receptor protein on cell viability using a combination of glutamate and glycine. In Fig. 9A we show that glutamate, from 10 μM–1 mM, had little effect by itself on the viability of the differentiated N18 cells. When added in combination with 10 or 50 μM glycine, however, glutamate was cytotoxic. The cytotoxic effect is dependent on the concentration of both glutamate and glycine: at 200 μM glutamate cell viability was 105%, 67% and 17% in the presence of 0, 10 and 50 μM glycine, respectively (Fig. 9A). Conditioning heat shock of the cells 24 h prior to the glutamate/glycine challenge blunted this cytotoxicity such that at 200 μM of glutamate cell viability was 101, 90 and 61% in the presence of 0, 10 and 50 μM glycine, respectively (Fig. 9B). The undifferentiated cells were insensitive to any combination of glutamate and glycine; viability of the cells was unaffected by the concentrations and combination of glutamate and glycine used. Together, the results in Figs. 8 and 9 demonstrated a vulnerability of the differentiated cells to stress induced cell death, a vulnerability that can be rectified at least in part by conditioning heat shock to

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

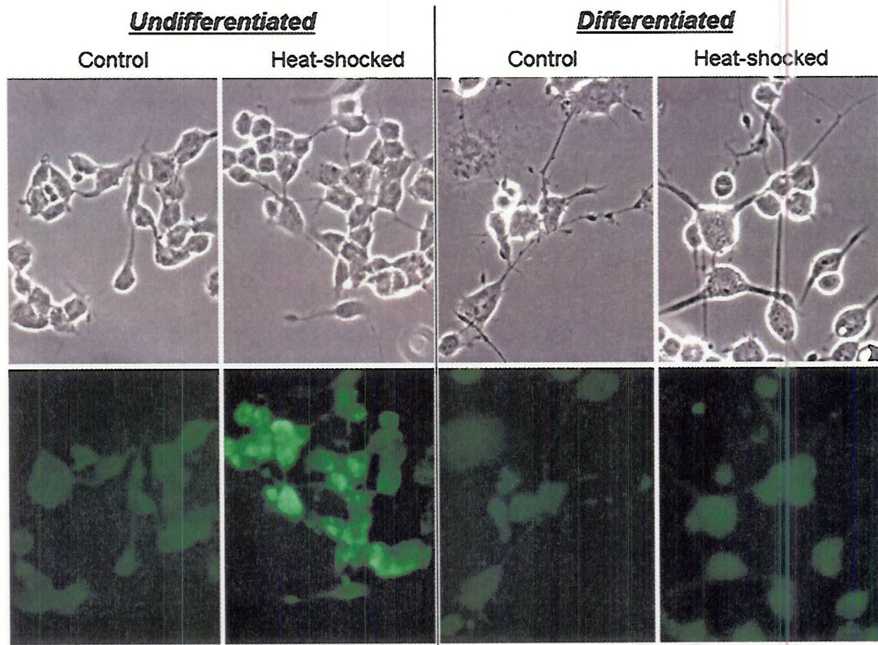

Fig. 7 – Phase contrast and HSP70 immuno-fluorescence photomicrographs of the control- and heat shocked-undifferentiated and differentiated NG108-15 cells. Undifferentiated and differentiated (1 mM dibutyryl cAMP in a 2% fetal bovine serum supplemented medium for 3 days at 37 °C) NG108-15 cells were incubated under control and heat shocked conditions (42 °C for 2 h followed by recovery at 37 °C for 6 h) and processed for immunocytochemical staining using the Stressgen anti-HSP70 polyclonal antibody (SPA812).

pre-induce the expression of HSPs or by gene transfer and increased expression of HSP70.

3. Discussion

There is a large body of evidence that induction of the heat shock transcriptional response and ability to up-regulate expression of the HSP chaperones provide important defense mechanisms against the dire consequences of protein misfolding and aberrant protein interactions (Forman et al., 2004; Morimoto, 2006; Muchowski and Wacker, 2005; Sherman and Goldberg, 2001). A corollary of this is that dysfunction of this cytoprotective mechanism is likely to have pathological consequences. Indeed, a notable patho-physiological manifestation of the blunting of this protective mechanism that has important biomedical implication is our observation of an attenuated heat shock response in aging cells: that as cells and organisms age, their ability to activate HSF1 and to mount the protective heat shock transcriptional response becomes markedly reduced (Liu et al., 1996).

In our present study of the regulation of HSR in neural differentiation, we observed that differentiation of neural progenitor cells is associated with an attenuated heat shock response. Our result is consistent with previous observations of differences in induction of the heat shock genes in regions of the mammalian brain — a robust response in glial and ependymal cells as compared to a null, delayed or diminished response in neurons (Batulan et al., 2003; Brown and Rush, 1999; Foster and Brown, 1997; Manzerra and Brown, 1996; Manzerra et al., 1997; Marcuccilli et al., 1996; Nishimura and Dwyer, 1996). The limited ability of neurons to mount the cytoprotective HSR likely contributes to their inherent vulnerability and selective neuron death in disease states.

The mechanism of this attenuated HSR in differentiated neurons is not entirely clear. In motor neurons, heat shock failed to elicit an activation of HSF1, and furthermore, while the transfection and expression of a wild type HSF1 failed to rectify the defective HSR, the transfection and expression of a constitutively active form of HSF1 did (Batulan et al., 2003). This would suggest changes in the sensing and/or signaling mechanism leading to the activation of HSF1 in the differentiated neuron. In previous studies from our lab, we showed that oxidation and intramolecular disulfide crosslinking of cysteine-SH of HSF1 locks HSF1 into a conformation that is recalcitrant to activation (Manalo and Liu, 2001; Manalo et al., 2002). Whether this or similar mechanisms contribute to the attenuated activation of HSF1 in the differentiated neurons remains to be determined. Consistent with this suggestion, differentiation is often associated with a shift towards a more oxidative intracellular environment, and redox has been suggested to be a central integrator of cell growth versus differentiation (Kamata et al., 2005; Noble et al., 2003; Smith et al., 2000).

The attenuated HSR in the differentiated neural cells is likely to contribute to their vulnerability to stress induced pathologies and death. We show in Fig. 8 that the differentiated NG108-15 cells are exquisitely sensitive to the cytotoxic

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

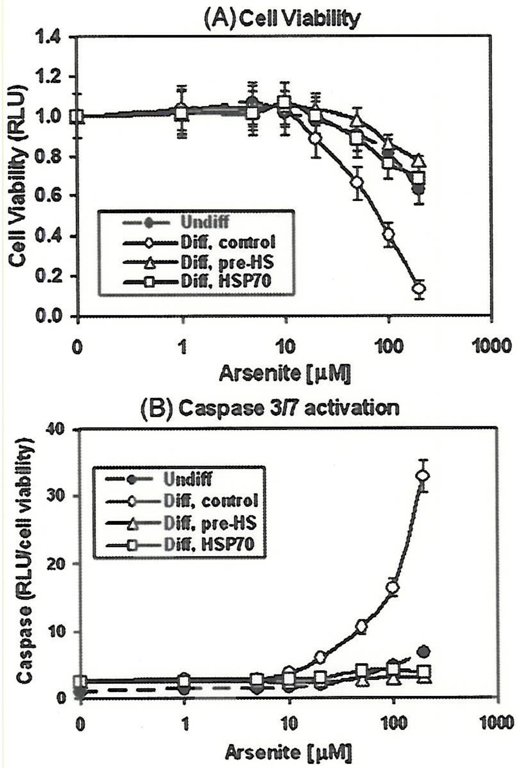

Fig. 8 – Vulnerability of the undifferentiated and differentiated NG108-15 cells towards oxidative stress induced cell death and the cytoprotective effects of conditioning heat shock and over-expression of HSP70 in the differentiated cells. Sodium arsenite was added to undifferentiated and differentiated NG108-15 cells in a 96 well plate to final concentrations of 1, 5, 10, 20, 50, 100 and 200 µM and incubated at 37 °C for 16 h. To test for the cytoprotective effects of conditioning heat shock and over-expression of hsp70 in the differentiated cells, cells were either heat shock at 42 °C for 2 h or transfected with a eukaryotic expression vector of hsp70 (pcDNA3–hsp70) 24 h prior to the challenge. (A) Cell viability. Viability was assayed using the CellTiter-Glo® reagent. Result presented is relative to that of the untreated (i.e. without arsenite) control of 1. Result represent average ± standard deviation, N=4. (B) Caspase 3/7 activity (RLU, normalized against cell viability signal) was assayed according to methods described in the text. Result represent average ± standard deviation, N=4. Solid symbol: undifferentiated cells; open symbols: differentiated cells.

effect of an oxidizer, sodium arsenite — much more so than the undifferentiated cells. Given that arsenite is both an inducer of the HSR and an elicitor of oxidative stress (Khalil et al., 2006), we inferred that the limited induction of HSPs in the differentiated cells coupled with their increased sensitivity to oxidative stress induced pathologies likely contributed to the demise of the differentiated cells in the presence of arsenite.

The cytotoxic effect of glutamate and glycine shown in Fig. 9 is likely due to activation of the NMDAR protein: (A) whereas glutamate plus glycine gave dose-dependent cytotoxic effects, glutamate alone was without effect. The NMDA receptors are heteromeric composed of NR1 subunits, which bind glycine, and the NR2 subunit, which binds glutamate; both NR1 and NR2 subunits are required to create a functional receptor (Waxman and Lynch, 2005); and (B) the cytotoxic effect of glutamate plus glycine was blocked by the NMDAR specific antagonist MK801 (data not shown). We note that expression and function of the NMDAR protein is modulated in neural differentiation: neurogenesis is correlated with the expression of various NMDA receptor subunits (Pizzi et al., 2002; Varju et al., 2001), and differentiation of the NG108-15 cells is

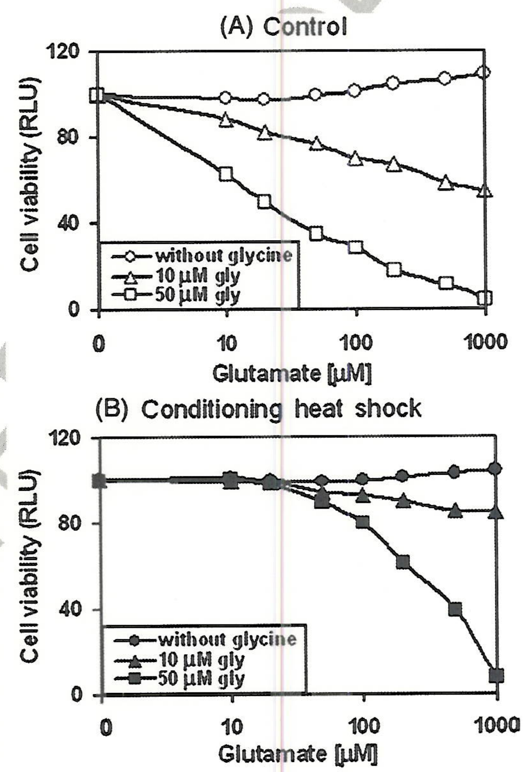

Fig. 9 – Susceptibility of the differentiated NG108-15 cells to the excitotoxic effects of glutamate/glycine. Differentiated NG108-15 cells were used. ~~To test for the protective effects of conditioning heat shock and of over-expression of hsp70, cells were either heat shocked at 42 °C for 2 h or transfected with a eukaryotic expression vector of hsp70 (pcDNA3–hsp70) 24 h prior to the challenge.~~ (A) To test for the effects of glutamate and glycine, cells were refurbished with Dulbecco's phosphate buffered saline without added amino acids. Glutamate was added to individual wells to final concentrations of 0, 10, 20, 50, 100, 200, 500 µM and 1 mM without (circle symbol) and with 10 and 50 µM glycine (triangle and square symbols, respectively). Cells were incubated at 37 °C overnight (16 h). (B) To test for the protective effect of conditioning heat shock, cells were heat shocked at 42 °C for 2 h 24 h prior to the addition of glutamate and glycine. The result presented is relative to that of the untreated control (i.e. without glutamate/glycine) of 100, and is representative of four separate experiments.

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

associated with an increase in the NMDAR mRNA level (Beczkowska et al., 1996, 1997). Indeed a comparison of the sensitivity of undifferentiated and differentiated NG108-15 cells toward the cytotoxic effects of glutamate and glycine reveal a selective vulnerability of the differentiated cells — the undifferentiated cells were not affected by the concentrations and combination of glutamate and glycine used.

The possibility that expression of the HSP chaperones may afford some protection against the cytotoxic effects of arsenite and of glutamate/glycine is supported by the observation that conditioning heat shock or forced expression of HSP70 conferred cytoprotection when the differentiated cells were challenged (Figs. 8 and 9). HSPs can suppress stress induced apoptosis by many and varied mechanisms including blocking cytochrome c release from mitochondria, preventing apoptosome formation, and inhibiting the activation of caspase 3 and downstream events (Gabai and Sherman, 2002; Mosser et al., 2000).

Our study provides evidence of an attenuated heat shock response as part of the neural differentiation program. This attenuated induction of HSPs likely contributes to neuronal vulnerability to stress induced pathologies and death. Our work may provide a framework for the development of a treatment regimen or a pharmacological agent to rectify the defective HSR in the differentiated neuron to mitigate the dire consequences of protein mis-folding and boost neuron survival under stress.

4. Experimental procedures

4.1. *Cell culture and induction of neural differentiation*

NG108-15 mouse neuroblastoma–glioma hybrid cell line was used as the prototype neural progenitor cells in this study. Other mouse neuroblastoma cell lines used include N2a, NB15, NS20 and N1E-115, and N-18 cell lines (Amano et al., 1972; Liu et al., 1988; Nelson et al., 1976; Nirenberg et al., 1983, 1984). For comparison, the PC12 pheochromocytoma cell line (Greene and Tischler, 1976) and the C17.2 surrogate neural stem cell line (Snyder et al., 1992) were also used to evaluate changes in regulation of the HSR in neural differentiation. Unless indicated otherwise, cells were grown in Dulbecco's Modified Eagle's Medium (Mediatech Inc.) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Inc.), 50 μg/ml streptomycin and 50 U/ml of penicillin. The C17.2 cell line was grown in DMEM supplemented with 10% FBS and 5% horse serum. Cells were subcultured at or near confluency by minimal trypsinization (0.25% trypsin; Mediatech Inc.) followed by dispersion of the cells into single cell suspension in new growth medium and plating onto new growing surfaces.

Differentiation of the NG108-15 neuroblastoma–glioma hybrid cells and the other neuroblastoma cell lines was induced by subculturing the cells into a low serum containing medium (2%, as opposed to the normal 10%, FBS) supplemented with 1 mM dibutyryl cAMP. Neural differentiation of the cells, can be scored by % of neurite-positive cells (neurite defined as processes >2× soma diameter) and >80% of the cells were neurite-positive 2 days after induction with dibutyryl cAMP, as compared to <10% of neurite-positive cells in the undifferentiated culture. Differentiation of the PC12 pheochromocytoma cells was induced by the addition of 50 ng/ml of nerve growth factor (NGF) for 2–3 days. Differentiation of the C17.2 surrogated neural stem cells was induced by replenishing cells with serum free medium; cell differentiation was observed 1–2 days after serum removal, although there was significant cell death.

To ascertain the "neural" specificity of this attenuated HSR, we treated a near confluent culture of the undifferentiated NG108-15 cells with 1 mM dibutyryl cAMP — when cells were recalcitrant to the neural inductive effect of dibutyryl cAMP due to cell crowding. We also tested the effects of other agents known to induce the neural differentiation process — forskolin and all-*trans*-retinoic acid.

Unless indicated otherwise, the condition for heat shock was at 42 °C for a specified time period. Cells were either harvested immediately for analysis of HSF1 or $mRNA^{hsp}$ or allowed to recover at 37° C for a specified time period for analysis of hsp70–firefly luciferase reporter gene expression, induction of HSP70 protein, and of the effects of conditioning heat shock on cell viability.

E16 rat embryonic neurons were obtained from the cortex, hippocampus, and spinal cord by tissue dissociation, and plating and culturing of the neurons according to methods described (Du et al., 2007; Magby et al., 2006; Nicot and DiCicco-Bloom, 2001). These embryonic neurons were maintained in the *in vitro* cell culture condition for 5–11 days (DIV, days *in vitro*) prior to biochemical analysis.

4.2. *Immunocytochemical staining of the cells for tubulin βIII, neurofilament and HSP70*

Cells were fixed with 4% paraformaldehyde for 30 min at 4 °C, and permeabilized with 0.1% TritonX-100 (30 min, 4 °C). Staining for tubulin βIII was done by overlaying cells with a 1:2000 dilution of mouse anti-tubulin antibody (β-III isoform; Chemicon MAB1637) and incubation at 4 °C for 60 min, followed by Texus Red-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Labs; 1:50 dilution, 60 min @ 4 °C). A rabbit anti-neurofilament 200 kDa polyclonal antibody (Chemicon AB1982; 1:500 dilution; 60 min, 4 °C) was used to probe for neurofilament, followed by incubation with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution, 60 min @ 4 °C). Nuclei were counter stained with 10 μM Hoechst 33342 at room temperature for 5 min.

For immunocytochemical staining of the 72 kDa heat inducible HSP70, we used the SPA-812 rabbit polyclonal antibody from Stressgen at 1:500 dilution and incubation at 4 °C for 1 h, followed by FITC-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution, 60 min @ 4 °C). Cells were viewed using a Nikon Diaphot 300 microscope and phase and fluorescent images captured with a SPOT camera system (Diagnostic Instruments, Inc., Sterling Heights, MI).

4.3. *Whole cell voltage clamp recordings of the undifferentiated and differentiated NG108-15 cells*

Undifferentiated and differentiated NG108-15 cells in 60 mm plates were used. Cells were held at a potential of −80 mV. The bath solution (pH 7.5) contained 1.67 mM $CaCl_2$, 0.98 mM $MgCl_2$, 5.36 mM KCl, 136.89 mM NaCl, 16.65 mM glucose, 10 M

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

HEPES and 50 mM sucrose. The pipette solution (pH 7.5) contained 112 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 11 mM EDTA, and 10 mM HEPES. The pipette resistance was between 3.6 and 4.2 MΩ. Cells were clamped at voltages as indicated and current signals were recorded with an Axopath 200A amplifier.

4.4. *Assay of hsp70 promoter driven firefly luciferase reporter*

The hsp70–firefly luciferase reporter gene was constructed by ligating a 1,036 bp *KpnI* and *NcoI* restriction enzyme fragment of the mouse hsp70 promoter–luciferase reporter, pLHSEU4 (Yanagida et al., 2000), to the *KpnI/NcoI* digested pGL3E (5,006 bp; Promega Inc.). For screening of the effects of heat shock on the hsp70–luciferase reporter gene activity, undifferentiated and differentiated cells in either 35 or 60 mm plates were transfected with the hsp70–reporter DNA along with the internal control of phRLSV40 (synthetic humanized *Renilla* luciferase DNA; Promega Inc. E6261). Unless indicated otherwise, the amount of each DNA used was 0.5 μg/35 mm plate or 1.5 μg/60 mm plate, and the amount of Lipofectamine 2000 used (in μl) was 3× that of the total amount of DNA (in μg). 6 h after DNA transfection, similar numbers of the undifferentiated and differentiated cells (~$2–4\times10^4$) were plated into individual wells of a 96 Stripwell™ plate (Corning/Costar 9102). To evaluate heat shock induction of the hsp70–luciferase reporter gene, strips of 8 wells of cells were placed in a 42 °C incubator for 2 h followed by recovery at 37 °C for 4 h prior to harvesting. Undifferentiated and differentiated cells were processed in parallel to minimize experimental noise due to variation in incubator temperature, quality/amount of the luciferase assay reagent, and decay of the luciferase luminescence signal.

The Dual-Glo luciferase assay reagent system from Promega Inc. (E2920) was used to assay for first the firefly then the *Renilla* luciferase activity according to manufacturer's instructions. We have also used the Bright-Glo luciferase assay reagent (E2610) from Promega Inc.; qualitatively similar results were obtained although the Bright-Glo reagent gave a stronger signal with a shorter half-life. Luciferase activity was measured using the Perkin Elmer Victor 2 multiplate reader equipped with dual injectors. As illustrated in Fig. 3, result on hsp70–firefly luciferase activity can be presented in one of three ways: (1) a direct read out from the Victor2 plate reader in relative luminescence unit, RLU; (2) normalized against that of the *Renilla* luciferase (RLU) to negate experimental noise due to variation in cell density, transfection efficiency, and non-specific toxic effects of the treatment condition; and (3) normalized against *Renilla* luciferase and relative to that of the undifferentiated control (ratio of firefly/*Renilla* activity set at 1) to facilitate comparison across experiments for statistical analysis. The hsp70–reporter gene assay is robust; in a given experiment, sample-to-sample (i.e. well-to-well) variation is <10% for a specified cell type and/or treatment condition. The magnitude of heat shock induction varied between experiments (the normal range being 10–40 fold over that of the control), this variation is largely due to differences in reporter gene expression under the basal 37 °C condition; perhaps, variations in cell handling, cell density, and other factors contributed to this. Nonetheless, the difference between the undifferentiated and differentiated cells of a given experiment is most reproducible over the entire 2-year duration of the study.

4.5. *Analysis of HSF1 DNA-binding activity by electrophoretic mobility shift assay (EMSA)*

Whole cell extract was prepared as described (Huang et al., 1994). EMSA was done according to methods described using 20 μg of whole cell extract protein, 0.5 μg of poly(dI-dC)·poly(dI-dC), and [^{32}P]labeled HSE in a total reaction volume of 10 μl. After 20 min of incubation at room temperature, 2 μl aliquot of a 5× loading buffer was added and samples analyzed by electrophoresis in a 4% acrylamide gel.

4.6. *Northern blot quantitation of hsp mRNAs*

RNA was isolated from undifferentiated and differentiated cells incubated under control (37 °C) and heat shocked (42 °C, 2 h) conditions following the Trizol reagent protocol for RNA isolation from Invitrogen Inc. For Northern blotting, 20 μg of the RNA sample was loaded onto a 1.2% formaldehyde agarose gel. RNA was transferred by capillary wicking onto GeneScreen Plus membrane and then UV-crosslinked at 0.3 J/cm^2. The membrane was pre-hybridized at 60 °C for 1 h in a pre-hybridization solution of 1% SDS, 10% dextran sulphate, 1 M NaCl, and 100 μg/ml of sheared salmon sperm DNA. Probing of the $mRNA^{hsp70}$, and the internal control RNA^{GAPDH} were done, respectively, by hybridization with [^{32}P]-labeled pH 2.3 (hsp70; Wu et al., 1985), and GAPDH cDNA at 60 °C overnight in a hybridization oven. The membrane was sequentially washed (60 °C, 15 min each) in 2× SSC (per liter: 17.5 gm NaCl, 2.76 g $NaH_2PO_4–H_2O$, 0.74 g EDTA, pH 7.4), 2× SSC, 1% SDS, 1× SSC, 1% SDS, 0.1× SSC, and 1% SDS, and exposed to X-ray film for signal detection.

4.7. *Immuno-Western blot detection of the heat inducible72 kDa HSP70 protein*

Immuno-Western blot detection and quantitation of the 72 kDa heat inducible HSP70 was done using a rabbit polyclonal antibody from Stressgen (SPA812; 1:10,000 dilution). Membrane was incubated with the primary antibody at 4 °C overnight followed by horseradish peroxidase (HRP) conjugated secondary antibody for 2 h at room temperature. The antibodies were diluted in Tris-buffered saline with 0.1% Tween 20 and 3% non-fat dry milk, and the immunoblot was probed using either the Amersham ECL-plus or the Millipore Immobilon Western blot reagent.

4.8. *Assay for cell viability and activation of caspase 3/7*

Cells in 96 well plates were used. To test for vulnerability to oxidative stress induced cell death, sodium arsenite was added to individual wells to final concentrations as indicated and incubated for time periods specified (12–24 h). The ability of glutamate to elicit excitotoxic cell death was evaluated in the presence of 0, 10 and 50 μM glycine and incubation at 37 °C for time periods indicated (12–24 h). Cell viability was determined using the CellTiter-Glo luminescent cell viability assay reagent from Promega Inc., and results were normalized against that of the untreated control (100%). Caspase 3 and 7 activity was

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

determined using the Caspase-Glo™ 3/7 assay reagent from Promega Inc., and the readouts were normalized against the signal from cell viability assay. To test for effects of conditioning heat shock in conferring protection against stress, cells were heat shocked at 42 °C for 2 h prior to challenge with either arsenite or glutamate/glycine 24 h later. To test for cytoprotective activity of the heat inducible HSP70, cells were transfected with pCep4hsp70, an episomal eukaryotic expression vector of hsp70, and cells were challenged with either arsenite or glutamate/glycine 24 h later.

Acknowledgment

This work was supported in part by grants from the NSF (MCB0240009) and NJ Commission on Spinal Cord Research (05-3037-SCR-E-0).

We are grateful to Dr. Mark Plummer of the Department of Cell Biology and Neuroscience for guidance in electrophysiological recording of the NG108-15 cells and for providing us with the rat embryonic hippocampal neuron culture (Magby et al., 2006). We thank Dr. Emanuel DiCicco-Bloom for providing us the embryonic cortical neuron culture (Nicot and DiCicco-Bloom, 2001), and Dr. Bonnie Firestein for the embryonic spinal cord neuron culture (Du et al., 2007). We thank Dr. Evan Snyder for the C17.2 surrogated neural stem cell line (Snyder et al., 1992).

REFERENCES

Amano, T., Richelson, E., Nirenberg, M., 1972. Neurotransmitter synthesis by neuroblastoma clones. Proc. Natl. Acad. Sci. U. S. A. 69, 258–263.

Batulan, Z., Shinder, G.A., Minotti, S., He, B.P., Doroudchi, M.M., Nalbantoglu, J., Strong, M.J., Durham, H.D., 2003. High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. J. Neurosci. 23, 5789–5798.

Beczkowska, I.W., Buck, J., Inturrisi, C.E., 1996. Retinoic acid-induced increase in delta-opioid receptor and N-methyl-D-aspartate receptor mRNA levels in neuroblastoma × glioma (NG108-15) cells. Brain Res. Bull. 39, 193–199.

Beczkowska, I.W., Gracy, K.N., Pickel, V.M., Inturrisi, C.E., 1997. Detection of delta opioid receptor and NMDA receptor like immunoreactivity in retinoic acid-differentiated neuroblastoma × glioma (NG108-15) cells. J. Neurosci. Res. 47, 83–89.

Brown, E.R., Rush, S.J., 1999. Cellular localization of the heat shock transcription factors HSF1 and HSF2 in the rat brain during postnatal development and following hyperthermia. Brain Res. 821, 333–340.

Du, Y., Chen, C.P., Tseng, C., Eisenberg, Y., Firestein, B.L., 2007. Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. Glia 55, 463–472.

Feige, U., Morimoto, R.I., Yahara, I., and Polla, B.S., eds., 1996. Stress-inducible Cellular Responses. Birkhauser/Springer, Basel/Boston/Berlin.

Foster, J.A., Brown, E.R., 1997. Differential induction of heat shock mRNA in oligodendrocytes, microglia, and astrocytes following hyperthermia. Mol. Brain Res. 45, 207–218.

Forman, M.S., Trojanowski, J.Q., Lee, V.M.-Y., 2004. Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. Nat. Med. 10, 1055–1063.

Gabai, V.L., Sherman, M.Y., 2002. Interplay between molecular chaperones and signaling pathways in survival of heat shock. J. Appl. Physiol. 92, 1742–1748.

Greene, L.A., Tischler, A.S., 1976. Establishment of a nonadrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428.

Hendrick, J.P., Hartl, F.U., 1995. The role of molecular chaperones in protein folding. FEBS J. 9, 1559–1569.

Huang, L.E., Zhang, H., Bae, S.W., Liu, A.Y.-C., 1994. Thiol reducing reagents inhibit the heat shock response: involvement of a redox mechanism in the heat shock signal transduction pathway. J. Biol. Chem. 269, 30718–30725.

Kamata, H., Oka, S., Shibukawa, Y., Kakuta, J., Hirata, H., 2005. Redox regulation of nerve growth factor-induced neuronal differentiation of PC12 cells through modulation of the nerve growth factor receptor, TrkA. Arch. Biochem. Biophys. 434, 16–25.

Khalil, S., Luciano, J., Chen, W., Liu, A.Y.C., 2006. Dynamic regulation and involvement of the heat shock transcriptional response in arsenic carcinogenesis. J. Cell. Physiol. 207, 562–569.

Liu, A.Y.-C., Lin, Z., Kamalakannan, V., Chen, K.Y., 1988. Induction and quantitation of the R_I cAMP-binding protein in clonal mouse neuroblastoma cell lines: evidence that the increase in R_I is not linked to neurite outgrowth. J. Neurosci. Res. 19, 303–311.

Liu, A.Y.-C., Lee, Y.K., Manalo, D., and Huang, L.E., 1996. Attenuated heat shock transcriptional response in aging: Molecular mechanisms and implications in the biology of aging. Stress-inducible Cellular Responses (Ulrich Feige, Richard I. Morimoto, Ichiro Yahara, and Barbara S. Polla, eds.), Birkhauser/Springer, pp. 393–408.

Magby, J.P., Bi, C., Chen, Z.Y., Lee, F.S., Plummer, M.R., 2006. Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. J. Neurosci. 26, 13531–13536.

Manalo, D.J., Liu, A.Y.-C., 2001. Resolution, detection, and characterization of redox conformers of human HSF1. J. Biol. Chem. 276, 23554–23561.

Manalo, D.J., Lin, Z., Liu, A.Y.-C., 2002. Redox-dependent regulation of the conformation and function of human heat shock factor 1. Biochemistry 41, 2580–2588.

Manzerra, P., Brown, I.R., 1996. The neuronal stress response: nuclear translocation of heat shock proteins as an indicator of hyperthermic stress. Exp. Cell Res. 229, 35–47.

Manzerra, P., Rush, S.J., Brown, I.R., 1997. Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. J. Cell. Physiol. 170, 130–137.

Marcuccilli, C.J., Mathur, S.K., Morimoto, R.I., Miller, R.J., 1996. Regulatory differences in the stress response of hippocampal neurons and glial cells after heat shock. J. Neurosci. 16, 478–485.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12, 378–3796.

Morimoto, R.I., 2006. Stress, aging, and neurodegenerative disease. N. Engl. J. Med. 355, 2254–2255.

Morimoto, R.I., Tissieres, A., Georgopoulos, C. (Eds.), 1994. The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, New York, p. 610.

Mosser, D.D., Carbon, A.E., Bourget, L., Merlin, A.B., Sherman, M.Y., Morimoto, R.I., Massie, B., 2000. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol. Cell. Biol. 20, 7146–7159.

Muchowski, P.J., Wacker, J.L., 2005. Modulation of neurodegeneration by molecular chaperones. Nat. Rev., Neurosci. 6, 11–22.

Nelson, P., Christian, C., Nirenberg, M., 1976. Synapse formation between clonal neuroblastoma × glioma hybrid cells and striated muscle cells. Proc. Natl. Acad. Sci. U. S. A. 73, 123–127.

Nicot, A., DiCicco-Bloom, E., 2001. Regulation of neuroblast mitosis is determined by PACAP receptor isoform expression. Proc. Natl. Acad. Sci. U. S. A. 98, 4758–4763.

Nirenberg, M., Wilson, S., Higashida, H., Rotter, A., Dureger, K., Busis, N., Ray, R., Kenimer, K., Adler, M., Fukui, H., 1983. Synapse formation by neuroblastoma hybrid cells. Cold Spring Harbor Symp. Quant. Biol. 48, 07–715.

Nirenberg, M., Wilson, S., Higashida, H., Rotter, A., Krueger, K., Busis, N., Ray, R., Kenimer, K., Adler, M., 1984. Modulation of synapse formation by cyclic adenosine monophosphate. Science 222, 794–799.

Nishimura, R.N., Dwyer, B.E., 1996. Evidence for different mechanisms of induction of hsp70i: a comparison of cultured rat cortical neurons with astrocytes. Mol. Brain Res. 36, 227–239.

Noble, M., Smith, J., Power, J., Mayer-Proschel, M., 2003. Redox state as a central modulator of precursor cell function. Ann. N.Y. Acad. Sci. 991, 251–271.

Pizzi, M., Boroni, F., Bianschetti, A., Moraitis, C., Sarnico, I., Benarese, M., Goffi, F., Valerio, A., Spano, P.F., 2002. Expression of functional NR1/NR2B-type NMDA receptors in neuronally differentiated SK-N-SH human cell line. Eur. J. Neurosci. 16, 2342–2350.

Sherman, M.Y., Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29, 15–32.

Smith, J., Ladi, E., Mayer-Proschel, M., Noble, M., 2000. Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. Proc. Natl. Acad. Sci. U. S. A. 97, 10032–10037.

Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartwig, E.A., Cepko, C.L., 1992. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 68, 33–51.

Varju, P., Schlett, K., Eisel, U., Madarász, E., 2001. Schedule of NMDA receptor subunit expression and functional channel formation in the course of *in vitro*-induced neurogenesis. J. Neurochem. 77, 1444–1456.

Waxman, E.A., Lynch, D.R., 2005. NMRA receptor subtypes: multiple roles in excitotoxicity and neurological disease. Neurosci. 11, 37–49.

Wu, B., Hunt, C., Morimoto, R.I., 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. Mol. Cell. Biol. 5, 330–341.

Yanagida, Y., Mizuno, A., Motegi, T., Kobatake, E., Aizawa, M., 2000. Electrically stimulated induction of hsp 70 gene expression in mouse astroglia and fibroblast cells. J. Biotechnol. 79, 53–61.

Please cite this article as: Yang, J., et al., Neural differentiation and the attenuated heat shock response, Brain Res. (2008), doi:10.1016/j.brainres.2008.01.082

Varju P, Schlett K, Eisel U, Madarász E (2001) Schedule of NMDA receptor subunit expression and functional channel formation in the course of in vitro-induced neurogeneisis. J Neurochem 77:1444–1456

Voellmy R (1994) Transduction of the stress signal and mechanisms of transcriptional regulation of heat shock/stress protein gene expression in higher eukaryotes. Critical Rev Eukaryot Gene Expr 4:357–401

Waxman EA, Lynch DR (2005) *N*-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. Neuroscientist 11:37–49

Welch WJ, Gambetti P (1998) Chaperoning brain diseases. Nature 392:23

Westerheide SD, Morimoto RI (2005) Heat shock response modulators as therapeutic tools for diseases of protein conformation. J Biol Chem 280:33097–33100

Yanagida Y, Mizuno A, Motegi T, Kobatake E, Aizawa M (2000) Electrically stimulated induction of hsp 70 gene expression in mouse astroglia and fibroblast cells. J Biotechnology 79: 53–61

Yenari MA, Fink SL, Sun GH et al (1998) Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy. Ann Neurol 44(4):584–591

Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK (1999) The neuroprotective potential of heat shock protein 70 (Hsp70). Mol Med Today 5(12):525–531

Young JC, Barral JM, Hartl FU (2003) More than folding: localized function of cytosolic chaperones. Trends Biochem Sci 28:541–547

Zinsmaier KE, Bronk P (2001) Molecular chaperones and the regulation of neurotransmitter exocytosis. Biochem Pharmacol 2001(62):1–11

BRAIN RESEARCH XX (2008) XXX–XXX

ELSEVIER

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Neural differentiation and the attenuated heat shock response

Jingxian Yang[a,b], *Jay Oza*[a,b], *Kristen Bridges*[a,b], *Kuang Yu Chen*[c], *Alice Y.-C. Liu*[a,b,*]

[a]*Department of Cell Biology and Neuroscience, Rutgers State University of New Jersey, Piscataway, New Jersey, USA*
[b]*Division of Life Sciences, Rutgers State University of New Jersey, Piscataway, New Jersey, USA*
[c]*Department of Chemistry and Chemical Biology, Rutgers State University of New Jersey, Piscataway, New Jersey, USA*

ARTICLE INFO

Article history:
Accepted 21 January 2008

Keywords:
Neuronal cell differentiation
Neuronal vulnerability
Neuroprotection
Heat shock response
Heat shock protein
HSP70

ABSTRACT

Differentiation of neural progenitor cells of neuroblastoma, pheochromocytoma, and surrogate stem cell lineages from a state resembling stem cells to a state resembling neurons is accompanied by a marked attenuation in induction of the heat shock protein 70 promoter driven-luciferase reporter gene, and induction of the reporter gene in primary embryonic neurons from hippocampus, cortex, and spinal cord is lower still when compared to the differentiated cells. Neural specificity of this phenotype is demonstrated by a negative correlation of hsp70–reporter gene expression and neurite extension under various experimental conditions. Analysis of biochemical events involved in induction of the heat shock response (HSR) reveal a blunted activation of HSF1 DNA-binding activity, and decreased induction of the $mRNA^{hsp70}$ and the 72 kDa HSP70 protein. Immunocytochemical staining for HSP70 demonstrates a cytoplasmic staining pattern; heat shock greatly increased the HSP70 staining intensity in the undifferentiated cells and less so in the differentiated cells. Vulnerability of the differentiated cells towards the oxidizer, arsenite, and the excitotoxic glutamate/glycine is demonstrated by the dose-dependent cytotoxic effects of these agents on cell viability and activation of caspase 3/7. Importantly, conditioning heat shock as well as increased expression of HSP70 by gene transfer conferred protection against such cytotoxicity. Together, our results show that neural differentiation is associated with a decreased induction of the heat shock response and an increased vulnerability to stress induced pathologies and death.

1. Introduction

The heat shock response (HSR; aka stress response) is a primary, evolutionarily conserved, and homeostatic genetic response to many stressors. The response is initiated by activation of the heat shock transcription factor HSF1, and culminates in the induction of a family of heat shock proteins (HSPs) that function as molecular chaperones to help in the folding and re-folding of non-native proteins, proteases to help in the disposition of damaged proteins, and other proteins essential for recovery from

* *Corresponding author*. Department of Cell Biology and Neuroscience, Rutgers State University of New Jersey, 604 Allison Road, Piscataway, NJ 08854, USA. Fax: +1 732 445 5870.
E-mail address: liu@biology.rutgers.edu (A.Y.-C. Liu).

Abbreviations: dibutyryl cAMP, N^6,2′-O-dibutyryl adenosine 3′:5′-cyclic mono-phosphate; FBS, fetal bovine serum; HSF1, heat shock factor 1; HSR, heat shock response; HSP, heat shock protein; HSP70, the 72 kDa heat shock protein; HSC70, the 73 kDa constitutively expressed heat shock cognates; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; SDS, sodium dodecyl sulfate

0006-8993/$ – see front matter
doi:10.1016/j.brainres.2008.01.082