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Research Report

Neural differentiation and the attenuated heat shock response

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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

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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

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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,

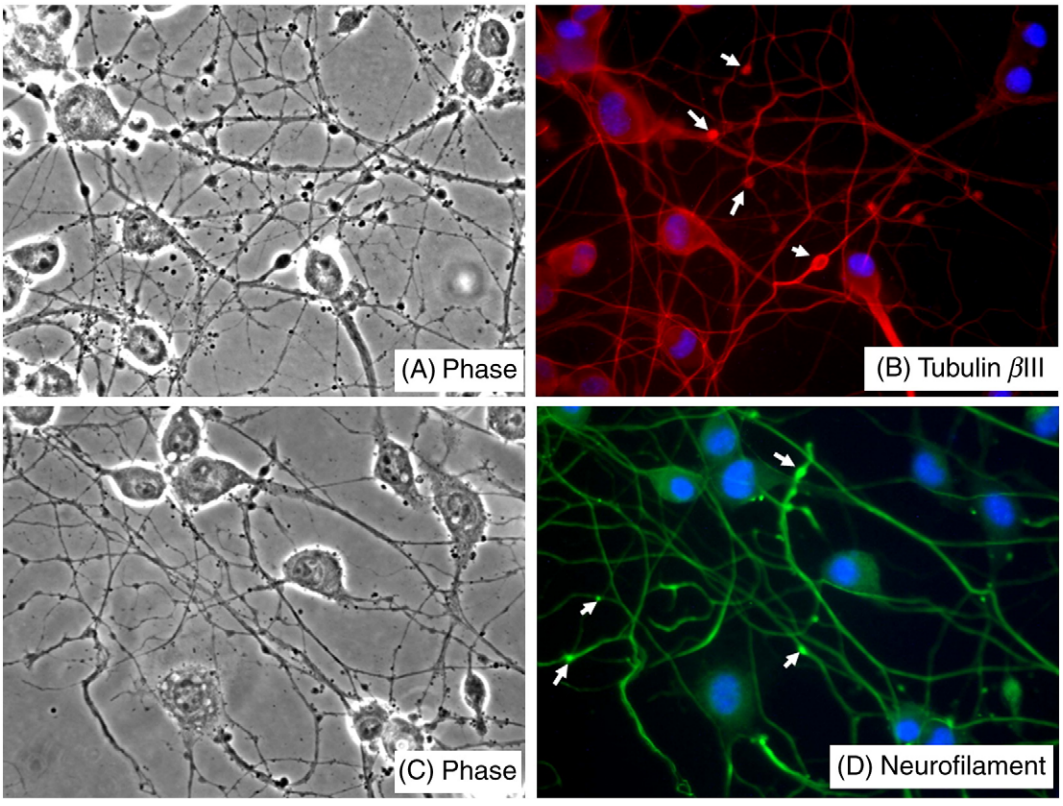

Fig. 1 – Immunocytochemical staining of differentiated NG108-15 cells for tubulin βIII and neurofilament. Dibutyryl cAMP-induced (1 mM, 3 days) differentiated NG108-15 cells in 35 mm plates were fixed and stained according to methods described in the text. The secondary antibody used for the staining 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.

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 functional synapses with co-cultured 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.

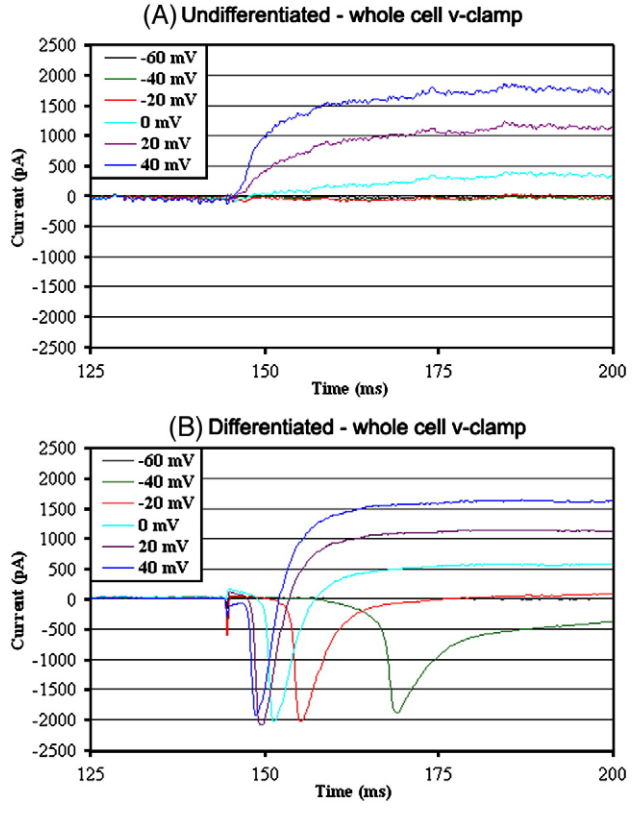

Fig. 2 – Whole cell voltage clamp recordings of the (A) undifferentiated and (B) differentiated NG108-15 cells. Undifferentiated and differentiated NG108-15 cells in 60 mm plates were used. For voltage clamp recording, cells were clamped at voltages as indicated. Signals were recorded with an Axopath 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

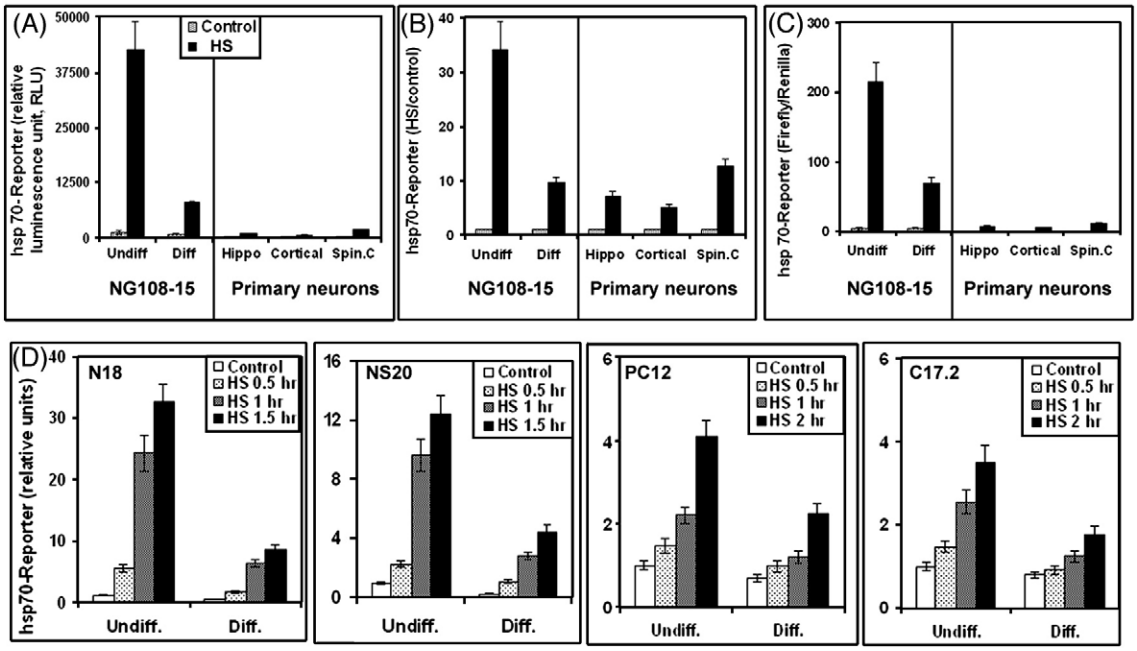

Fig. 3 – An attenuated induction of the hsp70–firefly luciferase reporter is a feature of the differentiated neuron. (A) A comparison of the basal (37 °C) and heat shock-induced (42 °C) hsp–firefly luciferase activity (in relative luminescence unit, RLU) in the undifferentiated versus differentiated 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 1.5/2 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-

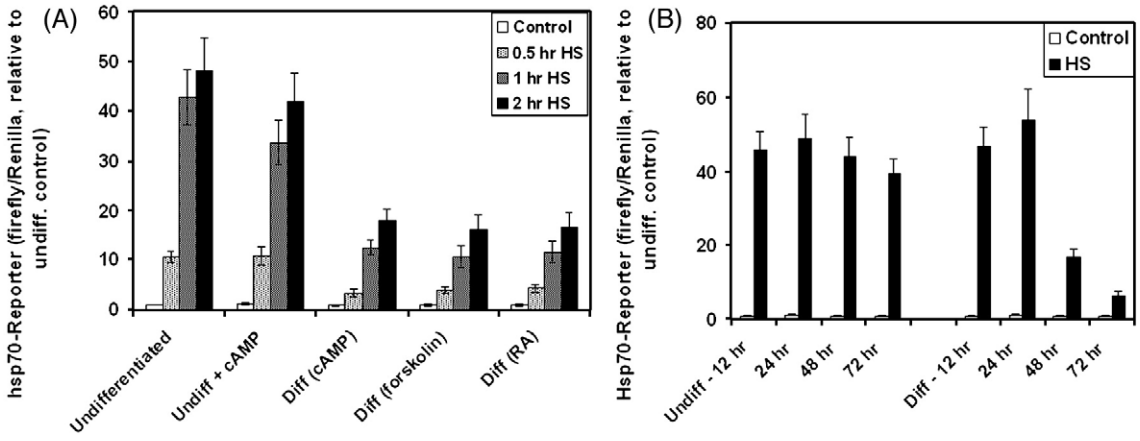

Fig. 4 – Specificity and time course of change in hsp70–reporter gene expression in neural differentiation. (A) Specificity of the attenuated induction of hsp70–reporter gene. Plates of NG108-15 cells were induced to differentiate by the addition of dibutyryl cAMP [Diff (cAMP); 1 mM, 48 h], forskolin [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.

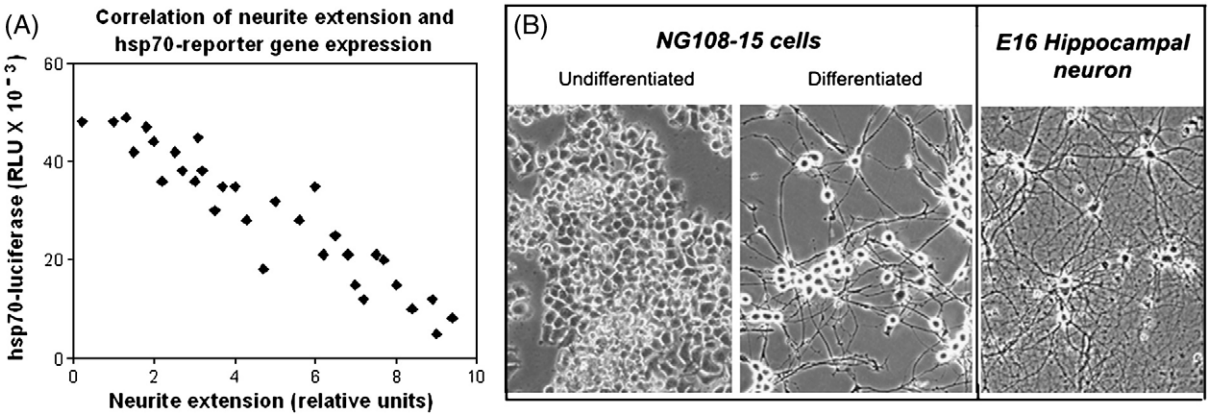

Fig. 5 – (A) Induction of the hsp70–reporter is negatively correlated with morphological differentiation. NG108-15 neuroblastoma cells were transfected with the hsp70–firefly luciferase DNA. 6 h after DNA transfection cells were subcultured and plated in 24 well plates at varying plating density in DMEM 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.

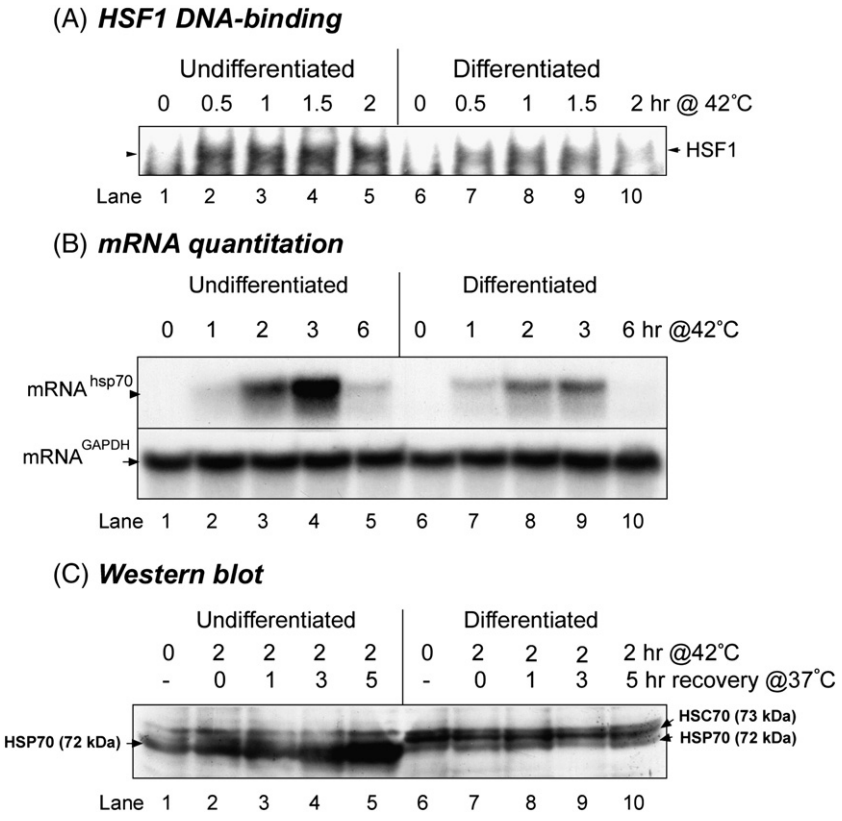

Fig. 6 – Activation of the HSF1 DNA-binding activity, and induction of hsp70 mRNA and protein in the undifferentiated and differentiated NG108-15 cells. (A) Heat shock-dependent activation of the HSF1 DNA-binding activity. Electrophoretic mobility shift assay was used to assess the HSF1 DNA-binding activity in the control- and heat shocked- (42 °C, 0.5, 1 and 2 h) undifferentiated (lanes 1–5) and differentiated (lane 6–10) 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

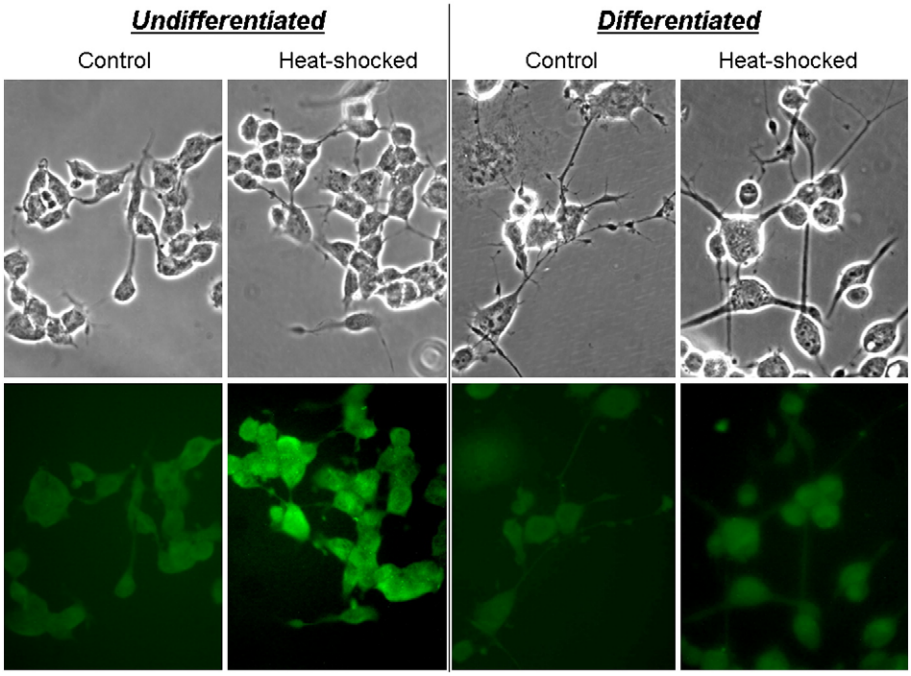

Fig. 7 – Phase contrast and HSP70 immuno-fluorescence photomicrographs of the control- and heat shocked-undifferentiated and differentiated NG108-15 cells. Undifferentiated and differentiated (1 mM dibutyryl cAMP in a 2% fetal bovine serum supplemented medium for 3 days at 37 °C) NG108-15 cells were 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

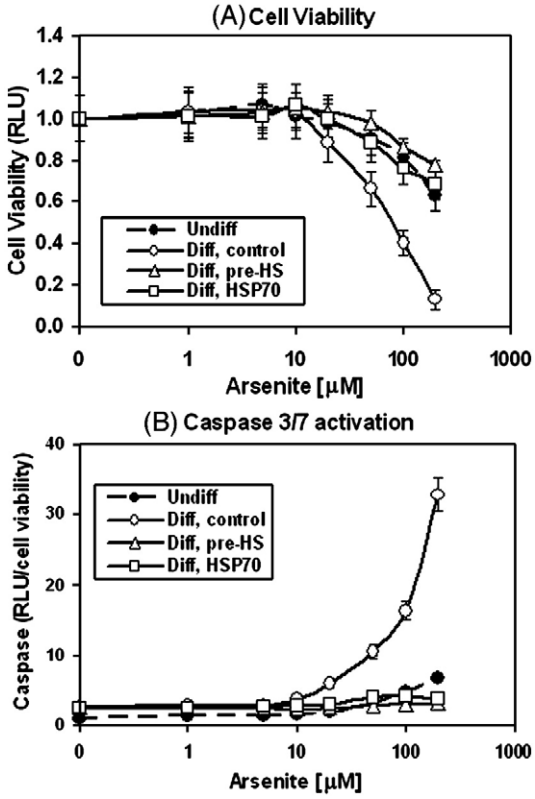

Fig. 8 – Vulnerability of the undifferentiated and differentiated NG108-15 cells towards oxidative stress induced cell death and the cytoprotective effects of conditioning heat shock and over-expression of HSP70 in the differentiated cells. Sodium arsenite was added to undifferentiated and differentiated 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 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

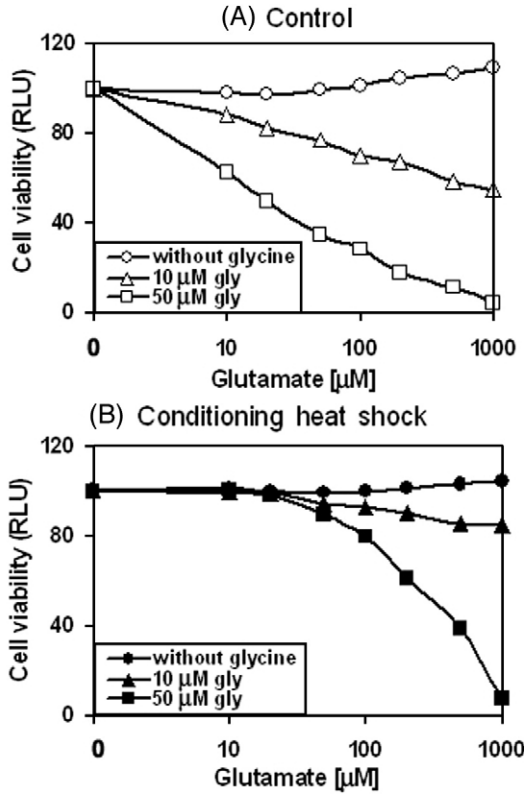

Fig. 9 – Susceptibility of the differentiated NG108-15 cells to the excitotoxic effects of glutamate/glycine. Differentiated NG108-15 cells were used. (A) To test for the effects of glutamate and glycine, cells were refurbished with Dulbecco's phosphate buffered saline without added amino acids. Glutamate 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.

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.

E15 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 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 (~2–4×10^4) 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 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.

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