

The Systemic Amyloid Precursor Transthyretin (TTR) Behaves as a Neuronal Stress Protein Regulated by HSF1 in SH-SY5Y Human Neuroblastoma Cells and APP23 Alzheimer's Disease Model Mice

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Increased neuronal synthesis of transthyretin (TTR) may favorably impact on Alzheimer's disease (AD) because TTR has been shown to inhibit A β aggregation and detoxify cell-damaging conformers. The mechanism whereby hippocampal and cortical neurons from AD patients and APP23 AD model mice produce more TTR is unknown. We now show that *TTR* expression in SH-SY5Y human neuroblastoma cells, primary hippocampal neurons and the hippocampus of APP23 mice, is significantly enhanced by heat shock factor 1 (HSF1). Chromatin immunoprecipitation (ChIP) assays demonstrated occupation of *TTR* promoter heat shock elements by HSF1 in APP23 hippocampi, primary murine hippocampal neurons, and SH-SY5Y cells, but not in mouse liver, cultured human hepatoma (HepG2) cells, or AC16 cultured human cardiomyocytes. Treating SH-SY5Y human neuroblastoma cells with heat shock or the HSF1 stimulator celastrol increased *TTR* transcription in parallel with that of *HSP40*, *HSP70*, and *HSP90*. With both treatments, ChIP showed increased occupancy of heat shock elements in the *TTR* promoter by HSF1. *In vivo* celastrol increased the HSF1 ChIP signal in hippocampus but not in liver. Transfection of a human *HSF1* construct into SH-SY5Y cells increased *TTR* transcription and protein production, which could be blocked by shHSF1 antisense. The effect is neuron specific. In cultured HepG2 cells, HSF1 was either suppressive or had no effect on *TTR* expression confirming the differential effects of HSF1 on *TTR* transcription in different cell types.

Key words: Alzheimer's disease; celastrol; heat shock; HSF1; transgenics; transthyretin

Introduction

The majority of cortical and hippocampal neurons in human Alzheimer's disease (AD) and APP23 transgenic mouse model brains stain with antibodies to transthyretin (TTR) (Schwarzman and Goldgaber, 1996; Stein and Johnson, 2002; Li et al., 2011). Studies in transgenic models of human A β deposition have indicated that TTR suppresses the AD-like neuropathologic changes characteristic of the disease (Stein et al., 2004; Choi et al., 2007; Buxbaum et al., 2008b). *In vitro* experiments have documented interactions between TTR and A β _{1–40/1–42}, which result in inhibition of A β aggregation and cytotoxicity (Giunta et al., 2005; Liu and Murphy, 2006; Costa et al., 2008; Du and Murphy, 2010; Du et al., 2012; Cascella et al., 2013). Hence, neuronal *TTR* expres-

sion could represent a cellular defense to aggregated A β or reactive oxygen species that are part of the neurodegenerative process.

Heat shock factor 1 (HSF1), the major regulator of cellular stress responses, is a post-translationally regulated stimulator of transcription of chaperones, chaperone-like proteins, and a variety of molecules responsible for rapid cellular responses to multiple environmental stresses including heat (Calabrese et al., 2010). Cytoplasmic HSF1 is a monomer, in complex with an inhibitor, perhaps Hsp70 and/or Hsp90 (Raychaudhuri et al., 2014). It is released on exposure to stress and trimerizes and translocates to the nucleus where it binds to heat shock elements (HSEs) in the promoters of its target genes (Morimoto et al., 1997; Morimoto, 1998; Neef et al., 2011). Activation does not require synthesis of new HSF protein. Post-translational modifications may vary in different cells. Silencing the *Hsf1* gene in mice has pleiotropic effects, including failure to induce heat shock protein (Hsp40, Hsp70, Hsp90) expression in response to stress (McMillan et al., 1998; Zhang et al., 2002; Homma et al., 2007). Studies in yeast, *Drosophila melanogaster*, HeLa, HT1080, HEK293, mouse embryo fibroblasts, and a variety of tumor cells have revealed that HSF1 has multiple targets in addition to the classical heat shock proteins (Hahn et al., 2004; Trinklein et al., 2004; Page et al., 2006; Mendillo et al., 2012; Ryno et al., 2014). None of the

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studies suggested that *TTR* was subject to regulation by HSF1. The promoter regions of genes regulated by HSF1 contain one or more HSEs with at least three nGAA repeats (Perisic et al., 1989; Ankar and Sistonen, 2011). The presence of such sequences in both the human and murine *TTR* promoter regions suggested that HSF1-driven increased *TTR* expression could play a role in its apparent neuroprotective activity (Stein and Johnson, 2002; Buxbaum et al., 2008b; Li et al., 2011).

The experiments reported here examine the effect of HSF1 on *TTR* gene expression and protein production in cultured human cells of hepatic, neuronal, and cardiac origin and in murine liver and hippocampus *in vivo*. The cell lines were chosen to represent liver, the major site of systemic TTR synthesis *in vivo* (HepG2, HuH-7), heart, a tissue that is a target of TTR deposition in the systemic TTR amyloidoses, and is not known to synthesize TTR (AC16) and the tissue of primary interest (SH-SY5Y neuroblastoma cells) representing a cell lineage that is the target of neurodegenerative disease.

Materials and Methods

Genomic sequence analysis. Searches for potential transcription factor binding sites in both the human and murine *Ttr* promoter sequences were performed using Jaspar, Transcription Element Search System, and Transcription Factor Finder online databases (Schug, 2008; Cui et al., 2010; Portales-Casamar et al., 2010).

Animals and drug treatment. C57BL/6J, APP23, APP23/*Ttr*^{-/-} (APP23 mice on *Ttr* knock-out background) mouse strains were established and maintained as described previously according to a protocol approved by the institutional animal care and use committee at The Scripps Research Institute (Buxbaum et al., 2008b). *Ttr*^{-/-} mice were obtained from M. Gottesman (Columbia University College of Physicians and Surgeons, New York) (Episkopou et al., 1993). Male mice were used in all the *in vivo* experiments.

The *in vivo* effects of celastrol were assessed in 13-week-old C57BL/6J mice injected intraperitoneally (1 mg/kg body weight) with celastrol ($n = 5$) or with 100 μ l of vehicle (35% DMSO in PBS, $n = 5$) daily for 4 d (Paris et al., 2010). One hour after the last injection, hippocampus and livers of the animals were collected, snap frozen in liquid nitrogen, and stored at -80°C .

Cell culture. SH-SY5Y human neuroblastoma cells (Biedler et al., 1973; Montgomery et al., 1983), obtained from the ATCC, were cultured in DMEM/F12 (1:1) medium (Invitrogen), supplemented with 10% (v/v) FBS, 50 U/ml penicillin, and 100 μ g/ml streptomycin, as were the AC16 human cardiomyocyte-derived cells obtained from Dr. M. Davidson, Columbia University College of Physicians and Surgeons (Davidson et al., 2005). HepG2 (Knowles et al., 1980) cells and HuH-7 (Nakabayashi et al., 1982) cells, from human hepatocarcinomas, were grown in DMEM with the same supplements as the SH-SY5Y cells.

Heat shock treatment. Cells were subjected to heat shock by incubation at 42°C in a water bath from 30 min to 2 h before RNA extraction.

Celastrol treatment. Cells were treated with different concentrations (range, 1–6 μM) of the HSF1 activator celastrol or with the same volume of vehicle for 24 h. After treatment, the cells were collected and RNA extracted using the RNeasy Plus Mini Kit (QIAGEN).

Primary neuron cultures. Primary hippocampal neuron cultures were established from C57BL/6J and APP23 mice, following previously established protocols (Kaech and Banker, 2006; Li et al., 2011). For transfection experiments, 7 d *in vitro* (DIV7) neurons were used.

Table 1. Potential transcription factor binding sites in human and murine *Ttr* promoter region^a

Name	Effective threshold	<i>p</i>
HNF1	14.14	5.92E-02
HSF1	6.00	1.34E-06

^aSites were identified using Jaspar, Transcription Element Search System–Tess, and Transcription Factor Finder. HSF1 binding sites are present in both the human and murine sequences, suggesting that they are evolutionarily conserved.

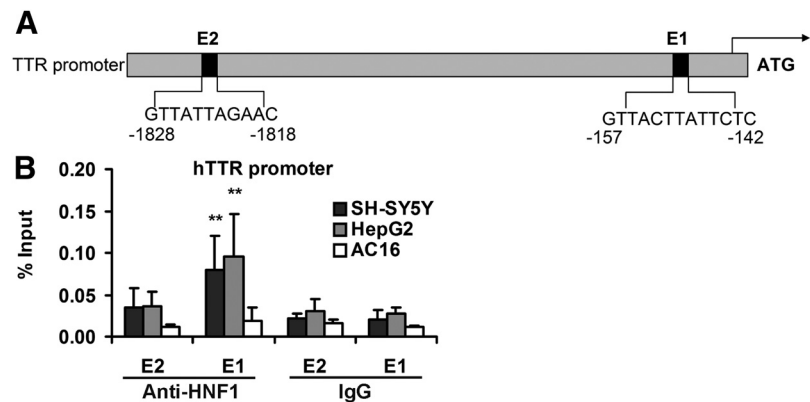


Figure 1. ChIP analysis of HNF1 binding sites in the human *TTR* promoter. **A**, Schematic representation of known HNF1 sequences in the human *TTR* promoter. ChIP–qPCR primers flank the predicted HNF1 binding sites. **B**, Extracts of SH-SY5Y (black), HepG2 (gray), and AC16 (white) cells (1×10^6 cell equivalents per IP) were subjected to a ChIP assay with antibodies specific for HNF1 and nonimmune IgG. Precipitated DNA was amplified by real-time qPCR with primers flanking the predicted HNF1 binding sites (shown) of the *TTR* promoter. ChIP assays were performed in triplicate. Data are presented as percentage of input DNA. Error bars indicate mean \pm SD. **Statistical significance of differences in binding between anti-HNF1 antibody and nonspecific IgG is indicated (from ≥ 3 independent experiments, Student's *t* test); $p < 0.01$.

Plasmid preparation and transfection. A novel, *Gussia* luciferase-based transcriptional reporter construct was generated by replacing the firefly luciferase gene in the promoterless pGL4.17 vector (Promega) with an enhanced *Gussia* luciferase (GLuc) (eGLuc2) gene (mutated at two oxidation-prone methionines, M43I and M110I), creating pGL4.17-eGLuc2 (Hulleman et al., 2011). A 2 kb fragment of the human *TTR* promoter region immediately upstream of the initiation codon was inserted into the pGL4.17 vector and used to drive eGLuc2 transcription (pGL4.17-*TTR*-eGLuc2) (Hulleman et al., 2011). Single clone colonies of SH-SY5Y or HuH-7 cells harboring the pGL4.17-*TTR*-eGLuc2 plasmid were generated by transfection according to the manufacturer's protocol (X-tremeGENE 9, Roche), followed by selection in G418. The stable SH-SY5Y or HuH-7 cell lines were propagated in media supplemented with G418 (400 μ g/ml). All cell lines were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C .

Plasmids containing constitutively active HSF1 and short hairpin antisense HSF constructs were obtained from Professor Richard Morimoto, Northwestern University (Zuo et al., 1995).

GLuc luminescence assay of *TTR* promoter activity. The luminescence assay was performed as previously described (Hulleman et al., 2012). The secretion of eGLuc2 was monitored by adding 50 nl of substrate diluted in 10 μ l of neat GLuc buffer (BioLux *Gussia* Luciferase Assay Kit; New England Biolabs) to 45 μ l aliquots of conditioned media (typically $\sim 1/10$ of the total volume). Immediately after mixing, luminescence was measured in a 96-well Costar flat-bottomed black assay plate (Corning) in a Safire II microplate reader (Tecan). For celastrol treatment, we normalized the luminescence results using total cell protein as determined by a Bradford assay (Bio-Rad) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) analysis. ChIP was performed on the HepG2, AC16, or SH-SY5Y cell lines and on extracts of hippocampus dissected free of choroid plexus and livers of the same mice. For analysis of transcription factor binding site occupancy on the *TTR* promoter, nuclear proteins were cross-linked to the DNA with 1% formaldehyde. Antibodies for HNF1 (sc-8986, Santa Cruz Biotechnology) and

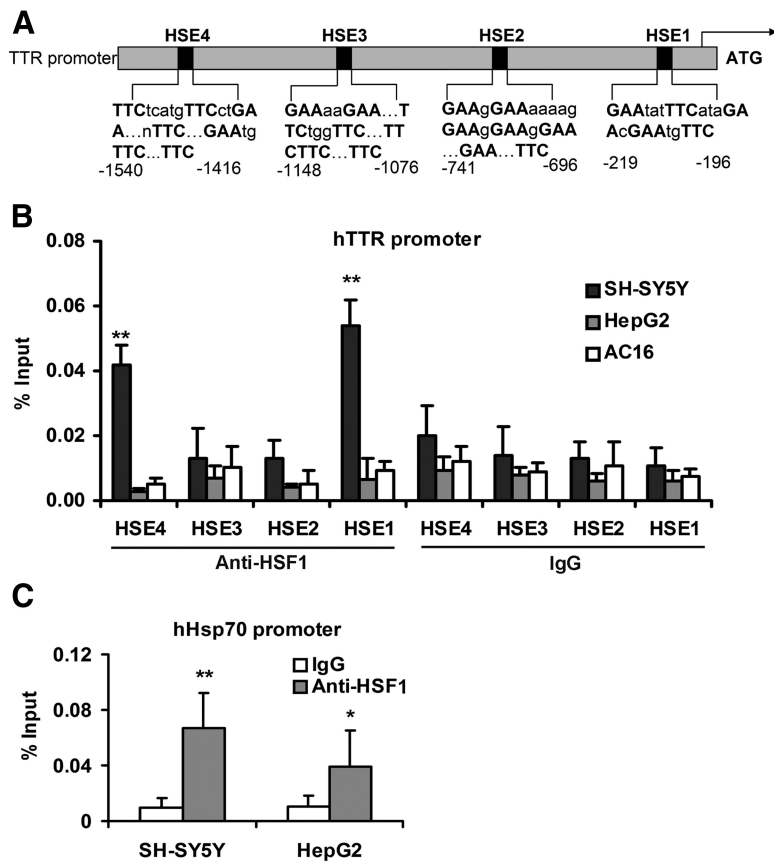


Figure 2. ChIP analysis of HSF1 binding sites in human *TTR* and *HSP70.1* promoters. **A**, Schematic representation of predicted HSEs in the human *TTR* promoter. ChIP-qPCR primers flank the predicted HSF1 binding sites. **B**, Extracts of SH-SY5Y (black), HepG2 (gray), and AC16 (white) cells (1×10^6 cell equivalents per IP) were subjected to ChIP assay with antibodies specific for HSF1 and nonimmune IgG. Precipitated DNA was amplified by real-time qPCR with primers flanking the predicted HSF1 binding sites (shown) of the *TTR* promoter. There were significant differences between nonimmune IgG and HSF1 antibody binding to the HSE1 and HSE4 sites of the *TTR* promoter in SH-SY5Y but not in HepG2 or AC16 cell DNA. **C**, Binding of HSF1 to the positive control *Hsp70.1* promoter in SH-SY5Y and HepG2 cells. Fold enrichment (as percentage of input DNA) is relative to binding by nonimmune IgG. Multivariate analysis reveals statistically significant HSF1 binding (IgG vs HSF1 antibody) to the promoter both in the SH-SY5Y and the HepG2 cells. Error bars indicate mean \pm SD. Statistical significance is indicated (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$, ** $p < 0.01$.

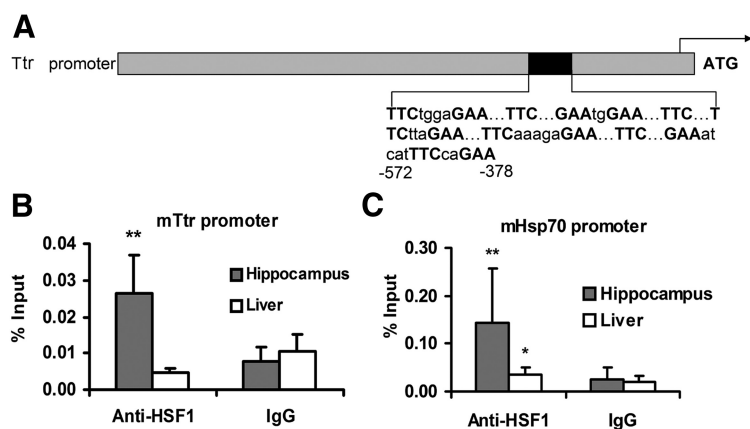


Figure 3. ChIP analysis of HSF1 binding sites in the murine *Ttr* and *Hsp70.1* promoters. **A**, A schematic depiction of the single HSE in the murine *Ttr* promoter. **B**, The histogram shows the results of ChIP by HSF1 Ab or normal mouse IgG in the hippocampus and liver of WT C57BL/6 mice ($n = 4$) using the *Ttr* HSE-specific primers. **C**, A similar analysis using murine *Hsp70* specific primers. Percentage of input DNA is relative to nonimmune IgG. Multivariate analysis reveals statistically significant HSF1 binding (IgG vs HSF1 antibody) to both promoters in the hippocampus but not the liver of C57BL/6 mice ($n = 4$). Error bars indicate mean \pm SD. * $p < 0.05$ (Student's *t* test). ** $p < 0.01$ (Student's *t* test).

HSF1 (sc-17757, Santa Cruz Biotechnology) were used with magnetic protein A/G beads to immunoprecipitate the protein-DNA complexes. As a control, samples were immunoprecipitated with 5 μ g nonimmune mouse IgG or anti-RNA polymerase II (Millipore Biotechnology). A protocol suggested by the manufacturer (Millipore Biotechnology) for ChIP was used, with some modifications. The beads were washed and eluted, and the cross-linked protein/DNA complexes were dissociated by heating at 62°C for 2 h with shaking. The chromatin DNA was purified using a spin column. The DNA fragments were analyzed by qPCR, using primer pairs designed to amplify the region on the *TTR* promoter surrounding the specific transcription factor (TF) binding site identified in the analysis. The amount of input DNA used in each reaction was normalized using a primer set designed to recognize the exon 1 of the *GAPDH* gene before immunoprecipitation. All reactions were performed in triplicate with samples derived from three experiments.

Positive primers provided controls for successful ChIP and gene transcription. In the HSF1 ChIP experiments, we used the human or murine *Hsp70.1* promoter as a positive control. Negative primers provided a reference for the amount of nonspecific genomic DNA that coimmunoprecipitates during the procedure. In this protocol, we used ChIP-qPCR Human 1GX1A Negative Control primer (GPH 00001C (-) 01A, SA Biosciences; all primer sequences are available upon request).

Real-time qPCR. qPCRs were performed in a total volume of 10 μ l containing 1 μ g of reverse-transcribed RNA, 5 μ l of FastStart Univ Syber Green Master (Rox) (Roche), 300 nM forward and reverse primers. The primers to amplify the human genes were as follows: *β -Actin*, forward 5'-CCATCATGAAGTGTGACGTGG-3' and reverse 5'-GTCCGCCTAGAAGCATTGCG-3'; *TTR*, forward 5'-ATGGCTTCTCATCGTCTGCT-3' and reverse 5'-TGTCATCAGCAGCCTTTCTG-3'; *HSP90*, forward 5'-ACCGATTGGTGACATCTCCATGCT-3' and reverse 5'-CCAGGTGTTCTTGTGCTGCCATGT-3'; *HSP40*, forward 5'-CCCTCATGCCATGTTTGCTGAGTT-3' and reverse 5'-CCAAAAGTTCA CGTTGGTGAAGCCA-3'; *HSP70*, forward 5'-AGAGCCGAGCCGACAGAG-3' and reverse 5'-CACCTTGCCGTGTTGGAA-3'; *HSF1*, forward 5'-CCGCGGGAGCATAGACGAGAG-3' and reverse 5'-GACGGAGCGGGGGCAGGTTCACT-3'. The primers used to amplify the mouse genes were as follows: *β -Actin*, forward 5'-CAACGAGCGTTCGGATG-3' and reverse 5'-GCCACAGGATTCCATACCCA-3'; *TTR*, forward 5'-AAAAGACCTCTGAGGGTCTCT-3' and reverse 5'-GGTACAATGGGATGCTACTGC-3'; *HSF1*, forward 5'-AGTGGGAACAGCTTCCACG-3' and reverse 5'-CCACGCAAGAAACAAGGATGC-3'. qPCR amplifications were performed using the Opticon Monitor 3 Detection System (Bio-Rad), and

the data were analyzed with iCycler iQ software (Bio-Rad).

Measurement of TTR production in cultured cells by ELISA. Cells were grown to a density of $\sim 8 \times 10^6$ cells/15 cm plate. Medium and detached cells were removed, and the cell layer washed twice with PBS. Reduced-serum OPTI-MEM was added, and the cells were incubated at 37°C for 24–48 h. The medium from SH-SY5Y cells was collected, centrifuged ($1000 \times g$, 10 s, 4°C) to pellet-detached cells and large debris, then concentrated by Millipore Ultra-4 centrifugal filter units ($4000 \times g$, 30 s, 4°C). No media concentration step was necessary for HepG2 or HuH-7 cells. ELISA plates (Immulon 4 HBX 96-well plates) were coated with rabbit anti-human TTR antibody (Dako A0002, 1:1000) in 50 mM carbonate buffer, pH 9.6, overnight at 4°C (Buxbaum et al., 2008a). The plates were then washed with 25 mM Tris, 75 mM NaCl, 0.05% Tween 20, pH 7.5 (TBST) 5 times with 200 μ l and blocked with 200 μ l/well of 5% nonfat dry milk in TBST. Standards were prepared with recombinant wild-type (WT) human TTR diluted in blocking buffer. Samples were diluted in blocking buffer if necessary. The standards and samples were added to each well in triplicate (100 μ l), covered, and incubated for 1.5 h at 37°C. Plates were then washed with TBST buffer using a SkanWasher 300. Conjugated antibody (goat anti-human prealbumin alkaline phosphatase, EY Laboratories, AA-2112-1) was diluted 1:1000, and 100 μ l was added to each well and incubated at 37°C for 1.5 h. The plate was washed with TBST and 100 μ l of NPP substrate (2 mg/ml in 10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.8) was added and the plate placed in the dark for 15–30 min and then read at 405 nm, using a Spectramax 384 Plus (Molecular Devices) and SoftmaxPro software (Buxbaum et al., 2008a). The TTR concentration was normalized for the number of cultured cells, using the amount of DNA extracted from the attached cells to provide an estimate of the amount of TTR secreted per cell per time of incubation.

Electrophoretic mobility shift assay (EMSA). EMSAs were performed to measure the DNA-binding ability of heat induced purified HSF1 (Abcam) using *in vitro* reactions containing purified recombinant HSF1 and double-stranded PCR-based oligonucleotide probes specific for HSEs in the promoter of the *hTTR* target gene (for sequence location information, see Fig. 2A). A total of 2–5 μ l of the *in vitro* heat shock reaction (250 ng purified HSF1 protein at 35°C for 30 min) was incubated in a total volume of 10 μ l at room temperature for 30 min in a buffer containing 10 mM Tris, pH 7.4, 150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, and a 40 ng double-stranded oligonucleotide. A total of 10 μ l of the EMSA reaction was loaded onto a native 6% polyacrylamide gel, and electrophoresis was performed in 0.5 \times TBE buffer at room temperature. The gel was stained with SYBR Green dye by using a fluorescence-based EMSA kit (Invitrogen). To detect total protein, the same gel was stained with SYPRO Ruby dye (Invitrogen) according to the manufacturer's protocol. Gels stained with fluorescent dyes were visualized using a Typhoon laser scanner. The proximal HSE from the human *hsp70.1* gene promoter served as a positive binding DNA probe for HSF1 protein.

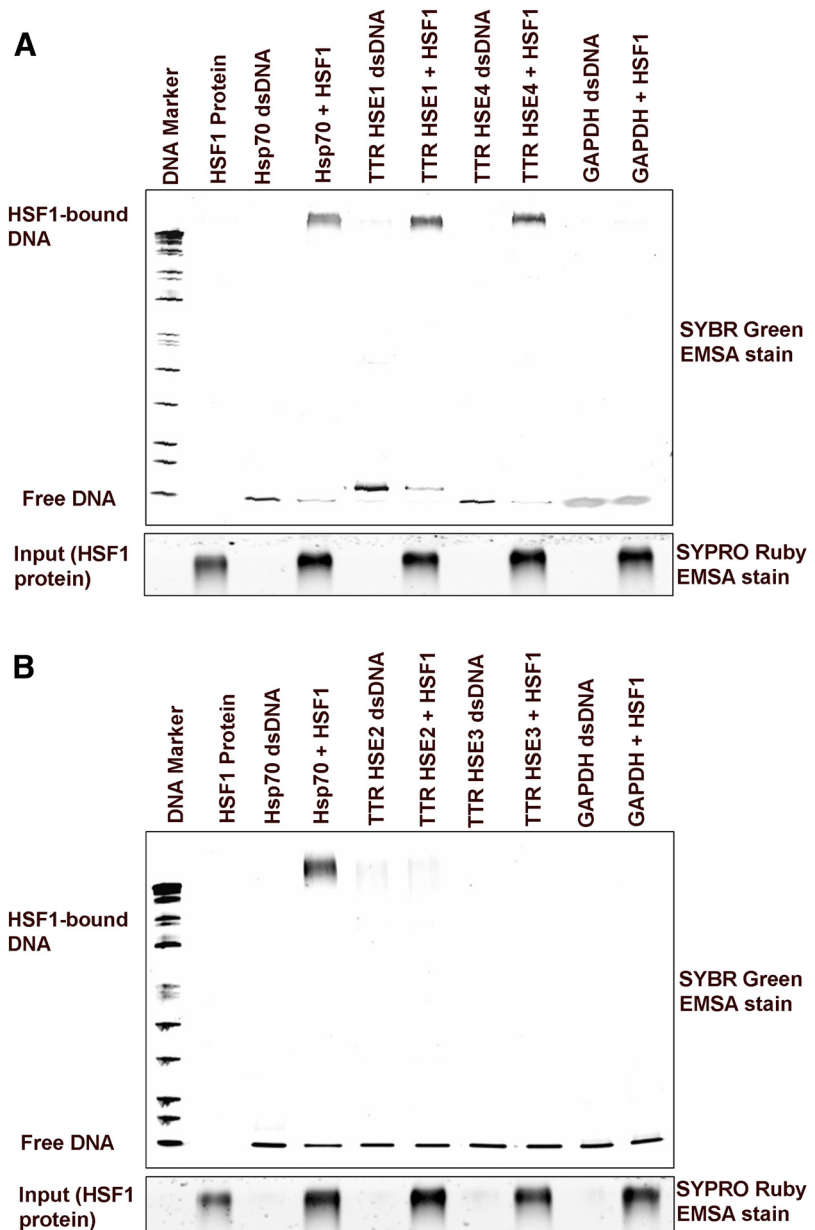


Figure 4. EMSA results obtained with 250 ng HSF1 protein and double-stranded oligonucleotides (40 ng) representing various segments of the human *TTR* promoter (shown in Fig. 2A). Image of the EMSA gel stained with SYBR Green EMSA DNA stain. The same gel was stained with SYPRO Ruby EMSA protein stain and destained before taking an image. HSE1 and HSE4 oligonucleotides have HSF1 DNA binding activity, as does the HSE oligonucleotide of the human *Hsp70* promoter (positive control) (A). B, HSE2 and HSE3 show no binding.

Western blotting of cell extracts and secreted media. Cell lysates or eluates of immunoprecipitates were boiled in SDS sample buffer for 10 min. The samples were separated on 12% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBST) for 1 h, washed with TBST, and incubated with anti-Hsp70 (Cell Signaling Technology, 1:1000), anti-Hsp90 (Cell Signaling Technology, 1:1000), anti- β -actin (Cell Signaling Technology, 1:1000), anti-HSF1 (Cell Signaling Technology, 1:1000), or anti-TTR (Dako 1:1000) primary antibodies overnight at 4°C. After washing in TBST, the membranes were incubated with antibody (IRDye secondary antibody), imaged and quantified using an Odyssey system (LI-COR Biosciences). To reprobe the membrane with different antibodies, they were stripped using stripping buffer (Thermo Scientific) for 20 min with rocking.

Immunoprecipitation (IP) of SH-SY5Y secreted protein. SH-SY5Y cells were grown to a density of $\sim 2 \times 10^6$ cells/10 cm plate. Medium and

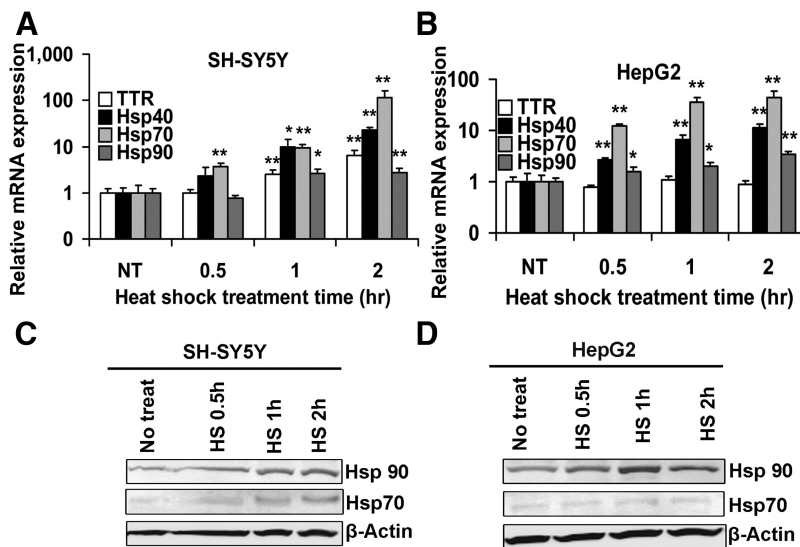


Figure 5. Effect of heat shock on *Hsp* and *TTR* expression in SH-SY5Y and HepG2 cells. SH-SY5Y (**A**) and HepG2 cells (**B**) were incubated at 42°C for the indicated periods. Samples were harvested for RNA isolation and analyzed by qRT-PCR analysis. **A**, Heat shock increases *Hsp40* (black), *Hsp70* (gray), *Hsp90* (dark gray), and *TTR* (white) mRNA levels in SH-SY5Y cells compared with untreated (NT) cells. **B**, Heat shock increases *Hsp* mRNA expression, but *TTR* mRNA levels do not change in HepG2 cells compared with the NT cells. Error bars indicate mean \pm SD. Statistical significance of differences between mRNA levels of treated and untreated cells (NT) is indicated (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$; ** $p < 0.01$. Protein extracts from heat shock-treated SH-SY5Y (**C**) or HepG2 (**D**) cells probed with antibodies to heat shock proteins.

detached cells were removed and the cell layer washed twice with PBS. Reduced-serum OPTI-MEM was added and cells incubated with celastrol or vehicle (DMSO) at 37°C for 24 h. The medium was collected, precleared by shaking with 20 μ l Protein A/G plus agarose beads (Santa Cruz Biotechnology) for 4 h at 4°C. The beads were removed and the cleared supernatants were incubated with anti-TTR (Dako) and Protein A/G plus agarose beads as per the manufacturer's protocol. The complexes were eluted and analyzed by Western blotting as above with the blots developed with the anti-TTR antibody.

Results

Computational analysis reveals HSF1 and HNF1 response elements within the *TTR* promoter

The regulatory elements for hepatic *TTR* expression have previously been established as being located within 2 kb of the transcriptional start site (TSS) of the human and murine *TTR* genes (Costa et al., 1990). Using Transcription Element Search System, Jaspur, and the Transcription Factor Search Database, we searched for TF binding sites conserved between the two genomes and sites that were either mouse or human genome-specific. We identified and scored binding sites for general TFs commonly present in the promoter regions of different genes: sites in the *TTR* gene that had been described and characterized by Costa et al. (1990) as liver-specific regulators and sites for new potential *TTR*-specific candidates based on the highest binding scores using the various search engines (Table 1).

In human and mouse genomes, there were binding sites for hepatocyte nuclear factor 1 (HNF1), a transcription factor known to be a major element in the regulation of hepatic *TTR* transcription (Fig. 1A) (Costa et al., 1990). In addition, we found four potential sequences consistent with HSEs within the 2 kb upstream of the 5' end of human *TTR* gene and a single site in the homologous region in the murine gene (Figs. 2A and 3A). All were located 5' to the promoter proximal binding sites for the transcription factors known to regulate hepatic *TTR* transcrip-

tion and 3' to the upstream hepatic *TTR* enhancer (Yan et al., 1990).

ChIP analysis of HSF1 and HNF1 binding activity within the *TTR* promoter

To test the occupancy of the computationally suggested sites in living cells, in the absence of a stress challenge, ChIP analysis of the *TTR* promoter in human cells of neuronal (SH-SY5Y) hepatic (HepG2) and cardiac (AC16) origin was performed. The two potential HNF1 binding sites identified were designated E1 and E2 (for elements 1 and 2) (Fig. 1A). HNF1 binding to the E1 site in the *TTR* promoter was readily demonstrated in the HepG2 and the SH-SY5Y cells (Fig. 1B). No binding was seen using the AC16 cardiac cell line DNA, a finding consistent with the absence of *TTR* gene transcription in cardiac tissue (Yan et al., 1990; Buxbaum et al., 2012). Hence, in these experiments, the AC16 cells served as a negative control.

The four potential HSF1 binding sites within the human *TTR* promoter were designated HSE1-HSE4 (Fig. 2A). In the SH-SY5Y cells, binding was detected at the -219 bp binding site (relative to the initiator ATG) (HSE1), with a significant signal also detected at the -1540 bp (HSE4) site. Little or no HSF1 binding was observed at the potential HSEs at -741 bp (HSE2) and -1148 bp (HSE3) upstream of the TSS, respectively. ChIP did not show binding for HSF1 at the HSEs in either the hepatic or cardiac cells (Fig. 2B). The HNF1 and HSF1 binding sites did not overlap. The endogenous *Hsp70.1* promoter, a known downstream target of HSF1, served as a positive control and showed appropriate binding in both cell lines (Fig. 2C). There was little nonspecific HSF1 binding to the human 1GX1A-negative control DNA (data not shown). There was demonstrable binding using a specific anti-RNA polymerase II antibody to DNA of all human promoters tested, including that of *TTR* (data not shown).

We also analyzed the sequence of the mouse *Ttr* promoter for conserved potential transcription factor binding sites that might be involved in neuronal *TTR* regulation. We found consensus sequence binding sites for the same two transcription factors in the murine *Ttr* promoter region (within 2 kb upstream of the TSS). The HNF1 binding site was at the previously identified -155 bp GTTACTTATTCTC site (Costa et al., 1986). In contrast to the human cells, only a single potential HSF1 binding site was identified between -572 bp to -378 bp upstream of the initiator ATG (Fig. 3A). In normal murine tissues, ChIP analysis revealed significant HSF1 binding activity to the putative binding site in DNA isolated from the hippocampus ($n = 5$) but not in that from the livers of C57BL/6J mice (Fig. 3B), indicating that HSF1 binding was a function of the tissue lineage of the cultured cells, not their derivation from human tumors (Mendillo et al., 2012). In the same extracts, HSF1 bound to the HSE in the endogenous *Hsp70.1* promoter (Fig. 3C).

Using a fluorescence-based EMSA with purified recombinant HSF1 protein, we identified the different HSE-containing oligonucleotide-protein complexes *in vitro*, further validating our *in*

in vivo results. Thus HSE1 and HSE4 (Fig. 4A), but not HSE2 and HSE3 (Fig. 4B), oligonucleotides of the human *TTR* promoter have HSF1 binding activity, as do the HSE oligonucleotides of the human *Hsp70* promoter, confirming our ChIP studies with cell and tissue extracts.

Heat shock effects on endogenous *TTR* gene expression

The functionality of the HNF1 site in the *TTR* promoter region in the hepatic regulation of the gene was established 20 years ago, but there were no data suggesting that *TTR* was an HSF1 target gene in any tissue (Costa et al., 1990). To determine whether the HSF1 binding sites identified by ChIP were functional and involved in the regulation of *TTR* expression, we exposed the neuronal and hepatic cell lines to a standard heat shock protocol. *TTR* transcription was increased in the SH-SY5Y cells after 1 h of heat shock (Fig. 5A). As a positive comparison, we measured the mRNA abundance of genes known to be HSF1 responsive (*Hsp40*, *Hsp70*, and *Hsp90*).

We performed the same experiment in the HepG2 hepatoma cells (Fig. 5B). As in the SH-SY5Y cells, there were comparable increases in the Hsp mRNAs over the course of the experiment. However, the *TTR* mRNA responses to increased temperature differed markedly in the two cell types, increasing in parallel with the Hsps in the neuronally derived SH-SY5Y cells and showing no change while the Hsp mRNAs increased in the HepG2 cells. Western blots of the heat shocked cells using antibodies to Hsp70 and Hsp90 showed increases in both proteins in a time-dependent manner (Fig. 5C,D). There were no changes in *TTR* levels in the HepG2 cells (data not shown), whereas the concentrations of *TTR* in the SH-SY5Y cells were below the limits of detection of the Western blots.

ChIP analysis showed increased occupation of HSE1 by HSF1 in response to heat treatment of the SH-SY5Y cells (Fig. 6A). The binding to HSE4 was only marginally enhanced. ChIP showed no enhanced binding to the HSEs in the HepG2 cells (Fig. 6B). Thus, in a neuronal but not in a hepatic context, *TTR* behaves as a responder to heat shock stress. HSF1 binding to the endogenous *Hsp70.1* promoter was increased in response to heat shock in both cell lines (Fig. 6C).

Celastrol induces *TTR* gene expression in neuronal cells

To determine whether a known small-molecule stimulator of the heat shock response also increased neuronal *TTR* transcription, we treated the SH-SY5Y and HepG2 cells with the plant-derived compound celastrol (Allison et al., 2001; Trott et al., 2008; Kanaiyan et al., 2011). The results (shown in Fig. 7A,B) were comparable with those obtained in the heat shock experiments.

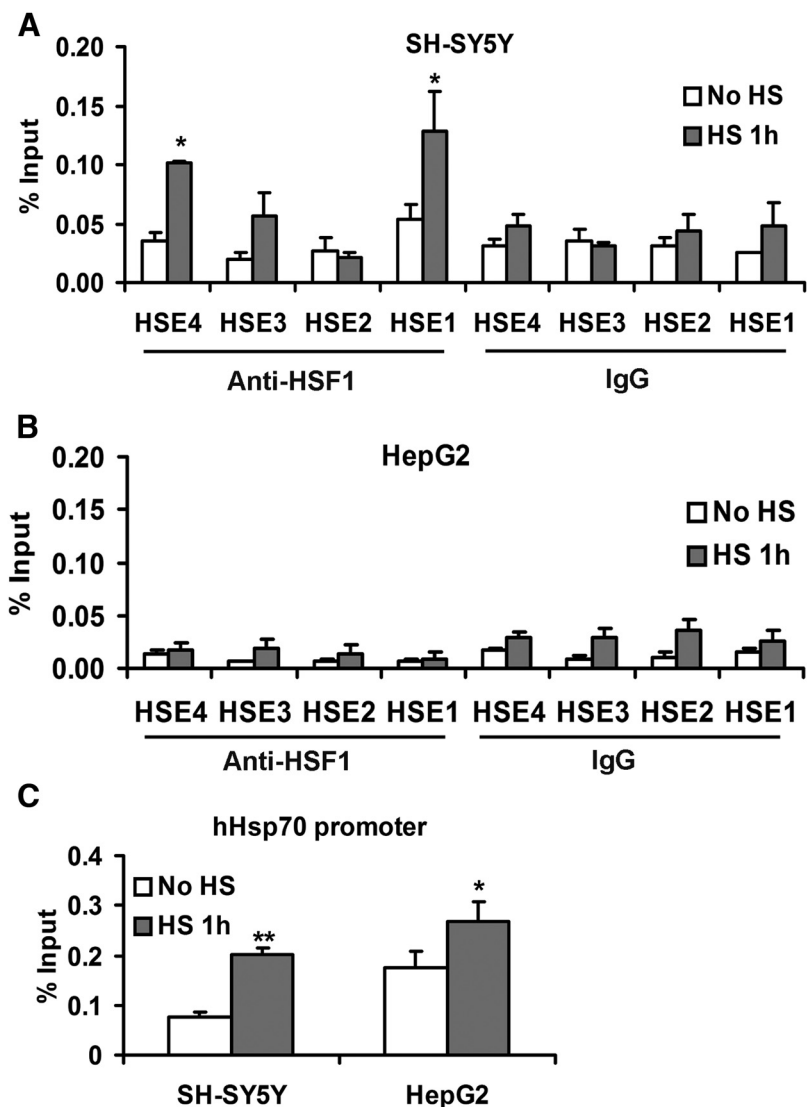


Figure 6. ChIP analysis of HSF1 binding sites in human *TTR* promoters after 1 h 42°C heat shock treatment. ChIP-qPCR results with no treatment (white) or heat shock induction (gray) are shown as percentage of input DNA in SH-SY5Y (A) or HepG2 (B) cells (1×10^6 cell equivalents per IP). C, ChIP-qPCR results with *Hsp70* promoter from same cells as target. Multivariate analysis reveals a significant effect of heat shock on HSF1-binding activity to the *TTR* promoter in the SH-SY5Y cells relative to that produced by no treatment (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$; ** $p < 0.01$. There was no significant binding to the *TTR* promoter in the HepG2 cells. Heat shock increased binding to the *HSP70* promoter in both cell lines.

Celastrol induces a “classical” transcriptional heat shock response, as indicated by increases in *HSP* mRNA levels in both SH-SY5Y and HepG2 cells. But, as in the heat shock experiments, there were different effects on *TTR* mRNA expression in the two cell types. *TTR* mRNA was induced after celastrol treatment in SH-SY5Y cells but did not change in HepG2 cells. The effect of celastrol on *TTR* mRNA expression was dose-dependent in SH-SY5Y cells.

The changes in *TTR* mRNA abundance after celastrol treatment were associated with changes in protein production. Because the absolute amount of *TTR* in the SH-SY5Y cells was too low to be detected by Western blot, we collected culture medium over the 24 h period, immunoprecipitated the *TTR*, and analyzed the precipitate by Western blotting; 1 μM celastrol resulted in a 1.4-fold increase in amount of secreted *TTR* (Fig. 7E,F). In the hepatoma cell line (HepG2), the amount of secreted *TTR* protein was significantly reduced compared with that seen with vehicle only treatment (Fig. 7F). These results indicated that, with re-

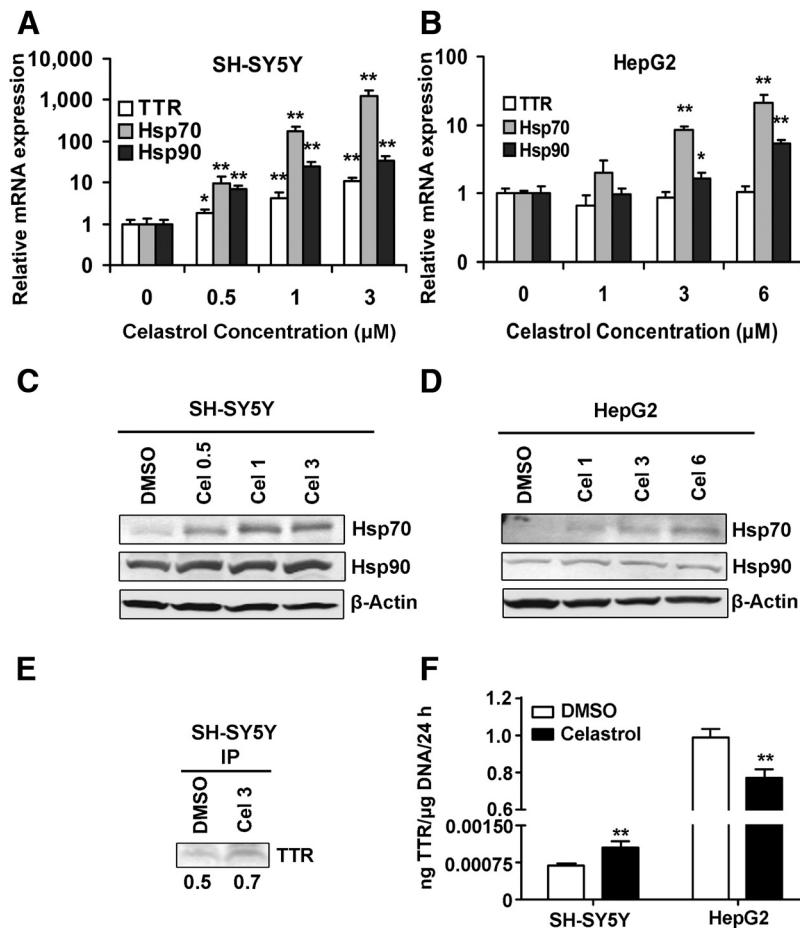


Figure 7. Dose-dependent effects of celastrol on *Hsp* (*Hsp70* and *Hsp90*) and *TTR* gene expression in SH-SY5Y and HepG2 cells. Cells were treated with celastrol at indicated concentrations for 24 h. Total RNA and protein were isolated, and the expression of *Hsp70* and *Hsp90*, *TTR*, and β -actin was determined by qRT-PCR. *Hsp70* and *Hsp90* protein levels were monitored by Western blot. **A**, Celastrol increases *Hsp70* (gray), *Hsp90* (black), and *TTR* (white) mRNA levels in SH-SY5Y cells. **B**, In HepG2 cells, celastrol increases *Hsp* mRNA, but *TTR* mRNA levels are unchanged. Protein extracts from celastrol-treated SH-SY5Y (**C**) or HepG2 (**D**) cells probed with antibodies specific for *Hsp70* and *Hsp90* proteins. **E**, Western blot of *TTR* immunoprecipitated from the medium of the SH-SY5Y cultures after treatment with DMSO or 1 μ M celastrol for 24 h. The relative amounts of *TTR* released by the SH-SY5Y cells after the two treatments were quantified using ImageJ software. **F**, ELISA analysis of *TTR* protein levels in concentrated media from SH-SY5Y cells or unconcentrated media from HepG2 cells after celastrol treatment (1 μ M, 24 h). The data were normalized with respect to total DNA in the cell cultures after removal of the *TTR*-containing medium. As shown on the ordinate baseline, *TTR* secretion is much higher in HepG2 than in SH-SY5Y cells. However, with the same treatment, secreted *TTR* is increased in the SH-SY5Y cells but reduced (relative to DMSO treatment in the HepG2 cultures). Error bars indicate mean \pm SD. Statistical significance of differences in mRNAs and secreted *TTR* between celastrol and DMSO-treated cells is indicated (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$; ** $p < 0.01$.

spect to *TTR* expression and synthesis, responses to both heat shock and celastrol depend on the cell type.

Celastrol induces HSF1 binding to the *TTR* promoter in cultured cells and in vivo

To confirm that the effect of celastrol treatment on *TTR* expression was mediated by HSF1 binding to the *TTR* promoter, we performed ChIP. The chromatin from SH-SY5Y or HepG2 cells treated with either DMSO or celastrol for 24 h were immunoprecipitated using a polyclonal antibody against human HSF1. qPCR analysis showed that celastrol-induced HSF1 binding to the *TTR* promoter at HSE4 and HSE1 in the cultured SH-SY5Y cells (Fig. 8A) but not in the HepG2 cells (Fig. 8B). DMSO treatment had little effect on the association with any region of the *TTR* promoter (Fig. 8A,B). HSF1 binding to the endoge-

nous *Hsp70.1* promoter was increased in both cell lines in response to celastrol (Fig. 8C).

To test the effect of celastrol on HSF1 binding activity to the *Ttr* promoter *in vivo*, C57BL/6J mice were injected intraperitoneally, daily for 4 d, with 1 mg/kg of body weight of celastrol or 100 μ l of vehicle (Paris et al., 2010). One hour after the last injection, mice were euthanized and their hippocampi and livers collected. We assessed binding by measuring specific *Ttr* enrichment in ChIP. We observed an ~ 2.5 -fold increase in HSF1 binding to the *Ttr* promoter in the hippocampus and no change in hepatic *Ttr* enrichment relative to vehicle-treated animals (Fig. 9A). HSF1 binding to the endogenous *Hsp70.1* promoter was increased in both tissues, again confirming the specificity of HSF1 for the neuronal *Ttr* promoter, whereas binding to the *Hsp70* promoter was not tissue specific (Fig. 9B).

TTR expression is increased by HSF1 activation in neuronal cells

To directly test whether the increase in *TTR* expression in the SH-SY5Y cells and primary hippocampal neurons was related to an increase in HSF1, we transfected the cells with a construct designed to constitutively express HSF1 (Zuo et al., 1995) or a short hairpin HSF1 silencing construct (shHSF1) (both generously provided by Prof. R. Morimoto Northwestern University). In the SH-SY5Y cells, HSF1 increased *TTR* mRNA abundance at least threefold over the control transfections, whereas in the in non-neuronal HepG2 cells only the antisense construct specific for HSF1 was associated with increased *TTR* mRNA (Fig. 10A,B). The increased expression of *TTR* mRNA in mouse primary neurons in response to HSF1 was confirmed independently by qPCR analysis of WT (C57BL/6J) hippocampal cells transfected with a human HSF1-encoding construct (hHSF1) (Fig. 10C). qPCR showed that, as expected, HSF1 transcripts were significantly increased and *Ttr* and *Hsp* transcripts were more abundant in primary cultured hippocampal neurons after transfection with hHSF1 than in those from parallel cultures transfected with a GFP-containing vector (Fig. 10C,D). Western blots of SH-SY5Y and HepG2 cells transfected with HSF1 and control constructs showed increased HSF1, *Hsp90*, and *Hsp70* protein production in both cell lines relative to the controls (Fig. 11C,D). The amount of secreted *TTR* measured by ELISA was increased when HSF1 was overexpressed in the SH-SY5Y cells (Fig. 11A). In the HepG2 cells, *TTR* transcription and protein levels were only increased in the cells transfected with the antisense construct (Figs. 10B and 11B), suggesting that, even under “nonstress” conditions, HSF1 may have a suppressive effect on hepatic *TTR* expression.

The results were reinforced by experiments in which SH-SY5Y or HuH-7 human hepatoma cells that were stably transfected with a construct in which 2 kb of the human *TTR* promoter was used to drive a modified *Gaussia* luciferase (GLuc) gene. In SH-SY5Y cells, HSF1 activates the *TTR* promoter-GLuc reporter construct (Fig. 12A). In similarly stably transfected HuH-7 cells, shHSF1 (antisense) increased the signal of the *TTR*-GLuc reporter (Fig. 12B). These experiments were consistent with the prior observations showing that HSF1 affected *TTR* expression differently in the two cultured cell types and the liver and hippocampus *in vivo* and further documented that the *TTR* promoter region contained the sequences responsible for the tissue specificity of the response.

We performed additional independent experiments in which SH-SY5Y cells stably expressing the *TTR*-GLuc reporter were transiently transfected with a constitutively expressed mutant FK506 binding protein (FKBP) fused to a constitutively active HSF1. In the absence of the FKBP stabilizing compound Shield 1, the fusion protein is degraded (Iwamoto et al., 2010; Shoulders et al., 2013). When Shield 1, the FKBP stabilizer, was added to the SH-SY5Y cells, the active HSF1 gene was expressed and *TTR*-regulated luciferase transcription was induced. Vehicle only control experiments showed no increase in the luciferase signal (Fig. 13).

Ttr mRNA is upregulated in the hippocampus but not the liver of APP23 and APP23/*Ttr*^{-/-} mice

Increased expression of *Ttr* mRNA in A β PP transgenic mice was independently confirmed by comparing qPCR analysis of *Ttr* mRNA abundance in hippocampal cells from WT (B6), APP23 (A β PP over-expressing), and APP23/*Ttr*^{-/-} (APP23 mice on *Ttr* knock-out background). As expected, no *Ttr* signal was found in the APP23/*Ttr*^{-/-} control cells (Fig. 14A). qPCR showed that *Ttr* transcripts were twice as abundant in hippocampal cells obtained from the APP23 mice than in those from WT animals. These results were consistent with previous findings from our laboratory that *Ttr* transcripts were more abundant in primary cultured hippocampal and cortical neurons obtained from the APP23 mice than in similar preparations from WT mice (Li et al., 2011). Hepatic *Ttr* mRNA abundance was decreased in the APP23 mice (Fig. 14B).

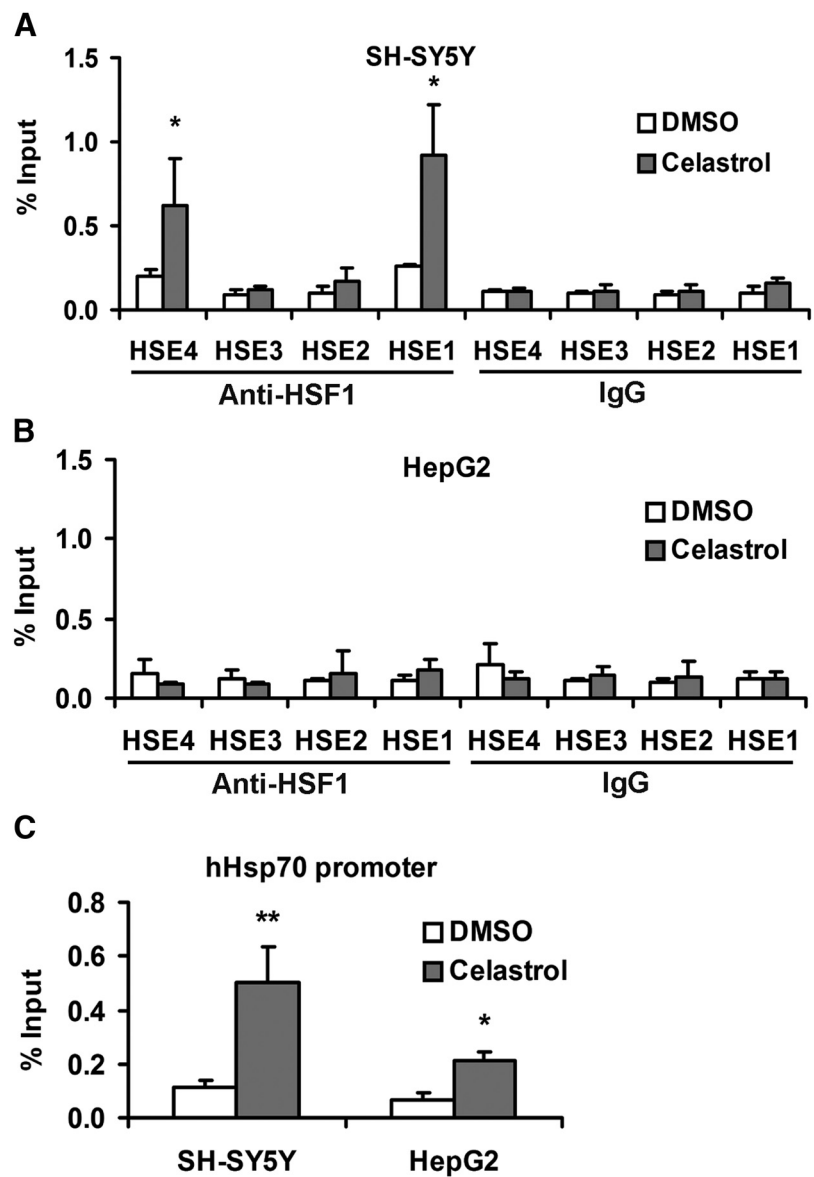


Figure 8. ChIP analysis of HSF1 binding sites in the human *TTR* promoter after celastrol treatment (1 μ M, 24 h). Celastrol increases HSF1-binding to the *TTR* promoter in human neuroblastoma but not in the hepatoma cells. ChIP-qPCR results with vehicle control (white) or celastrol induction (gray) are shown as percentage of input DNA in SH-SY5Y (**A**) or HepG2 (**B**) cells (1×10^6 cell equivalents per IP). **C**, ChIP-qPCR results from same cells with the *Hsp70* promoter as target. Multivariate analysis reveals a significant effect of celastrol on HSF1-binding activity to the *TTR* promoter relative to that produced by DMSO treatment (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$; ** $p < 0.01$.

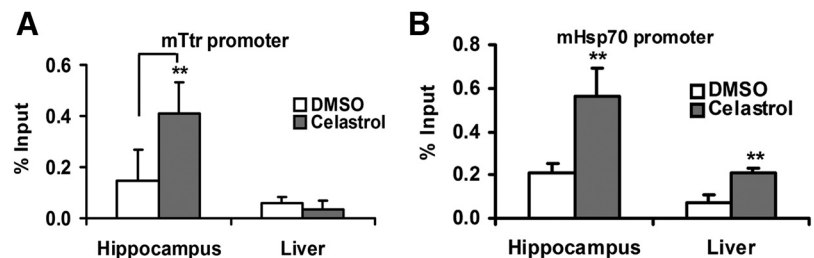


Figure 9. ChIP analysis of HSF1 binding sites in murine *Ttr* (**A**) and *Hsp70* (**B**) promoters in hippocampus and liver after *in vivo* celastrol treatment. ChIP-qPCR results from treatment of murine hippocampal or hepatic cells with vehicle control (white) ($n = 4$) or celastrol (gray bar) ($n = 4$) are shown as percentage of input. ChIP assays were performed at least in triplicate. Multivariate analysis reveals a significant effect of celastrol on HSF1-binding activity to the *Ttr* promoter in the hippocampus relative to that produced by DMSO treatment. There was also an increase in binding to the *Hsp70* promoter in both hippocampus and liver (Student's *t* test): ** $p < 0.01$.

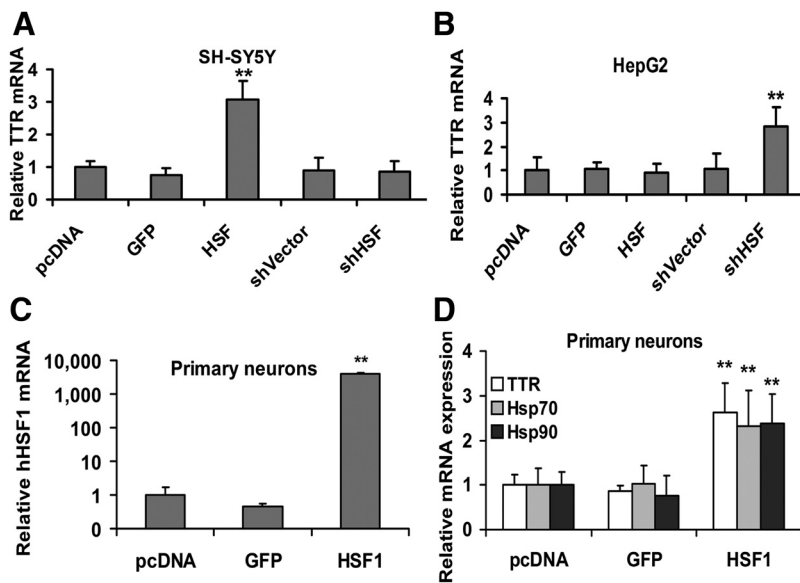


Figure 10. Analysis of *TTR* expression in SH-SY5Y, HepG2 cells, and WT C57BL/6 embryonic hippocampal neurons transiently expressing human HSF1 or shHSF1 plasmids. qRT-PCR of *TTR* mRNA (relative to endogenous actin mRNA) extracted from SH-SY5Y (**A**) and HepG2 (**B**) cells transfected with pcDNA empty vector, scrambled shRNA, a GFP construct serving as a marker of transfection efficiency (~20%, i.e., GFP positive cells vs total cells) and an HSF1 construct, SH-SY5Y (**A**) and HepG2 (**B**). The HSF1 construct significantly induces *TTR* mRNA in neuronal cells over that seen with any of the controls in the SH-SY5Y cells. Only the antisense HSF1 construct has any effect in the HepG2 cells (**B**). Quantitation of hHSF1 (**C**) or mTtr, hsp70, hsp90 mRNAs (**D**) in cultured primary neurons transfected with either pcDNA empty vector, GFP, or HSF1 ($n = 20$ embryos from 4 dams). Error bars indicate mean \pm SD. Statistical significance is indicated (from ≥ 3 independent experiments, Student's *t* test): $^{**}p < 0.01$.

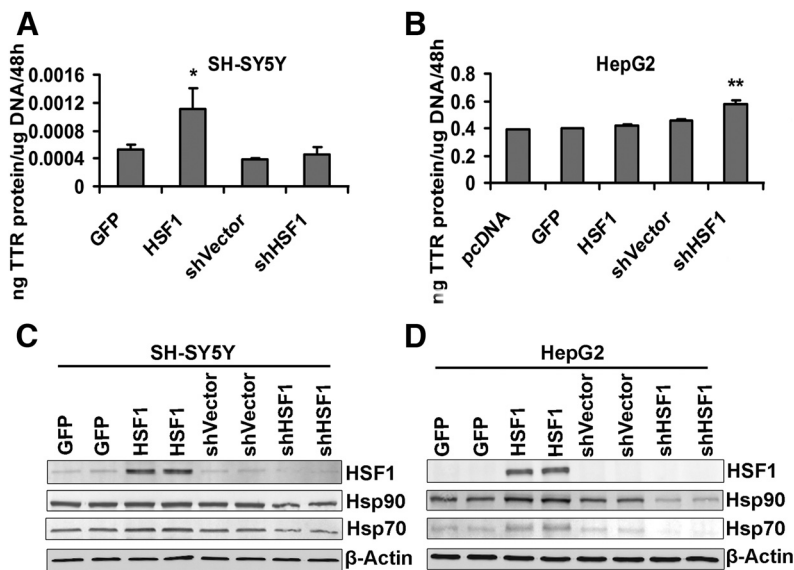


Figure 11. Analysis of *TTR* production in SH-SY5Y or HepG2 cells transiently expressing HSF1 or shHSF1 plasmids. **A**, ELISA analysis of *TTR* protein levels in concentrated media from SH-SY5Y cells shows that HSF1 transfection increases *TTR* protein levels in neuronal cells compared with control transfections. **B**, ELISA analysis of *TTR* protein levels in unconcentrated media from HepG2 cells shows that transfection of shHSF1, but not HSF1, increases *TTR* protein secretion. The results reflect the mRNA changes seen in Figure 10A, B. The data are normalized to total DNA in cell extracts. Error bars indicate mean \pm SD. Statistical significance is indicated (from ≥ 3 independent experiments, Student's *t* test): $^{*}p < 0.05$; $^{**}p < 0.01$. Western blots of HSF1 and heat shock proteins in SH-SY5Y (**C**) or HepG2 (**D**) cells transiently expressing HSF1 or shHSF1 plasmids.

Consistent with the increased *Ttr* transcription in the presence of a human $A\beta$ precursor gene, documented by measurement of APP mRNA in hippocampal neurons and hepatic cells of APP23 mice, we found that HSF1 binding to the hippocampal *Ttr* promoter in APP23 transgenic mice *in vivo* was increased twofold relative to WT mice of the same age and gender (Fig. 14C). Par-

allel studies in the livers of the same animals showed no significant HSF1 binding to the *Ttr* promoter (Fig. 14D). hAPP transcripts were significantly increased in hippocampal neurons and hepatic cells of APP23 transgenic mice (Fig. 15A, C). *HSF1* and *HSP* transcripts were also increased in APP23 hippocampi, but not in hepatic cells of the same animals relative to those of WT mice, independent of the presence of a functioning *Ttr* gene (Fig. 15B, D).

Discussion

Our interest in the regulation of *TTR* was stimulated by the observation that *in vitro* *TTR* inhibits $A\beta$ aggregation and cytotoxicity for a variety of cell targets (Li et al., 2011) and the finding of increased *TTR* expression in human AD and transgenic mouse models of human $A\beta$ deposition (Link, 1995; Schwarzman and Goldgaber, 1996; Stein and Johnson, 2002; Stein and Johnson, 2003; Buxbaum et al., 2008b; Li et al., 2011). Despite earlier reports indicating that the choroid plexus was the only source of *TTR* in the CNS (Dickson et al., 1986; Sousa et al., 2007), we thought the observations in AD were more likely to be related to neuronal synthesis of *TTR* (Stein and Johnson, 2002; Hovatta et al., 2007).

The human (and mouse) serum proteins *TTR* and albumin behave as “negative acute phase reactants” with the serum concentrations of both being reduced in the course of acute infectious or noninfectious inflammatory events (Schreiber et al., 1989; Kushner and Rzewnicki, 1994). The reductions are mediated by the inflammatory cytokines IL1, IL6, and TNF α (Wang and Burke, 2010). Costa et al. (1990) described the positive regulation of *TTR* transcription in hepatocytes by HNF1. They subsequently identified HNF3, HNF4, and HNF6 as participants in the downregulation of *TTR* expression in inflammation (Qian et al., 1995). They also noted the upstream enhancer activity of AP1 and C/EBP (Costa and Grayson, 1991; Herbst et al., 1991) but did not identify HSF1 as a regulator of *TTR* expression. Some of their experiments suggested neuronal expression of *Ttr*; however, no further characterization was reported (Yan et al., 1990).

The present data from experiments, stimulated by computational analysis of the *TTR* promoter, using ChIP, qPCR, and measurements of *TTR* secretion in several biologic models, show that the human and murine *TTR* promoter regions contain the previously identified potential binding site for HNF's and several potential consensus HSEs. The ChIP results showed occupancy of the HNF1 site in

HepG2 and to a lesser extent in the SH-SY5Y cells, confirming the earlier observations and providing a positive control for the current experiments. However, the findings in SH-SY5Y human neuroblastoma cells and hippocampal neurons differ from those in human hepatoma cell lines and murine hepatocytes. HSF1 binds to two of the potential HSEs in the *TTR* promoter in neuron-derived cells. *TTR* expression is increased in SH-SY5Y cells transfected with HSF1 constructs as is expression of the genes encoding HSP40, HSP70, and HSP90. HepG2 cells, transfected with the same construct, also show increases in *HSP* gene expression, but that of *TTR* is unaffected or reduced. A similar positive effect of HSF1 on *TTR* transcription was seen when neuronal cells transfected with a vector containing the *Gussia* luciferase gene driven by 2 kb of the normal human *TTR* promoter in the presence of an HSF1 coding sequence in the context of an FKBP-destabilized domain construct that could be activated by the pharmacologic chaperone Shield 1 (Shoulders et al., 2013). Consistent with these observations are the experiments in which the SH-SY5Y cells were subjected to heat shock or exposed to the HSF1 stimulator celastrol. With both treatments, *TTR* mRNA abundance increased, as did that of the HSF1-targeted HSPs. ChIP showed enhanced binding of HSF1 to HSE1 and HSE4 in both experiments.

Many publications have pointed out that neurons have a low heat shock or HSF1 response relative to other cell types, including glia (Blake et al., 1990; Marcuccilli et al., 1996; Batulan et al., 2003). It has also been observed that the response, whether stimulated by a standard heat shock protocol or celastrol, is more robust in cultured neuroblastoma cells (both murine and human) than when those cells have been differentiated (Hatayama et al., 1997) (Kaarniranta et al., 2002; Chow and Brown, 2007; Yang et al., 2008). However, even in the differentiated cells, the response, measured primarily in terms of Hsp70 expression, was never absent. Despite the discordance between differentiated and undifferentiated cultured neuroblastoma cells, the HSF1 response has been found to be salutary in tissue culture and mouse models of polyQ disorders and α -synuclein aggregation (Fujimoto et al., 2005; Fujikake et al., 2008; Liangliang et al., 2010; Malik et al., 2013). In addition, celastrol has been reported to reduce β -amyloid pathology in a PS1/Appsw transgenic model of AD and the G93A SOD1 model of ALS, although in the latter the effect was attributed to the anti-inflammatory activities of the compound rather than its HSF1 stimulatory properties (Kiaei et al., 2005; Trott et al., 2008; Paris et al., 2010). *TTR* expression was not measured.

Although HSF1 targets have been examined in flies, yeast, worms, and human cell lines, none of the studies in human or mouse cells has identified *TTR* as subject

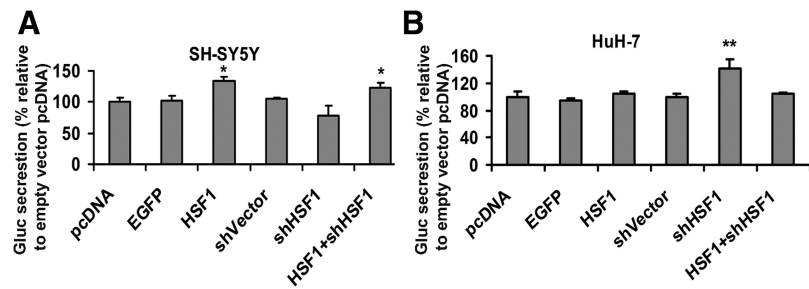


Figure 12. Effects of HSF1 on *TTR* promoter activity in SH-SY5Y and HuH-7 cells. Stable SH-SY5Y (**A**) or HuH-7 (**B**) cell line harboring the pGL4.17-*TTR* 2 kb promoter-eGLuc2 plasmid were transiently transfected with pcDNA empty vector control, HSF1, or shHSF1 plasmids. Luciferase activity was determined after 48 h. Error bars indicate mean \pm SD. Statistical significance is indicated (from ≥ 3 independent experiments, Student's *t* test): * $p < 0.05$; ** $p < 0.01$.

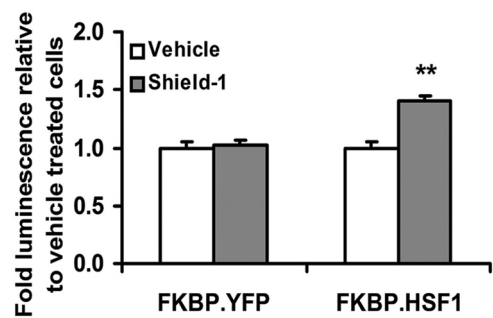


Figure 13. Constitutively active cHSF1 increases *TTR* promoter activity. SH-SY5Y cells stably transfected with *Gussia* luciferase regulated by the 2 kb *TTR* promoter were transiently transfected with FKBP.cHSF1 or FKBP.YFP and were treated for 36 h with or without the stabilizing compound Shield-1, which activates FKBP-mediated expression (4). A luminescence assay detecting luciferase in the conditioned media was performed. An increase in *TTR* promoter activity was observed when cHSF1 was activated. Error bars indicate mean \pm SD; $n = 3$. ** $p < 0.01$.

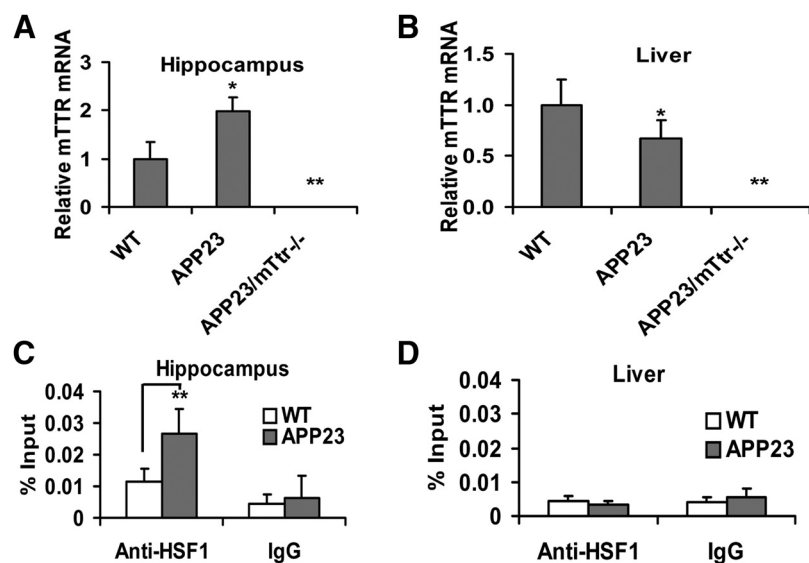


Figure 14. *Ttr* expression and HSF1 binding to the *Ttr* promoter in APP23 transgenic mice. **A**, *Ttr* mRNA was analyzed in hippocampal cells from WT ($n = 4$), APP23 ($n = 6$), and APP23/*mTtr*^{-/-} ($n = 3$) mice. **B**, Quantification of *Ttr* mRNA in hepatic cells from WT (6), APP23, and APP23/*mTtr*^{-/-}. **C**, HSF1 binding to the *Ttr* promoter in the hippocampus of APP23 mice ($n = 6$) compared with WT mice ($n = 4$) by ChIP assay. **D**, ChIP assays of HSF1 binding to *Ttr* promoter in hepatic cells of APP23 ($n = 6$) and WT mice ($n = 4$). The controls include input DNA before immunoprecipitation and a normal mouse IgG precipitation. Error bars indicate mean \pm SD. Statistical significance is indicated compared with WT mice: * $p < 0.05$; ** $p < 0.01$.

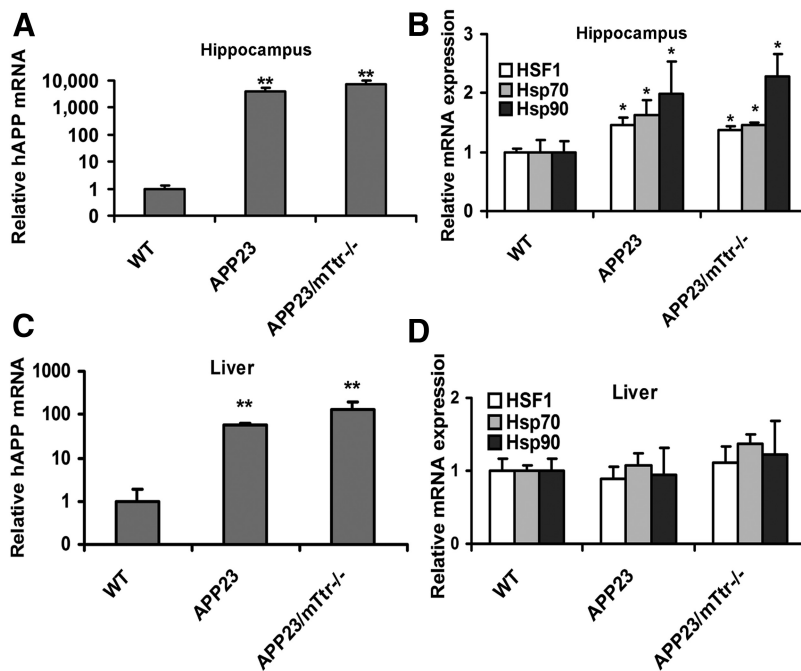


Figure 15. HSF1 and heat shock transcripts were expressed differently in hippocampal and hepatic cells of APP23 mice. **A**, *hAPP* mRNA was analyzed in WT ($n = 7$), APP23 ($n = 6$), and APP23/*mTtr*^{-/-} ($n = 5$) hippocampal cells. **B**, *HSF1* and heat shock gene mRNAs were analyzed in WT ($n = 7$), APP23 ($n = 6$), and APP23/*mTtr*^{-/-} ($n = 5$) hippocampal cells. **C**, *hAPP* mRNA was analyzed in WT ($n = 7$), APP23 ($n = 6$), and APP23/*mTtr*^{-/-} ($n = 5$) hepatic cells. **D**, Quantitation of *HSF1* and heat shock gene mRNAs in WT (B6), APP23, and APP23/*mTtr*^{-/-} hepatic cells. Error bars indicate mean \pm SD. Statistical significance of the differences between APP23 and WT mice is indicated in each panel: * $p < 0.05$; ** $p < 0.01$.

to regulation by HSF1 (Hahn et al., 2004; Trinklein et al., 2004; Page et al., 2006). The clear identification of functional binding sites in the *TTR* gene recognized by HSF1 in neuron-derived cells explains the effects in the SH-SY5Y cells. The lack of an increase or a quantifiable decrease in the expression of *TTR* mRNA in the presence of HSF1 and our failure to detect binding of HSF1 to the *TTR* promoter in the HepG2 cells would suggest that the possible suppressive effect of HSF1 on *TTR* promoter activity in the liver is indirect, mediated through interaction with other molecules, a notion consistent with the increase in *TTR* expression and protein production observed when the hepatoma cells are transfected with an HSF1 suppressor hairpin construct. It is not likely that differential post-translational modifications in the different cell types are responsible for our observations because we performed the transfections with a constitutively activated HSF1 construct (Perisic et al., 1989; Zuo et al., 1995; Fujimoto et al., 2005; Ankar and Sistonen, 2011).

Studies in mice heterozygous for an *Hsf1* gene that has been silenced by targeted disruption and carries a mutant human *TTR* gene associated with the human disorder familial amyloidotic polyneuropathy show an increased frequency of human *TTR* deposits relative to mice with two intact *Hsf1* alleles (Santos et al., 2010). It has been suggested that the increase in deposition is related to a diminished peripheral tissue response of genes regulated by HSF1. In the context of our observations, it is also possible that increased hepatic production of the mutant human *TTR* adds to the tissue load.

HSF1 has many gene targets. It increases expression of some and decreases the expression of others. The current example seemed to be unique in that HSF1 can have different effects on the same gene (*TTR*) under the same conditions in different cell types (neurons compared with hepatocytes).

We initiated these studies prompted by our observations and those of others suggesting increased production of *TTR* in neurodegenerative diseases, particularly AD (Link, 1995; Schwarzman and Goldgaber, 1996; Stein and Johnson, 2002; Stein et al., 2004; Li et al., 2011). It is quite clear from the data depicted in Figures 14 and 15 and other experiments (Li et al., 2011) that hippocampal transcription of the murine *Ttr* gene is increased relative to its expression in WT hippocampal cells when mutant human A β PP is overexpressed. The additional observation that HSF1 mRNA is elevated in the hippocampal cells of APP23 mice with and without a functional *Ttr* gene is somewhat unexpected because HSF1 activation usually involves trimerization of HSF1 monomers rather than increased transcription, although the latter has been reported in some circumstances (Ankar and Sistonen, 2011; Xue et al., 2012). We do not believe that HSF1 is the only TF involved in the regulation of neuronal *TTR* expression. Earlier experiments suggested that secreted fragments of A β PP (sAPP α or sAPP β), the AD precursor protein, could be positive regulators of *Ttr* expression, although the mechanism was not established (Stein and Johnson, 2003; Li et al., 2010). Similarly, a recent study suggested that the AICD fragment of APP could drive *TTR* expression on an epigenetic basis (Keridge et al., 2014). These possibilities and the results of our computational analyses indicating the presence of other TF binding sites of somewhat lower probabilities are a subject of continuing interest in our laboratory. Nonetheless, the current results were unexpected and provide a point of departure for studies attempting to gain further understanding of neuronal gene expression in response to potential neurodegenerative insults.

The stimulus to HSF1 activation in AD and transgenic models of human AD is unknown. In the context of the A β hypothesis of AD pathogenesis, the assembly of oligomers intracellularly or extracellularly with subsequent formation of reactive oxygen species could generate sufficient cytoplasmic “stress” to allow trimerization and nuclear localization of HSF1. Even if the initiation of AD does not involve increased A β formation and aggregation, any etiologic event that incites cytoplasmic stress could trigger both HSF1 activation and increased levels of A β _{1–40/42}. The current data suggest one possible mechanism that can account for the increased production of *TTR* as a potential protective molecule during the course of AD, giving further credence to the idea that the many studies indicating that *TTR* can inhibit both the aggregation and toxicity of A β and its oligomers may be relevant *in vivo*.

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