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Heat shock factor HSF1 regulates BDNF gene promoters upon acute stress in the hippocampus, together with pCREB

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Abstract

Heat shock factor (HSF1) is a master stress-responsive transcriptional factor, protecting cells from death. However, its gene regulation *in vivo* in the brain in response to neuronal stimuli remains elusive. Here, we investigated its direct regulation of the brain-derived neurotrophic factor (BDNF) gene (*Bdnf*) in response to acute neuronal stress stimuli in the brain. The results of immunohistochemistry and chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) showed that administration of kainic acid (a glutamate receptor agonist inducing excitotoxity) to young adult mice induced HSF1 nuclear translocation and its binding to multiple *Bdnf* promoters

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Contributions

HF, RW, and KS wrote the manuscript and other authors edited it. FFL and KS conceived the project and designed the experiments. HF, SO, KS performed stress treatments for tissue collection. HF, ML, and KS performed ChIP-PCR. HF, AW, TO and KS performed immunohistochemistry. RW and BW performed the luciferase assays and virus activation of HSF1. DY provided the AAV-H-HB/YFP virus. HF, BW, and KS analyzed data. KS directed the project.

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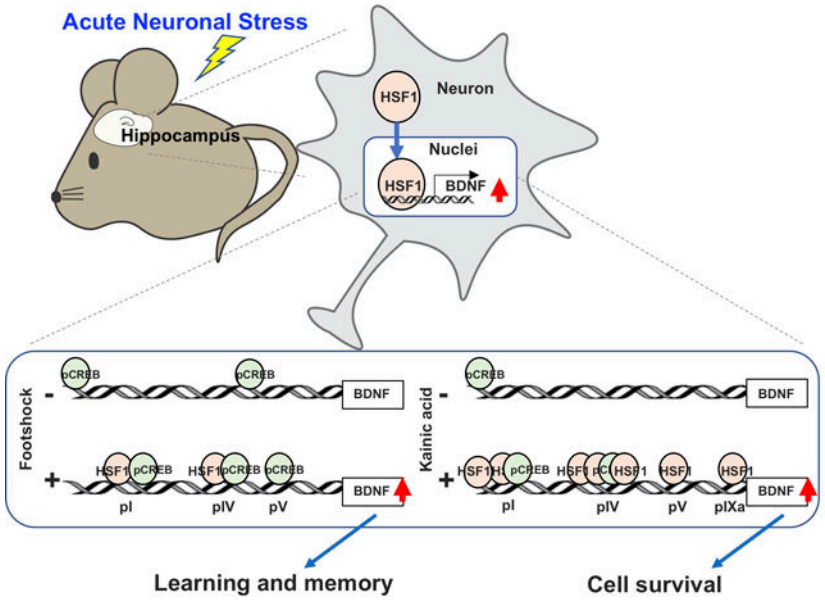
in the hippocampus. Footshock, a physical stressor used for learning, also induced HSF1 binding to selected *Bdnf* promoters I and IV. This is, to our knowledge, the first demonstration of HSF1 gene regulation in response to neuronal stimuli in the hippocampus *in vivo*. HSF1 binding sites (HSEs) in *Bdnf* promoters I and IV were also detected when immunoprecipitated by an antibody of phosphorylated (p)CREB (cAMP-responsive element-binding protein), suggesting their possible interplay in acute stress-induced *Bdnf* transcription. Interestingly, their promoter binding patterns differed by KA and footshock, suggesting that HSF1 and pCREB orchestrate to render fine-tuned promoter control depending on the types of stress. Further, HSF1 overexpression increased *Bdnf* promoter activity in a luciferase assay, while virus infection of constitutively active-form HSF1 increased levels of BDNF mRNA and protein *in vitro* in primary cultured neurons. These results indicated that HSF1 activation of *Bdnf* promoter was sufficient to induce BDNF expression. Taken together, these results suggest that HSF1 promoter-specific control of *Bdnf* gene regulation plays an important role in neuronal protection and plasticity in the hippocampus in response to acute stress, possibly interplaying with pCREB.

Graphical Abstract



Heat shock factor 1 (HSF1) is a master stress-responsive transcriptional factor, but its gene regulation in the brain remains elusive. This article firstly demonstrated HSF1 gene binding regulation of brain-derived neurotrophic factor (BDNF) in response to neuronal stress stimuli in vivo in the hippocampus. Different types of acute stress to animals produced different patterns of HSF1 biding to Bdnf promoters, while pCREB bound proximately to the HSF1 binding sites. HSF1 binding to Bdnf promoters was sufficient to induce gene transcription, suggesting the roles of HSF1 in stress-induced neuronal protection and plasticity, together with pCREB.

Keywords

HSF1; BDNF; promoter regulation; acute stress; hippocampus; pCREB

Introduction

Brain-derived neurotrophic factor (BDNF) is a major neuronal growth factor in the brain, critical for neuroprotection, synaptic plasticity, learning, and memory (Barde 1990; Figurov et al. 1996; Nagahara & Tuszynski 2011; Thoenen 1995; Poo 2001; Lu 2003; Linnarsson et al. 1997). However, its gene expression is decreased in many neurological and psychiatric disorders including Alzheimer's disease (Phillips et al. 1991; Connor et al. 1997; Murray et al. 1994; Ferrer et al. 1999; Holsinger et al. 2000; Garzon et al. 2002; Hock et al. 2000), schizophrenia (Weickert et al. 2003; Hashimoto et al. 2005; Wong et al. 2010) and depression (Dwivedi et al. 2003; Keller et al. 2010; Hing et al. 2012). Normalizing the deficiency of BDNF is a potential therapy to ameliorate the related neuronal damages. For this, understanding the mechanisms of the BDNF gene regulation is imperative.

BDNF gene regulation has been extensively studied over the past 30 years [see review in (Sakata 2011)]. BDNF gene expression is increased by neuronal activity, i.e., depolarization and Ca^{2+} influx, which is induced by kainic acid, an agonist of glutamate kainate receptors (Zafra et al. 1990), high potassium (Zafra et al. 1990; Tao et al. 1998), glutamate (Lindefors et al. 1992; Zafra et al. 1991), a GABA antagonist (bicuculine) (Zafra et al. 1991), and neuronal stimulation that induces long-term potentiation (LTP) (Patterson et al. 1992; Patterson et al. 1996). LTP is a synaptic model of memory (Bliss & Collingridge 1993). BDNF gene expression is also increased by *in vivo* conditions that increase neuronal activity in the brain, such as enriched environments (Falkenberg et al. 1992), learning tasks (Kesslak et al. 1998; Hall et al. 2000), and physical exercise (Neeper et al. 1995). Neuronal activity-induced BDNF expression is critical for neuronal survival (Ghosh et al. 1994) and hippocampal long-term synaptic plasticity (Sakata et al. 2013a).

Heat Shock Factor 1 (HSF1) is a master transcriptional factor that responds to various stress, such as heat shock, oxidative stress, and infections, and protects cells from cell death (Akerfelt et al. 2010; Anckar & Sistonen 2011; Barna et al. 2018). We recently discovered that HSF1 activation by heat shock protein 90 (Hsp90) inhibitors increased transcription of the BDNF gene (*Bdnf*) in the hippocampus and ameliorated synaptic and memory deficits in various Alzheimer's disease mouse models (Chen et al. 2014; Wang et al. 2017). Hsp90 inhibitors have been investigated as a treatment option for Alzheimer's disease (Blair et al. 2014), reducing abnormal tau protein (Luo et al. 2007; Dickey et al. 2007; Goryunov & Liem 2007; Kim et al. 2017) and Aβ toxicity (Chen et al. 2014; Wang et al. 2017; Ansar et al. 2007; Lu et al. 2009). Thus, HSF1 gene controls of *Bdnf* in response to stress may be one targeting mechanism to increase neuronal protection and resilience to stress. However, whether HSF1 directly regulates the BDNF gene was unknown. In particular, the gene regulation of HSF1 in the brain in response to neuronal stimuli remains elusive. Here, we present evidence that HSF1 responds to acute neuronal stress *in vivo* and binds to specific *Bdnf* promoters in the hippocampus. We also investigated a possible interplay of HSF1 and CREB (Ca^{2+}/cAMP Response Element Binding protein) because the HSF1-bindnding sites (HSEs) are located closely with the CREB-binding sites (CREs). CREB has been well studied for BDNF gene regulation in response to neuronal activity. Upon neuronal activity, phosphorylated (p)CREB binds to CREs in *Bdnf* promoters (Shieh et al. 1998; Tao et al. 1998) [see review (West et al. 2001)]. We found that HSF1 and pCREB bound to *Bdnf*

promoters upon stressful neural activity, which suggested that along with pCREB, HSF1 is a key transcriptional factor for neuroprotection and memory in the brain in response to stress.

MATERIALS AND METHODS

Animals

We used young adult (2–6 months old) male and female C57BL/6J mice (RRID: IMSR_JAX:000664, the Jackson Laboratory). All animal experiments were approved by the University of Tennessee Laboratory Animal Care and Use Committee (#20–0188.0) and were conducted in accordance with NIH guidelines. All animals were group-housed in a climate-controlled vivarium in a normal 12:12 hour (h)-dark-light cycle with food and water ad libitum. Mice were listed in a Microsoft Excel (RRID:SCR_016137) and sorted by the age and sex, and then arbitrarily assigned to control or stress treatment by the order (1 control and 2 stress). No blinding was performed because the measures were objectively done by a machine (e.g., qPCR).

Stress treatments

As stressors, we used kainic acid (KA) and footshock because these stressors have been shown to induce *Bdnf* mRNA levels utilizing multiple promoters (Aid et al. 2007; Lubin et al. 2008). For KA treatment, mice were administered KA (24 mg/kg, cat#0222, Tocris, cat#5806232, Biogems) by intraperitoneal (*i.p.*) injection. Control mice received saline (*i.p.*, 1 ml/kg). For footshock treatment, mice acclimated for 1 h in the testing room were placed in a fear-conditioning chamber (ActiMetrics, Wilmette, IL) for 200 sec, and then were given two footshock stimuli (set at 0.7 mA, actual delivery of 0.1–0.2 mA, 2 sec) separated by 2 mins. A control mouse was placed in the same conditioning chamber but did not receive footshock (0 mA). The mouse stayed in the chamber for additional 1 min and then returned to the home cage. Stress was given at the same time between the groups and tissues were collected between 14:00 and 18:00 h to avoid any effects of circadian rhythm; *Bdnf* gene regulation and HSF1 activity are rhythmic (Berchtold et al. 1999; Reinke et al. 2008).

Perfusion and Immunohistochemistry

To assess HSF1 activation by nuclear translocation, immunohistochemistry was performed as described previously (Jha et al. 2011; Sakata et al. 2013b). Briefly, at the time indicated after KA and footshock treatment, mice received *i.p.* injections of 90 mg/kg of ketamine and 10 mg/kg of xylazine for anesthesia, and were euthanized by transcranial perfusion with 4% paraformaldehyde fixative. The brain was cryoprotected, frozen, and then sliced using a cryostat (Leica, CM1850). Free-floating immunohistochemistry was conducted using primary antibodies of rabbit anti-HSF1 (1:200; cat#4356, Cell Signaling Technology, RRID: AB_2120258), mouse anti-NeuN (1:1000; cat#MAB377X, Millipore), and rabbit anti-pCREB (1:1000, cat#9198, Cell Signaling Technology, RRID:AB_2561044), and secondary antibodies, anti-rabbit Alexa Fluor 594 (1:500; cat#A32740, Invitrogen, RRID: AB_2762824) and anti-mouse Alexa Fluor 488 (1:500; cat#A32723, Invitrogen, RRID:AB_2633275). Images ware acquired on a microscope (IX50, Olympus) equipped with a video camera or on confocal microscope (FV1000, Olympus).

Promoter Sequence Analysis

We obtained the mouse genomic DNA sequence including *Bdnf* promoter regions from https://www.ncbi.nlm.nih.gov/gene/12064, and analyzed the promoter sequence, i.e., 1,500bp upstream from transcription starting site of each *Bdnf* exon using JASPAR (Portales-Casamar et al. 2010).

Chromatin immunoprecipitation (ChIP)-quantitative (q)PCR

To examine HSF1 binding to *Bdnf* promoters, ChIP-qPCR was conducted. Two hippocampi from one mouse were used for one CHIP sample. Two mice, one control and one stressed, were used for one ChIP experiment. A total of 8 independent ChIP experiments were conducted per treatment group. One hour (or indicated time) after KA or foot-shock treatment, mice were given isoflurane (1–3 mL/L vapor, for ~1 min) for rapid anesthesia and sacrificed by decapitation. The hippocampus was removed and diced into ~1 mm pieces and immediately frozen on dry ice within 3 min of death and stored at −80°C until further use for ChIP.

ChIP was performed using Chromatin Immunoprecipitation Assay Kit (cat#17–295, Millipore), following the manufactures instruction. Briefly, two hippocampi per tube were incubated with 1% formaldehyde in PBS for 10 min at 37°C to cross-link nuclear proteins to the DNA. Fixation was quenched by adding glycine at a final concentration of 125 mM and by quickly washing three times with ice-cold PBS. Then the tissues were homogenized by pipetting in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0) containing protease inhibitors (1 mM PMSF, 1 ug/mL aprotinin, 1 ug/mL pepstatin, 1ug/mL leupeptin). The tissues were placed on ice for 10 min and then sonicated on ice at 30% power for 8 cycles consisting of 3 sec pulse and 1 sec rest. This resulted in genomic DNA fragments ranging in size from 200–1,000 bp. The lysates were centrifuged at 13,000 rpm for 5 min at 4°C to remove insoluble material. Ten percent of the supernatant was kept for input. The remaining supernatant was 10 fold diluted with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) containing protease inhibitors (1 mM PMSF, 1 ug/mL aprotinin, 1 ug/mL pepstatin, 1ug/mL leupeptin) and then was divided three for immunoprecipitation with antibody detecting HSF1 (cat#4356, Cell Signaling Technology; cat#sc-9144, Santa Cruz Biotechnology, RRID:AB_2120276) or phospho-CREB (cat#9198, Cell Signaling Technology), or nonimmune rabbit IgG (cat#2729, Cell Signaling Technology, RRID:AB_1031062) as a negative control and incubated overnight at 4°C. The quality of the antibody and immunoprecipitation were verified by Western blotting (Supplementary Fig. 1). Chromatin-antibody complexes were collected with protein A agarose beads (cat#16–157C, Millipore) and were washed sequentially with low-salt, high-salt, LiCl buffers (Millipore) once, and then TE buffer (10 mM Tris, 1mM EDTA ph 8.0) twice. Chromatin was eluted with freshly made 1% SDS/ $NAHCO_3$ buffer. Chromatin samples and input were incubated at 65°C for 4 h in high-salt conditions with shaking to reverse crosslinking. DNA fragments were then treated with proteinase K followed by extraction with phenol/chloroform/ethanol. The DNA samples were then subjected to qPCR. Primer pairs (see Supplementary Table 1) were designed to amplify the region of the predicted putative HSF1 binding sites (HSEs) in the *Bdnf* promoters (Table 1). An HSE in the *Hsp70* promoter was used as a positive control for

HSF1 binding (Abravaya et al. 1991). qPCR was carried out using SYBR Green Master Mix (cat#1725271, BioRad). The fluorescence intensity of each amplicon was measured by the real-time PCR system (Realplex2, Eppendorf). PCR conditions were 95°C for 2 min followed by 40 cycles at 95°C for 15 sec, 60°C for 15 sec, and 68°C for 20 sec. ChIP data were normalized to the input DNA from each sample and the fold differences were calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak & Schmittgen 2001). The amplicons were visualized by running on a 2% agarose gel in TBE buffer and AlphaImager™ System (Alpha Innotech).

Luciferase assay of Bdnf promoter activity

Primary cortical and hippocampal neurons were prepared from E17 embryos of Sprague Dawley rats as described previously (Chen et al. 2014). Briefly, pregnant rats were given isoflurane (1–5 mL/L vapor, for 3–5 min) for rapid anesthesia and decapitated. The embryos were decapitated and the pooled brain tissues from 4–6 pups were digested to prepare neurons. The primary neurons were maintained in neurobasal medium supplemented with 0.8 mm l-glutamine and B27. The neurons at 13 days *in vitro* (DIV13) were co-transfected with plasmids containing HSF1 constructs and plasmids containing luciferase constructs with *Bdnf* promoters for 48 hours using lipofectamine 2000 (Invitrogen). HSF1 constructs containing either wild-type *Hsf1* for HSF1 overexpression, HSF1 trimerization deletion (HSF1Δ) for blocking HSF1 nuclear translocation, or HSF1 shRNA for *Hsf1* knockdown, as described previously (Wang et al. 2017). The HSF1 trimerization deletion (HSF1Δ) plasmid was subcloned into G0345 vector (University of Iowa viral vector core). The *Bdnf* promoter-luciferase plasmids were created by inserting *Bdnf* promoters upstream of exons IV, V, and VI into pGL3-Basic vector (cat#E1751, Promega). The *Bdnf* promoters were amplified by PCR from rat genomic DNA with primers listed in Supplementary Table 2. The luciferase reporter assay was performed according to the manufacturer's instructions (cat#E2920, Promega) on a Turner Designs TD-20/20 luminometer. The promoter activity was analyzed by the luciferase activity relative to protein applied.

Adeno associated virus (AAV) treatment.

Primary rat hippocampal neurons were incubated with 1×10^5 adeno associated virus (AAV) at 7 d *in vitro* (DIV7). AAV1-H-BH (6.11×10^{12} viral genomes/mL), a constitutively active form of HSF1 (Jung et al. 2008), and AAV1-dYFP vector control (2.64×10^{12} viral genomes/mL), were gifted from Dr. Deborah Young, the University of Auckland, New Zealand. Construction of shRNA-*Hsf1* was designed using the following sequence 5'-GCCCAAGTACTTCAAGCACAA-3'. AAV-shRNA-*Hsf1* (AAV2/5CMVeGFPhU6shRNAhsf1) was packaged at the Viral Vector Core Facility, University of Iowa at the titers of 1×10^{12} viral genomes/mL. All custom-made materials will be shared upon reasonable request. After 7 days of incubation, neurons were harvested for analyses of BDNF expression.

mRNA analysis by qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen). Single-stranded cDNA was synthesized from 1 μg of total RNA using High-Capacity cDNA reverse transcription kits (cat# 4368814, Applied Biosystems). Quantitative real-time PCR was performed with RealMasterMix

SYBR ROX (5 Prime) according to the manufacturer's protocols. Primers used are listed in Supplementary Table 3.

Western blotting

To measure BDNF levels and to verify immunoprecipitation and antibody specificity, Western blotting was performed as described previously (Sakata et al. 2009; Sakata et al. 2013a; Wang et al. 2013). Briefly, eluates of immunoprecipitates or tissue lysates were boiled in SDS sample buffer for 10 min. The samples were separated on SDS-PAGE gel 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 primary antibodies [rabbit anti-HSF1 (1:1000; Cell Signaling, 4356 or Santa Cruz Biotechnology, sc-9144); rabbit anti-BDNF (1:500; Santa Cruz Biotechnology, sc-546); mouse anti-β-actin (1:10000; Sigma-Aldrich)] overnight at 4°C. After washing in TBST, the membranes were incubated with secondary antibody (anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated Abs, 1:5000; Sigma-Aldrich), imaged and quantified using Image J (NIH).

Statistical analysis

Data from two groups (e.g., control vs. treatment) were analyzed using Student-*t* test (two-tailed). Data from three groups and more were analyzed using one-way ANOVA followed by Tukey's *post-hoc* test using Prism (GraphPad Software, San Diego, CA). We removed outliers that were more than two standard deviations away from the mean, as they likely resulted from technical errors. Otherwise, no exclusion criteria were pre-determined. The Jarque-Bera normality tests were performed to analyze the data distribution for each group. Most of the data were normally distributed ($p>0.05$). The data not normally distributed were analyzed by the Mann–Whitney U test. The statistical reports are provided in Supplementary Table 4. The sample size was calculated by power analyses and based on the hypotheses. Promoters I and IV data from ChIP-PCR with kainic acid were used to estimate effect size using a priori power analyses (G*Power software) (Faul et al. 2007) with 0.8 power and 0.05 error probability. We obtained 5–8 sample numbers and thus collected N=8 per group for all ChIP-PCR data. Statistical significance was set at $p<0.05$*. Values were presented as means mean ± SEM.

Results

1. Kainic acid (KA) induced nuclear translocation of HSF1 at peak of 1 h

First, we verified whether and when HSF1 was activated by the neuronal stress stimulus, KA, *in vivo* in the hippocampus by measuring its nuclear translocation; HSF1 moves to nuclei for gene transcription upon activation (Morimoto 1998), such as by Hsp90 inhibitors (Wang et al. 2017). KA is an agonist of glutamate receptors and a potent excitant and neurotoxin, which causes seizures and neuronal death by Ca^{2+} overload, ER stress, and oxidative stress, primarily within the hippocampus (Sokka et al. 2007; Wang et al. 2005). While neuronal death by KA varies between mouse strains, C57BL/6 mice are relatively resistant to the neuronal death (McLin & Steward 2006). Thus, we used C57BL/6 mice since we hypothesized that the neuroprotection process might involve HSF1 activation.

Immunohistochemistry revealed HSF1 co-staining with NeuN, a marker of neurons, and DAPI, a maker of nuclei, in the CA1 region of the hippocampus strongest at 1 h after KA treatment (Fig. 1a). The result indicated that HSF1 was rapidly activated for nuclear translocation by the neuronal stress stimulus. Therefore, we selected 1 h after the stress stimuli to examine binding of the transcriptional factors to *Bdnf* promoters in the hippocampus.

In the same CA1 region, pCREB staining was also observed at 1 h after KA treatment (Fig. 1b), suggesting KA-induced co-regulation of these transcriptional factors in the CA1 region. KA also increased staining of HSF1 and pCREB in other hippocampal regions and the cortex, where specific cells (e.g., CA3, dentate gyrus, and cortical shallow layer neurons) show double staining with DAPI (Supplementary Fig. 2). These regions including the CA1 region corresponded with the regions showing KA-induced BDNF expression reported previously (Sakata et al. 2009; Sakata et al. 2013b), suggesting that HSF1 and pCREB were involved in the KA-induced BDNF expression. As a note, expression pattern of HSF1 and BDNF mRNA is similar in the brain (except for cerebellum) with the common high expression at the hippocampus and cortex (AllenBrainAtlas).

2. HSF1 binding sites in *Bdnf* promoters

To determine the direct HSF1 control of the BDNF gene (*Bdnf)* upon neuronal stress stimuli, we first identified HSF1 binding elements (HSEs) in the promoter regions of *Bdnf*. *Bdnf* has nine promoters to control its gene expression in both rodents and human (Aid et al. 2007; Liu et al. 2005). Each promoter drives small exon which is spliced to a common BDNF protein-coding exon (Aid et al. 2007; Sakata 2011) (Fig. 2a, top). Our sequence search has identified putative HSEs in the *Bdnf* promoter regions (Table 1). Interestingly, some HSEs are located in proximity to the CREB binding sites (CRE) (Table 1), indicating potential coregulation of these transcriptional factors for BDNF expression in response to stress-inducing neuronal stimuli.

3. HSF1 binding to *Bdnf* promoters by kainic acid

Next, we determined whether HSF1 directly controls the BDNF gene by measuring its binding to the *Bdnf* promoters using ChIP-qPCR. ChIP-qPCR detected HSF1 binding to almost all HSEs in the *Bdnf* promoters 1 h after KA administration (Fig. 2a). Quantitative analyses from 8 independent experiments showed significant increases by KA observed at pI-HSE^{-914}, pI-HSE^{-754}, pIV-HSE^{-1095}, pIV-HSE^{-417}, pIV-HSE$^{-203/-181}$, pV-HSE$^{-328/-334}$, and pIXa-HSE^{-992} (saline control vs. KA: $p<0.05$, Fig. 2b). When the same chromatin complex (input) was immunoprecipitated with an anti-pCREB antibody, qPCR detected DNA containing pI-HSE^{-914}, pI-HSE^{-754}, pI-HSE^{-122}, pIII-HSE^{-287} and pIV-HSE^{-417} (Fig. 2a), where significant increases by KA were observed at pI-HSE^{-754} and pIV-HSE^{-417} (saline control vs. KA: $p<0.05$, Fig. 2c). These results suggested that KA induced regulation of both HSF1 and pCREB at pI-HSE^{-754} and pIV-HSE^{-417}. Interestingly, pCREB binding to DNA including pI-HSE^{-914} was observed with saline injection, which was significantly decreased by KA (saline control vs. KA: $p<0.05$, Fig. 2c). The data suggested a possibility that saline injection also caused a stress response to increase the pCREB DNA binding, but pCREB was dissociated and shifted to the CRE (pI-CRE^{-701})

closed to the HSE that HSF1 bound (pI-HSE^{-754}). Collectively, these results indicated that KA increased the overall binding of HSF1 to the HSEs in multiple *Bdnf* promoters, some of which were also bound by pCREB, while the location of pCREB could be shifted among the *Bdnf* promoter regions.

4. HSF1 binding to *Bdnf* promoters by footshock

Further, we examined HSF1 binding to *Bdnf* promoters using footshock, a neuronal stress stimulus that is commonly used to form fear memory associated to the context and cue in rodents (Phillips & LeDoux 1992; Izquierdo et al. 2016). ChIP-qPCR revealed HSF1 binding to promoter DNA at *Bdnf* promoter (p)I-HSE^{-122}, pIV-HSE^{-417}, and pIXa-HSE^{-922} 1 h after footshock, while the binding at pIXa-HSE^{-922} was also observed without footshock (Fig. 3a). Quantitative analyses showed significant increases by footshock at pI-HSE^{-122} and pIV-HSE^{-417}(FS- vs. FS+: p<0.05, Fig. 3b). We verified that HSF1 binding to *Bdnf* promoter at pI-HSE^{-122} peaked at 1 h (Supplementary Fig. 3). We also verified the nuclei staining of HSF1 induced by footshock; very slight increases were observed specifically in the CA1 pyramidal neurons of the hippocampus (Supplementary Fig. 4). When the same chromatin complex was immunoprecipitated with the anti-pCREB antibody, qPCR detected DNA containing the same HSEs as HSF1 bound, at pI-HSE^{-122} and pIV-HSE^{-417} (Fig. 3a), of which levels were significantly increased by footshock (FS- vs. FS+: p<0.05, Fig. 3c). These results suggested that footshock induced regulation of both HSF1 and pCREB to these *Bdnf* promoters. The qPCR also detected increased pCREB binding to DNA containing pV-HSE$^{-328/-334}$ after footshock (Fig. 3a and 3c). As a note, these sites, pI-HSE^{-122} and pIV-HSE^{-417}/pV-HSE$^{-328/-334}$, are closely located to pCREB binding sites, pI-CRE^{-78} and pIV-CRE^{-38}/pV-CRE^{-176}, respectively (Table 1). Further, ChIP-qPCR detected DNA containing at pIII-HSE^{-283} and pIV-HSE$^{-203/-181}$ without footshock (Fig. 3c), possibly because exposure to a new context increased pCREB binding to CRE closely located to the HSEs in the *Bdnf* promoters.

5. Comparisons between different neuronal stress stimuli: footshock and KA

To understand whether stress-induced HSF1 binding to *Bdnf* promoters differ by the types of stressors, we compared the fold increases in the binding induced by KA and footshock. The fold increases in HSF1 binding to *Bdnf* promoters were generally larger by KA than by footshock, where statistically significant differences were observed at pI-HSE^{-754}, pIV-HSE^{-1095}, and pV-HSE$^{-328/-334}$ (KA vs. footshock: p<0.05, Fig. 4). These results suggested a greater dependence of HSF1 controls for the *Bdnf* gene transcription in response to a more severe stress (KA) which causes neurotoxicity, than to a milder stress (footshock). By contrast, the fold changes in pCREB binding to the *Bdnf* promoters were rather similar between these two treatments, and statistically larger by footshock than by KA at pV-HSE$^{-328/-334}$ (footshock vs. KA: p<0.05, Fig. 4). Notably, the HSE is located closely to the CREB binding site, pIV-CRE^{-38} (Table 1). These results suggest that pCREB regulates the *Bdnf* gene transcription upon mild stress for hippocampus-dependent learning and memory, while HSF1 plays a larger role in the *Bdnf* gene transcription in response to severe neurotoxic stress for neuronal protection.

One interesting note was that pCREB binding to *Bdnf* promoters containing pI-HSE^{-914} and pIV-HSE$^{-203/-181}$ decreased by either KA or footshock, with significance at pI-HSE^{-914} (Fig. 4). It is possible that pCREB was constitutively bound to the DNA region and shifted to the binding sites, which are closer to the transcription starting site in response to neuronal stimuli.

6. HSF1-mediated BDNF transcription

Previously, it has been reported that HSF1 binding to promoter DNA does not always induce transcription (Trinklein et al. 2004; Mahat et al. 2016). Therefore, we next examined whether HSF1 binding to *Bdnf* promoters indeed induced gene transcription. We created a construct containing luciferase linked to the *Bdnf* promoter IV-V regions (Fig. 5a, top) because these regions contain multiple HSEs (pIV-HSE^{-417}, pIV-HSE-$^{203/-181}$, and pV-HSE$^{-328/-334}$) to which HSF1 bound in response to KA or footshock (Fig. 2 and 3). The construct significantly increased luciferase activity when HSF1 was over expressed in primary cultured neurons (p<0.05, Fig. 5a). This activity was blocked by deletion of HSF1 transcriptional activity (HSF1Δ) which inhibits HSF1 trimerization/nuclear translocation (p<0.05, Fig. 5a). Further, downregulation of endogenous HSF1 levels by shRNA-*Hsf1* significantly reduced the luciferase activity (p<0.05, Fig. 5b). These results indicated that HSF1 indeed induced gene transcription when bound to the *Bdnf* promoter. Further, we verified that the activation of HSF1 induced transcription of the endogenous *Bdnf* gene. In rat primary cultured hippocampal neurons, direct activation of HSF1 by AAV containing constitutively active form of HSF1 (AAV-H-BH) (Jung et al. 2008) significantly increased mRNA levels of *Bdnf* exon IV (the transcript driven by promoter IV) and *Hsp70* (p<0.05 for both), and the increases were abolished when HSF1 was downregulated by AAV-shRNA-*Hsf1* (Fig. 5b). The AAV-H-BH treatment also significantly increased the protein levels of both mature- and pro-forms of BDNF (p<0.05, Fig. 5c). We also verified the specificity of the used antibody against BDNF by using BDNF knockout mice and measuring KA-induced BDNF protein levels in the hippocampus (Supplementary Fig. 1b). Together, these results indicated that HSF1 binding to the *Bdnf* promoter indeed induced transcription to increase BDNF protein levels.

Discussion

Results of this study demonstrated that: 1) HSF1 responded to neuronal stress stimuli and translocated into the nucleus in the hippocampus; 2) HSF1 bound to selective *Bdnf* promoters differently in response to different stress stimuli, and several of these HSF1 biding sites were also detected by ChIP for pCREB; and 3) activation of HSF1 was sufficient to increase *Bdnf* promoter activity and its gene and protein expression. Altogether, these findings suggest that HSF1 directly upregulates *Bdnf* in response to neuronal stress stimuli in the hippocampus, together with pCREB.

Despite the gene regulation of HSF1 has been extensively studied in various cells and organs mostly *in vitro* in response to heat shock, its gene regulation in the brain *in vivo,* particularly in response to neuronal stress stimuli, remains elusive. To our knowledge, this is the first study that showed HSF1 gene binding regulation in response to neuronal stress

stimuli *in vivo* in the hippocampus. The hippocampus is an important region required for learning and memory (Milner et al. 1998) and emotional control (Fanselow & Dong 2010), but is vulnerable to damage from various types of stress (Kim & Diamond 2002; McEwen & Magarinos 1997; McEwen 2001; Schmidt & Duman 2007). Neuronal atrophy and dysfunction in the hippocampus are observed widely in neurological and psychiatric conditions, such as Alzheimer's disease (Hyman et al. 1984; West et al. 1994), depression (Sheline et al. 1996), and schizophrenia (Lieberman et al. 2018). Previous studies have shown that HSF1 reduction causes impaired contextual memory in response to footshock (Wang et al. 2017) and depression-like behavior (Zhu et al. 2008; Uchida et al. 2011), while decreasing synapse formation and neuronal maturations in the hippocampus (Uchida et al. 2011). Our finding suggests that HSF1, a master stress-responsive transcriptional factor, may contribute to synaptic plasticity and learning in the event of stress (e. g., footshock) and to neuroprotection in the event of excitotoxicity by inducing expression of BDNF in the hippocampus. Any dysregulation of this HSF1-BDNF control may cause neuronal atrophy and dysfunction, while activating or rescuing the control may be a target mechanism to increase neuronal resilience to stress events, preventing neurological and psychiatric conditions.

1. HSF1 controls *Bdnf* promoters by neuronal activity.

Our result showed that KA increased HSF1 binding to the majority of HSEs in *Bdnf* promoters I, IV, V, and IXa, but not III, VI. This result corresponds with the previous reports that KA increases the levels of *Bdnf* mRNA driven by almost all promoters, but not by promoters III and VI (Aid et al. 2007; Lubin et al. 2007). On the other hand, footshock increased HSF1 binding to selected HSEs in *Bdnf* promoters I and IV, corresponding with the previous reports that footshock increases levels of *Bdnf* mRNA driven by promoters I and IV (Lubin et al. 2008; Mizuno et al. 2012). Our results indicate that HSF1 is a transcriptional factor directly regulating these *Bdnf* promoters for the KA- or footshock-induced transcription. Footshock has also been shown to increase levels of *Bdnf* transcription driven by promoters VI and VII (Mizuno et al. 2012). Our results of no HSEs or no change in HSF1 binding in these *Bdnf* promoters suggest that the transcription by these *Bdnf* promoters is regulated by transcriptional factors other than HSF1.

It should be noted that both KA and footshock induced HSF1 binding to *Bdnf* promoters I and IV. Promoters I and IV (classified as promoter III before 2007) have previously been characterized as activity-dependent promoters, most responsive to KA (Metsis et al. 1993; Timmusk et al. 1993) and neuronal depolarization (Tao et al. 1998; Tabuchi et al. 2000; Pruunsild et al. 2011). KA- or footshock-induced *Bdnf* transcription requires Ca^{2+} signals evoked via non-NMDAR (Zafra et al. 1991) or N-methyl-d-aspartate glutamate receptor (NMDAR) (Lubin et al. 2008), respectively. Ca^{2+} signals evoked via L-type voltage-dependent calcium channel (L-VDCC), non-NMDAR, activate both promoters I and IV, while those via NMDAR activate promoter IV (Tabuchi et al. 2000). Thus, HSF1 likely mediates the Ca^{2+} signals evoked via L-VDCC by KA and NMDAR by footshock to activate *Bdnf* promoters I and IV (Fig. 6).

The pathways of how HSF1 is activated by neuronal stimuli and Ca^{2+} signals in the brain remain unstudied. However, previous studies have identified signal pathways that activate HSF1 upon heat shock in non-neuronal cells; for example, temperature-sensitive transient receptor potential channels (e.g., TRPV) evoke Ca^{2+} signals in epithelial cells (Bromberg et al. 2013), and calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates serine 230 of HSF1 to increase its transcriptional activity in cultured cancer cells (Holmberg et al. 2001). Several other pathways (e.g., Rac1, GSK-3β, SIRT1, mTOR) also activate HSF1 upon heat shock [see review in (Torok et al. 2014; Hooper et al. 2016)]. Similar mechanisms may apply to neuronal activity-induced HSF1 activation in the brain.

2. Interplay of HSF1 and pCREB induced by neuronal activity

By contrast to the understudied HSF1 regulation by neuronal activity in the brain, pCREB regulation by neuronal activity is well established. Neuronal depolarization and Ca^{2+} influx activate CaM kinase IV to phosphorylate CREB, which binds to *Bdnf* promoter I and IV (Tao et al. 1998; Shieh et al. 1998). Our results of binding of both HSF1 and pCREB in the proximal locations in promoters I and IV (Fig. 2 and 3) suggest that these transcriptional factors co-regulate in response to stress stimuli and perhaps amplify the *Bdnf* gene induction (see the model in Fig. 6). It remains to be elucidated to what extent these transcriptional factors co-bind to the same DNA fragments and whether they functionally facilitate *Bdnf* gene transcription.

We verified the colocalization of HSF1 and pCREB specifically in the CA1 region 1 h after KA (Fig. 1b). The CA1 region corresponds to the region where KA increases levels of BDNF transcription and protein driven by promoter IV (Metsis et al. 1993; Yan et al. 1997; Sakata et al. 2013a; Rudge et al. 1998). Interestingly, in this region, Hsp70, the major HSF1-regulating neuroprotective chaperone (Zatsepina et al. 2021), is also induced by KA (Krueger et al. 1999). The hippocampal CA1 region is particularly vulnerable to KA and shows delayed cellular death (Liu et al. 1999). This cell-death vulnerability to KA depends on the mouse genetic background; the used C57/B6 mice are relatively resistant to KA-induced cell death (McLin & Steward 2006). How well the HSF1 and pCREB co-regulate BDNF gene expression may explain how well CA1 neurons survive and preserve the function in response to excitotoxic stress.

The *Bdnf* gene is also regulated by other transcriptional factors. For example, KA or neuronal activity induces binding of NF-κB to *Bdnf* promoter I (Lubin et al. 2007) and Npas4 to promoters I and IV (Bloodgood et al. 2013; Pruunsild et al. 2011). Multiple transcriptional factors may aid or inhibit *Bdnf* transcription, while numerous binding sites overlap, often being competitive for binding. It is possible that upon neuronal stress stimuli, HSF1 interplays with these transcriptional factors together with pCREB for neuronal protection and plasticity. The functional interplays can be further elucidated, which may explain how neuronal activity controls physiological (e.g., alteration of synaptic formation and plasticity, apoptosis) and pathological processes (e.g., epilepsy or ischemic injury).

3. Different types of neuronal stimuli activate HSF1 to bind to different DNA sites.

Our results showed that HSF1 responded differently to KA or footshock in the hippocampus. HSF1 responded to KA robustly in the CA1 neurons (Fig. 1) and moderately in the CA3 and dentate gyrus regions (Supplementary Fig. 2), binding to almost all HSEs in *Bdnf* promoters (Fig. 2). HSF1 responded to footshock slightly and selectively in the CA1 neurons (Supplementary Fig. 4), binding to selective HSEs in *Bdnf* promoter I and IV. The different HSF1-*Bdnf* promoter control by these stressors may originate from differences in cell responses and intracellular signaling in the CA1 neurons, and/or from differences in cell populations in the CA3/dentate gyrus regions. Whether HSF1-*Bdnf* promoter control is different depending on the sub-region of the hippocampus can be elucidated in the future. Our results also suggest that different types of neuronal stimuli differently activate HSF1-BDNF control to produce different consequences. For example, KA, which elevates intracellular Ca^{2+} levels via non-NMDA-R/L-VDCC and from ER, may robustly activate HSF1 transcription of *Bdnf* to prevent neuronal death in the hippocampus. On the other hand, footshock, which increases Ca^{2+} influx via NMDA-R, may activate HSF1 transcription of specific *Bdnf* isoforms selectively in the CA1 neurons to enhance synaptic plasticity and memory associated to the context (Fig. 6). In addition to BDNF, HSF1 can also control other neuroprotective and synaptic molecules (e.g., chaperones, PSD95, receptors) in response to neuronal stimuli. A previous study has shown that HSF1 binds to various genes (e.g., for vesicle trafficking and cytoskeletons) in response to heat shock in Huntington's disease model cells (Riva et al. 2012). Studies using over expression or knockdown of HSF1 have also shown that HSF1 can regulate many genes directly or indirectly to promote longevity, intracellular signal transduction, and neurotrophin4, as shown in *C. elegans,* cerebellar granule neurons, cultured depolarized cerebellar granule neurons, and in heated mouse muscles (Sural et al. 2019; Qu et al. 2018; Neueder et al. 2017). Depending on the types of neuronal stress stimuli, HSF1 may produce different consequences, such as neuronal survival or synaptic plasticity, by transcribing different combinations of genes. To fully understand this mechanism, the genes regulated by HSF1 in response to different neuronal stimuli are to be further studied.

4. Neuronal activity-induced HSF1 control: beyond heat shock response

Classically, heat shock is used to activate HSF1 (Brown & Rush 1996; D'Souza et al. 1998; Higashi et al. 1995) and to induce its transcription (Barna et al. 2018). However, earlier studies reported that HSF1 activation by heat shock is limited in neurons (Marcuccilli et al. 1996; Batulan et al. 2003; Brown & Rush 1999). It is possible that HSF1 in neurons is more responsive to neuronal stress stimuli, rather than to heat shock. HSF1 induction of heat shock proteins may also be limited in response to neuronal stimuli; our results showed that HSF1 bound to *Hsp70* promoter in the control conditions (Fig. 2a and 3a), and its induction in response to either KA or footshock was less compared to the induction of HSF1 to *Bdnf* promoters (Fig. 4). Our results agree with the previous studies that have shown a high threshold to activate HSF1 binding to Hsp70 promoters in rat cultured neurons (Marcuccilli et al. 1996; Batulan et al. 2003). It is possible that neuronal activity-induced HSF1 transcriptional regulation has been underestimated because of the limited changes in HSF1 responses to Hsp70 promoters. Our results of HSF1 binding to *Bdnf* promoters in response to neuronal stress stimuli in the adult hippocampus suggest the new roles of HSF1

in activity-dependent neuroprotection and synaptic plasticity, which can be a treatment target mechanism for many central nervous system (CNS) disorders.

5. Activating HSF1-BDNF axis as a target mechanism for CNS therapy

The neuroprotective roles of HSF1 have been reported in various CNS disease models (Kim et al. 2016; Das & Bhattacharyya 2015; Kondo et al. 2013; Hashimoto-Torii et al. 2014), mainly focusing on its transcribing chaperones (e.g., Hsps) and clearing toxic proteins [see reviews (Gomez-Pastor et al. 2018)]. Our results suggest expanding the roles of HSF1 in proteostasis to stress stimuli-induced neuronal protection and plasticity via *Bdnf*. Traditional view of HSF1 activation by heat shock is that HSF1 in cytosol is released from chaperones, such as Hsp90, as these chaperones shift to interact with aggregated and misfolded proteins (Hartl et al. 2011; Wu 1995; Zou et al. 1998), receives phosphorylation, and then translocates to nuclei to bind to its target promoters (Morimoto 1993). Hsp90 inhibitors, which release HSF1, increase expression of BDNF and synaptic molecules, enhance synaptic plasticity in the hippocampus, and ameliorate memory deficit in model mice of Alzheimer's disease (Chen et al. 2014; Wang et al. 2017; Thirstrup et al. 2016), while reducing aggregation of toxic proteins, i.e., huntingtin, α-synuclein and tau related Huntington's disease, Parkinson's disease and Alzheimer's disease (Luo et al. 2007; Putcha et al. 2010; Labbadia et al. 2011; Baldo et al. 2012). Our results of the activity-induced HSF1-*Bdnf* regulation suggest that it may be a good strategy to use the combination of drugs that release HSF1 from chaperones and neuronal stimuli that activates HSF1 possibly via Ca^{2+}-dependent phosphorylation, to enhance transcriptional activity of HSF1.

While the specific types of neuronal stimuli that activate HSF1 remain largely unknown in the brain, our results provided evidence on two different stimuli, KA and footshock, activated HSF1-*Bdnf* regulation. We foresee that HSF1 is activated by other neuronal stimuli that induce *Bdnf* gene transcription, such as environmental enrichment (Falkenberg et al. 1992), learning training (Hall et al. 2000), and physical exercise (Neeper et al. 1995), as well as the acute neuronal stress, such as ischemia (Schmidt-Kastner et al. 2001) and brain injury (Truettner et al. 1999). Especially, HSF1 may be strongly affected by environmental enrichment provided during early life, which provides larger and persisting induction of BDNF and neurotransmitter-related genes (Jha et al. 2016; Dong et al. 2020; Dong et al. 2018). HSF1 may also be activated by healthy diets (e.g., omega 3-fatty acids) and dietary restriction that induce BDNF (Wu et al. 2008; Duan et al. 2001; Mattson et al. 2004). Augmenting the HSF1-BDNF control by such positive behavioral and environmental conditions, together with drugs that release HSF1, may prevent neuronal loss and memory deficit caused by aging and disease conditions.

6. Possible dysregulation of HSF1-BDNF controls by chronic stress and disease conditions

Future studies can also elucidate whether any disruption in the HSF1-BDNF controls upon acute stress may cause neuronal dysfunction or memory impairment. This situation is predicted under chronic stress, which cause epigenetic modifications at *Bdnf* promoters. For example, chronic social defeat (Tsankova et al. 2006), early-life maltreatment (Roth et al. 2009), and perinatal methylmercury exposure (Onishchenko et al. 2008) modify histone

acetylation and DNA methylation to cause long-lasting repressive state of promoter IV. Reduced activity of different *Bdnf* promoters are also reported in CNS disease conditions [e.g., I, II and IV in Alzheimer's disease (Garzon et al. 2002), I, II, IV, and VI in Schizophrenia (Wong et al. 2010; Reinhart et al. 2015), II in bipolar disorders (Reinhart et al. 2015), IV in suicide subjects (Keller et al. 2010)]. Such epigenetic modifications may block binding of HSF1 and pCREB to *Bdnf* promoters in response to acute neuronal stress stimuli. We identified multiple HSEs across BDNF promoters I-IXa (Table 1). It remains to be elucidated how chronic stress and disease conditions epigenetically modify these HSEs. In addition, reduced levels of HSF1 (trans control) are observed in aged and disease conditions [e.g., AD patients and rodent models (Kim et al. 2017; Jiang et al. 2013) and Huntington's disease and aged rat striatum (Gomez-Pastor et al. 2017; Zarate et al. 2021)]. How such conditions affect acute stress-induced HSF1-BDNF control in the brain remains to be elucidated.

Conclusion

Our findings indicate that HSF1 directly regulates the BDNF gene in response to acute neuronal stress stimuli in the hippocampus, together with pCREB. The differential degrees and locations of promoter binding of HSF1 by different stress stimuli suggest that this HSF1-BDNF axis may contribute to neuronal protection and plasticity according to the types of neuronal stimuli. Future studies can elucidate the mechanisms of how this acute-stress induced HSF1-BDNF control is affected by the positive and negative behavior and environments. This may facilitate our understanding of why some people are resilient to acute stress and improve learning, and some are not, and thus be instrumental to our future therapeutic design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AAV	Adeno-associated virus
AD	Alzheimer's disease
ANOVA	Analyses of variance
BDNF	Brain-derived neurotrophic factor
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CREB	cAMP-responsive element-binding protein

Ct	Cycle threshold
ER	Endoplasmic reticulum
HSF1	Heat shock factor 1
Hsp	Heat shock protein
KA	Kainic acid
NIH	National Institute of Health
qPCR	Quantitative polymerase chain reaction
RRID	Research Resource Identifiers

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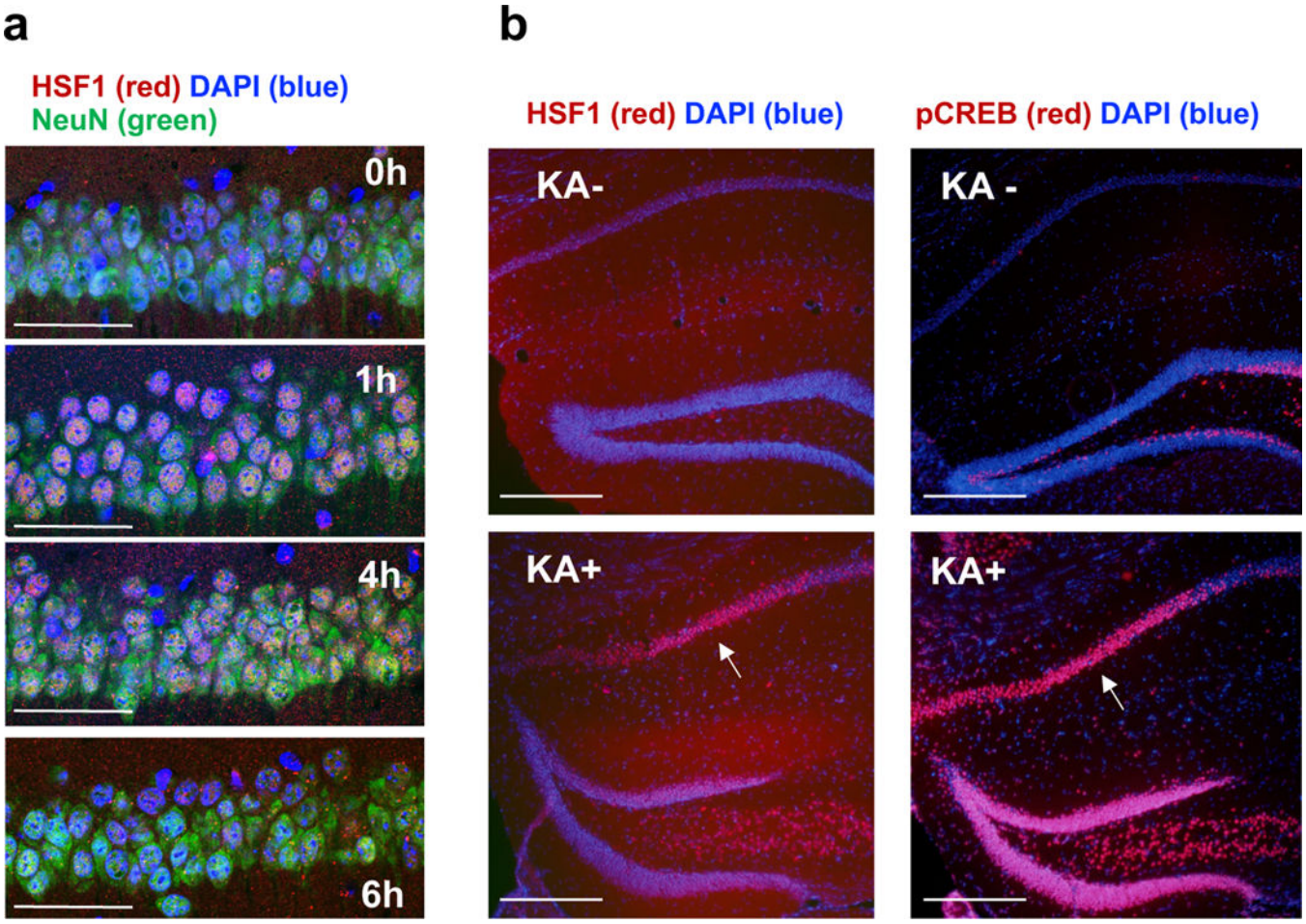

Fig. 1. HSF1 nuclear translocation in the CA1 of the hippocampus by kainic acid.

a. Time course. Immunohistochemistry was conducted with anti-HSF1 (red), anti-NeuN (green, neuronal marker), and DAPI (blue, nuclei maker) at 0, 1, 4, and 6 h after administration of kainic acid (24 mg/kg, *i.p.*). Note the greatest HSF1 nuclear staining (purple by red and blue) at hippocampal CA1 neurons 1 h after kainic acid treatment. Scale bar, 50 μm. **b.** Co-nuclear translocation of HSF1 and phosphorylated (p)-CREB at the CA1 region of the hippocampus 1 h after kainic acid treatment. Scale bar, 250 μm. HSF1: heat shock factor 1. CREB: cAMP-responsive element-binding protein. CA: cornu Ammonis. NeuN: neuronal nuclear protein. DAPI: 4’,6-diamidino-2-phenylindole.

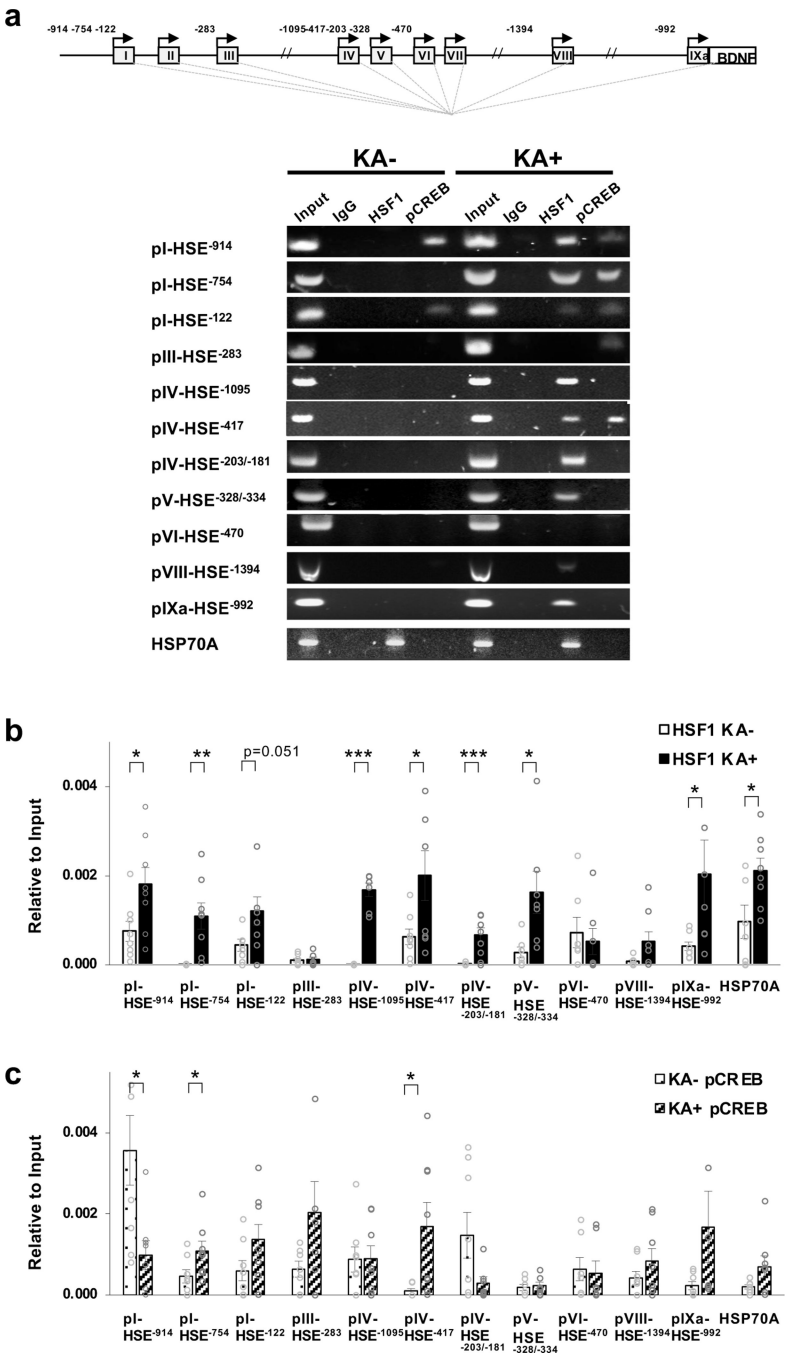

Fig. 2. HSF1 and pCREB binding to *Bdnf* promoters in response to kainic acid (KA) in the hippocampus.

ChIP-qPCR assay was conducted 1 h after KA administration (24 mg/kg, *i.p.*). Chromatin complex containing transcriptional factors and DNA was immunoprecipitated with an anti-HSF1 antibody or an anti-pCREB antibody, and then purified DNA was subjected to qPCR with the primers detecting HSF1 binding site, heat shock elements (HSEs) in *Bdnf* promoters (see Table 1). **a.** The schematic of the BDNF gene showing the location of HSEs counted from the transcriptional starting site of each exon (top) and representative gel images of ChIP-qPCR (bottom). **b** and **c.** Quantification of the levels of HSF1-bound (b)

or pCREB-bound (c) DNA containing each HSEs, normalized to input. *p<0.05, **p<0.01, ***p<0.001 by t-test between saline vs. KA. N=8 mice per group from 8 independent ChIP-qPCR experiments. HSF1: heat shock factor 1. pCREB: phosphorylated cAMP-responsive element-binding protein. BDNF/*Bdnf*: brain-derived neurotrophic factor. ChIP: chromatin immunoprecipitation. qPCR: quantitative polymerase chain reaction.

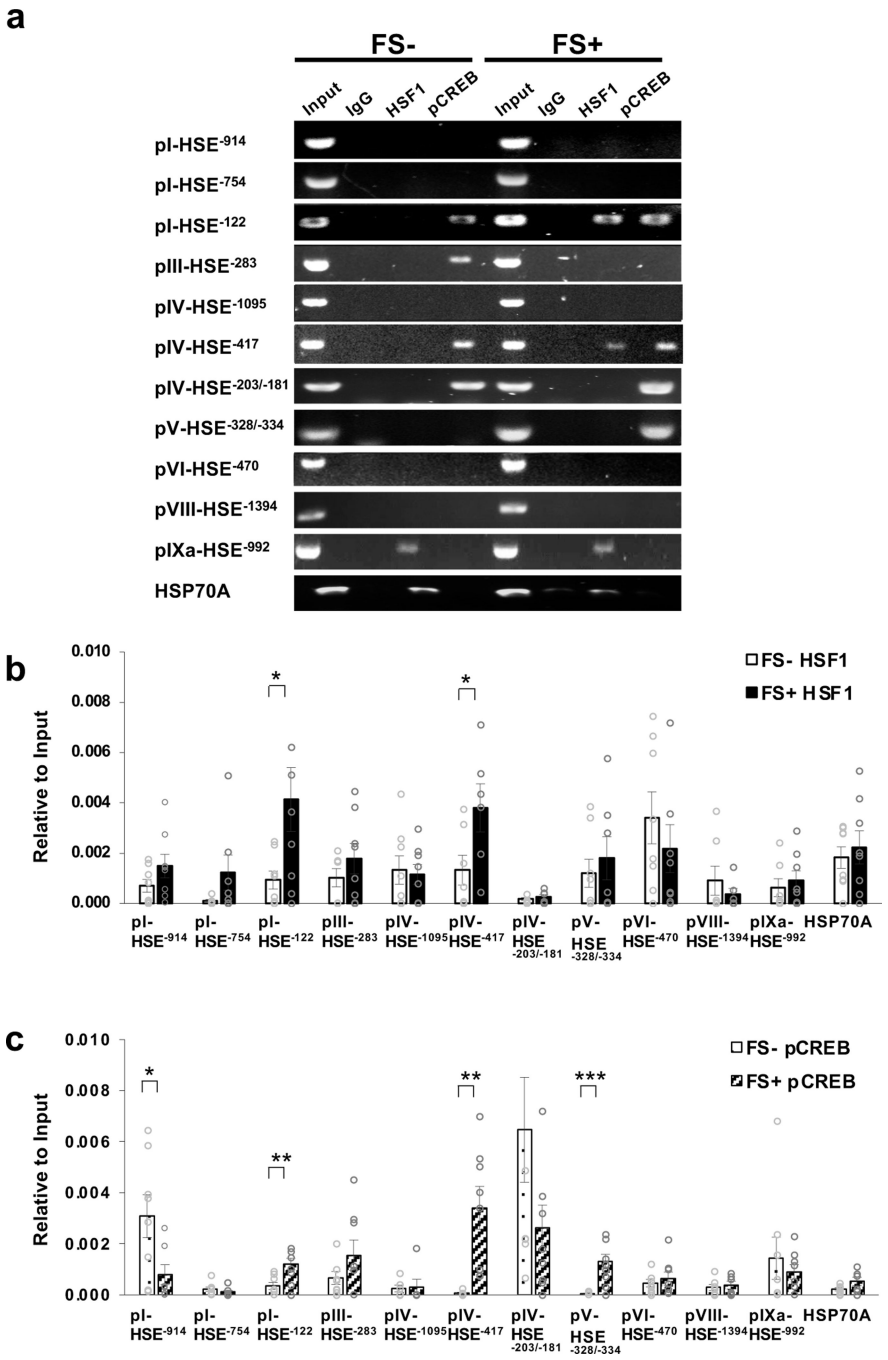

Fig. 3. HSF1 and pCREB binding to *Bdnf* promoters in response to footshock (FS) in the hippocampus.

ChIP assay was conducted 1 h after FS stress (0.2 mA x 2). **a.** Representative gel images of ChIP-qPCR. **b** and **c.** Quantification of the HSF1-bound (b) or pCREB-bound (c) DNA levels containing each HSEs, normalized to input. *p<0.05, **p<0.01, ***p<0.001 between FS- vs. FS+. N=8 mice per group from 8 independent ChIP-qPCR experiments. HSF1: heat shock factor 1. pCREB: phosphorylated cAMP-responsive element-binding protein. *Bdnf*:

brain-derived neurotrophic factor. ChIP: chromatin immunoprecipitation. qPCR: quantitative polymerase chain reaction.

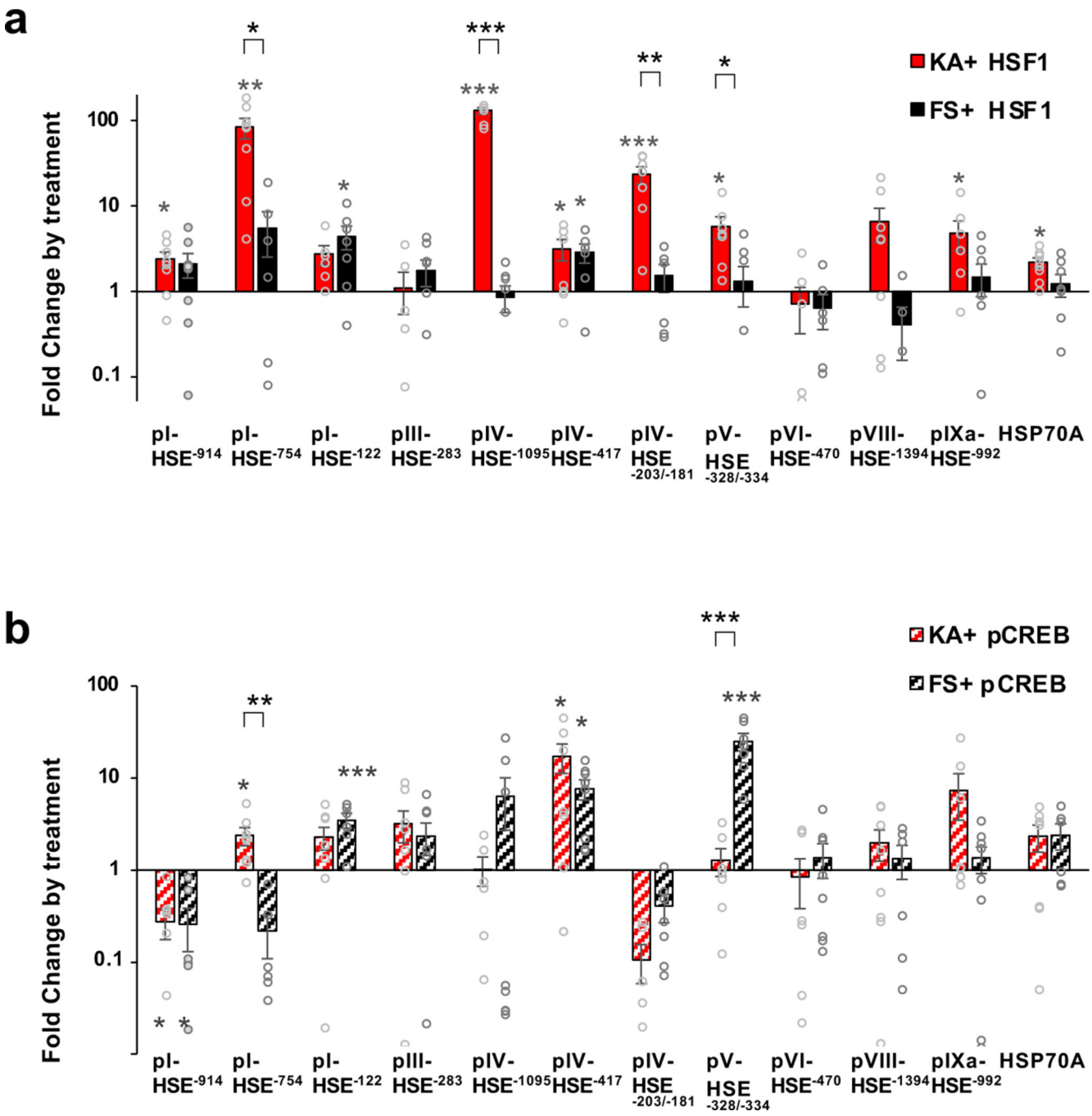

Fig. 4. Comparing effects of stressors on HSF1-binding (a) and pCREB-binding (b) to *Bdnf* promoters.

ChIP-qPCR assay was conduct 1 h after kainic acid injection (24 mg/kg, *i.p.*) or footshock (0.2mA, 2 sec, x2) to mice, measuring DNA levels containing HSEs in *Bdnf* promoters in the hippocampus. Fold changes by the treatments compared to controls, i.e., to mice with saline administration or to mice without footshock, are presented. Asterisks in gray on the column indicate statistical significance in binding compared to controls. Asterisks in black indicate statistically significant differences between KA and footshock. **P*<0.05, ***P*<0.01, ****P*<0.001, *t*-test. N=6–8 mice per group from 8 independent ChIP-qPCR experiments.

HSF1: heat shock factor 1. pCREB: phosphorylated cAMP-responsive element-binding protein. *Bdnf*: brain-derived neurotrophic factor. ChIP: chromatin immunoprecipitation. qPCR: quantitative polymerase chain reaction.

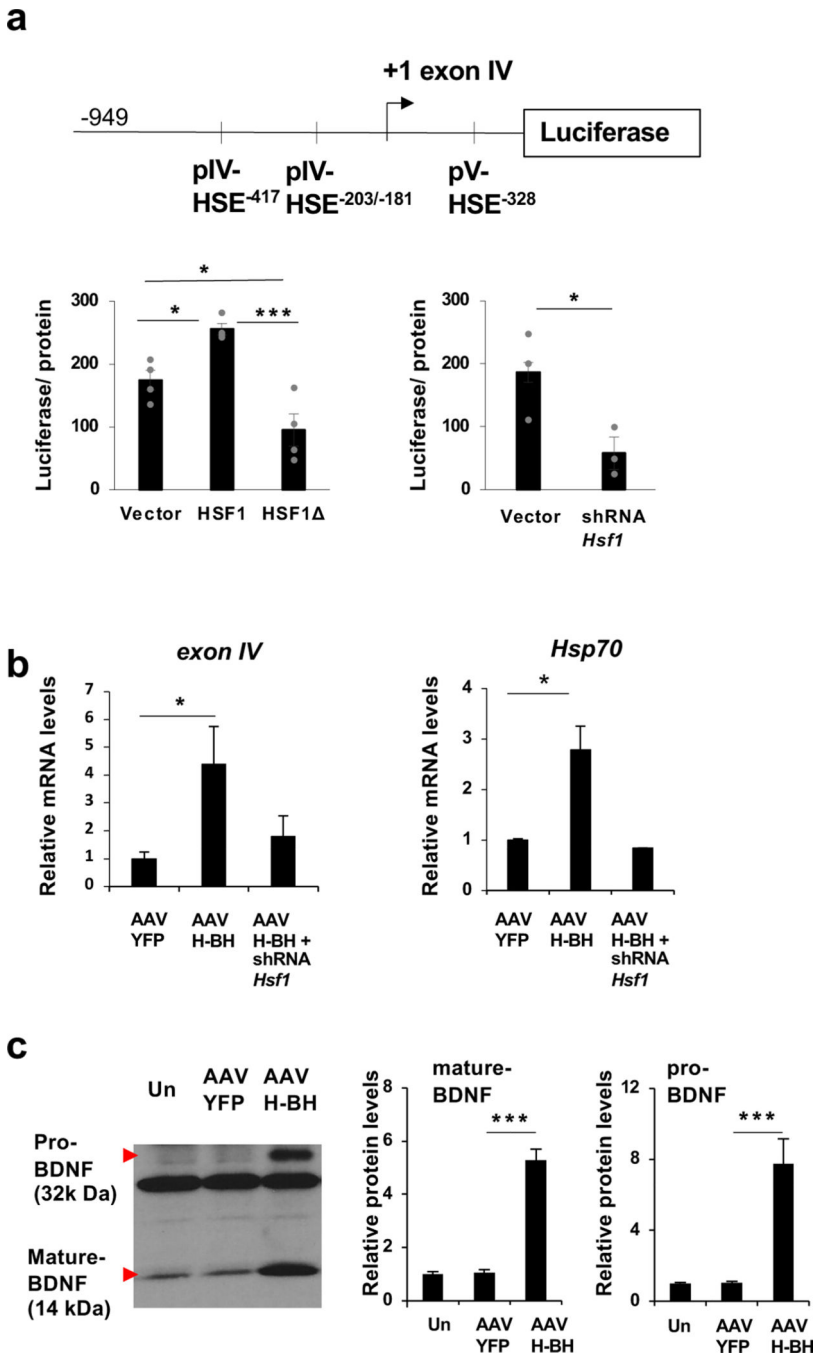

Fig. 5. HSF1 activated *Bdnf* promoter-driven transcription to increase BDNF levels.

a. Luciferase assay showing HSF1 induced transcription through *Bdnf* promoter. The schematic of the used construct containing rat *Bdnf* promoter IV-V linked to luciferase is presented at the top. Rat primary cultured neurons were co-transfected with luciferase plasmids containing the *Bdnf* promoter and plasmids with HSF1, HSF1 dimerization deletion mutant (HSF1Δ), or HSF1 knockdown (shRNA) for 48 hours. Promoter activity was analyzed with luciferase activity relative to vector control. Note that HSF1 overexpression significantly increased the *Bdnf* promoter activity, while dominant negative

HSF1 and knock-down of HSF1 significantly reduced the *Bdnf* promoter activity. N=3–4 samples per group from 2 independent assays. **b.** RT-qPCR detected HSF1 induced transcription of endogenous *Bdnf* gene *in vitro*. Primary cultured hippocampal neurons were infected with adeno-associated virus (AAV) containing constitutively active form of HSF1 (H-BH) or control yellow fluorescent protein (YFP) for 7 days. Note that increased mRNA levels of *Bdnf* exon IV (which is derived by promoter IV) and hsp70 by AAV-H-BH, of which induction was abolished by shRNA-*Hsf1*. N=3 cell preparations per group. **c.** Western blot analysis detected increased protein levels of both precursor BDNF (pro-BDNF) and mature BDNF by AAV-H-BH. Un: untreated. The Western blot data are from Dr. Bin Wang's thesis with his permission (Bin 2016). N=3 cell preparations per group. $*p<0.05$, $***p< 0.001$ by one-way ANOVA with Tukey's post hoc test, except *t*-test used for Fig. 5a right. HSF1: heat shock factor 1. BDNF/*Bdnf*: brain-derived neurotrophic factor. RT-PCR: reverse transcription quantitative polymerase chain reaction. ANOVA: analyses of variance.

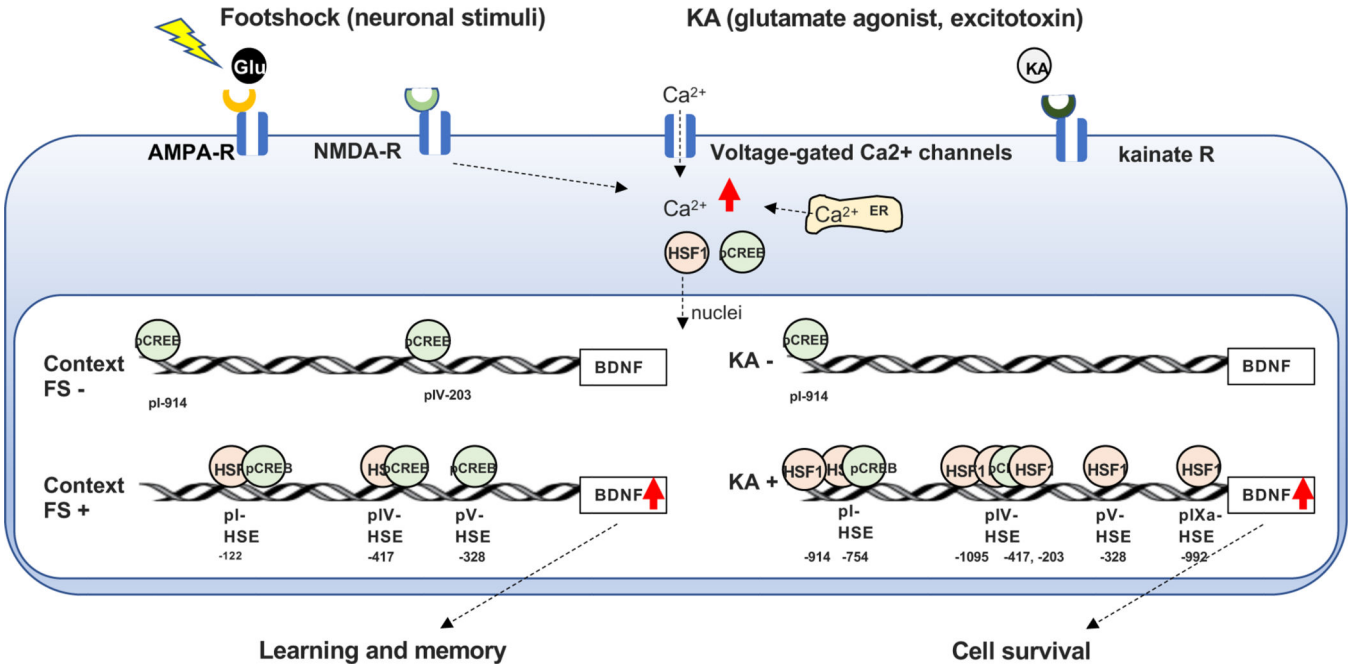

Fig. 6. A model of how HSF1 and pCREB control BDNF gene transcription in response to footshock and kainic acid (KA).

Neuronal activity by foot shock induces N-methyl-D-aspartate glutamate receptor (NMDAR)-dependent Ca^{2+} influx, nuclear translocation of HSF1, and its binding to *Bdnf* promoters I and IV. pCREB may reside at *Bdnf* promoter I and IV which may shift the binding locations close to the HSEs in promoter I and IV. HSF1, together with pCREB, induces gene transcription of BDNF, which contributes to synaptic plasticity and footshock related contextual memory. On the other hand, kainic acid (KA) acts on non-NMDA glutamate receptors (kainite/AMPAR), which activate voltage-gated calcium channel to increase Ca^{2+} influx, releases Ca^{2+} released from ER, and induces nuclear translocation of HSF1, which binds to multiple HSEs in *Bdnf* promoters, together with pCREB to induce gene transcription of BDNF, which contributes to neuroprotectant to the excitotoxity caused by KA. HSF1: heat shock factor 1. pCREB: phosphorylated cAMP-responsive element-binding protein. *Bdnf*: brain-derived neurotrophic factor. NMDA: N-methyl-D-aspartate. AMPAR: The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

Table 1.

The HSF1-biding heat shock elements (HSEs) and pCREB-biding cAMP response elements (CRE) in promoter(p) regions of the mouse BDNF gene. The promoter sequence was obtained from 1,500bp upstream of the reference genes and was analyzed using JASPAR with the threshold of 75% (except IXa HSE, 70%). The position is from the transcriptional start site (+1) of the exon that each promoter drives.

BDNF Promoter	Reference Gene	HSE sequence	Location	HSE#	Primer#	CRE sequence	Location
I	NM_007540	tttctagagatttccggaca	−914	pI-HSE^{-914}	1		
		tgactcttcttgaag *	−754 *	pI-HSE^{-754}	2	tgacctct	−701
		ttctaagaagtttcc	−122	pI-HSE^{-122}	3	tcacgtaa	−78
II	NM_001285416					tcacgtaa	−861
						tgacttca	−647
III	NM_001285419	ttacacaacattc	−283	pIII-HSE^{-283}	4		
IV	NM_001048141	agcaggttttggaag *	−1,095 *	pIV-HSE$^{-1,095}$	5	tgaggtct	−1,192
		cctccagaacctagt	−417	pIV-HSE^{-417}	6		
		tgaatttgctaggac *	−203 *	pIV-HSE^{-203}	7		
		gaaaacatctacaaa *	−181 *	pIV-HSE^{-181}	7	tcacgtca	−38
V	NM_001285420	gtctagaaccttg	−328	pV-HSE^{-328}	8	tgaggtct	−176
		gtaagagtctagaac *	−334 *	pV-HSE^{-334}	8	agaggtca	−52
VI	NM_001285417	ggaaggtgcgggaag *	−470 *	pVI-HSE^{-470}	9		
VII	NM_001316310					tgacccca	−811
						tgactcca	−543
VIII	NM_001285421	cttccagcaaaatct	−1,394	pVIII-HSE$^{-1,394}$	10	tggtgtca *	−1364 *
						ttacctca *	−1080
						ttacttca *	−1067
		tgtccaaaaagttcc	−992	pVIII-HSE^{-992}	11	tgacacct	−960
						tgacgact	−450
						tcacatca	−403
IXa	NM_001285422	tttccagctttctct	−327	pIX-HSE^{-327}	12	tgatgtaa	−758
		gttccttaagtttct	−187	pIX-HSE^{-187}	12	tgaggtat	−653

(*)Asterisk indicates that the elements are on the'- strand.'