Detection of vulnerable neurons damaged by environmental insults in utero

Masaaki Torii[a,b,c,d,1], Masanori Sasaki[e,f,g,1], Yu-Wen Chang[e,f], Seiji Ishii[a], Stephen G. Waxman[e,f], Jeffery D. Kocsis[e,f], Pasko Rakic[c,d,2], and Kazue Hashimoto-Torii[a,b,c,d,2]

[a]Center for Neuroscience Research, Children's Research Institute, Children's National Medical Center, Washington, DC 20010; [b]Department of Pediatrics, Pharmacology and Physiology, School of Medicine and Health Sciences, The George Washington University, Washington, DC 20052; [c]Department of Neurobiology, School of Medicine, Yale University, New Haven, CT 06510; [d]Kavli Institute for Neuroscience, School of Medicine, Yale University, New Haven, CT 06510; [e]Department of Neurology, Yale University School of Medicine, New Haven, CT 06510; [f]Center for Neuroscience and Regeneration Research, VA Connecticut Healthcare System, West Haven, CT 06516; and [g]Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Hokkaido 060-8556, Japan

Contributed by Pasko Rakic, December 22, 2016 (sent for review October 3, 2016; reviewed by Mark F. Mehler and Ben-Ari Yehezkel)

Development of prognostic biomarkers for the detection of prenatally damaged neurons before manifestations of postnatal disorders is an essential step for prevention and treatment of susceptible individuals. We have developed a versatile fluorescence reporter system in mice enabling detection of Heat Shock Factor 1 activation in response to prenatal cellular damage caused by exposure to various harmful chemical or physical agents. Using an intrautero electroporation-mediated reporter assay and transgenic reporter mice, we are able to identify neurons that survive prenatal exposure to harmful agents but remain vulnerable in postnatal life. This system may provide a powerful tool for exploring the pathogenesis and treatment of multiple disorders caused by exposure to environmental stress before symptoms become manifested, exacerbated, and/or irreversible.

cerebral cortex | development | environmental stress | heat shock signaling | reporter

Prenatal exposure to numerous harmful factors, ranging from chemicals and infectious agents to genetic defects, can injure developing brain cells. Most cells have intrinsic systems enabling adaptation against such damage by achieving new steady states that preserve function and viability (1). However, if the adaptive capability of individual cells is overcome by the strength of the external insult, they can be irreversibly damaged, resulting in functional defects that ultimately present as clinical deficits. A more complete understanding of the early molecular events in affected cells is required to facilitate the development of prognostic biomarkers and effective treatments that might rescue damaged cells before they become symptomatic. The ability to distinguish normal from latently damaged cells at various pre- and postnatal stages of development provides an opportunity to further advance our knowledge of pathogenic mechanisms governing neurological disorders.

The damage of neural stem cells and young postmitotic neurons during brain development by various prenatal environmental factors (such as psychological stress, drugs, smoking, alcohol, radiation, and hypoxia) may become irreversibly retained in progeny neurons and glia via epigenetic mechanisms, thereby potentially increasing the vulnerability of exposed individuals to psychiatric disorders (2, 3). That such a diverse group of risk factors cause similar disorders suggests fundamental cellular mechanisms that exert adverse effects on brain development irrespective of the type of stress (4). Such mechanisms remain poorly understood, partly because of difficulties in distinguishing differentially affected cells in the brain before damage manifests clinically (5–7). However, no tools or methods currently exist that efficiently and specifically detect vulnerable cells. The availability of an appropriate biomarker capable of identifying vulnerable neurons during various stages of brain development is needed to significantly enhance our ability to identify and study mechanisms underlying neurological disease.

One potential candidate for such a marker is the Heat Shock Factor 1 (HSF1), a part of the Heat Shock Protein (HSP) signaling pathway known for stress-induced chaperone activity (8–10). A universally conserved cytoprotective mechanism present in organisms ranging from bacteria to humans, the heat shock response is induced by multiple forms of cellular stress (8–10). HSF1 and HSPs are essential mediators of this response. Whether by heat stress or other stimuli, HSF1 is activated through sequential protein modification events including phosphorylation, sumoryzation, trimerization, and nuclear translocation. Activation then triggers the transcription of *HSP*s and other downstream target genes. More recently, HSF-1 has been identified as a key player in the initial steps of pathological brain development shortly after prenatal exposure to environmental risk factors, some of which have been implicated in the development of late-onset psychiatric disorders (4).

In the present study, we use the molecular characteristics of the HSF1–HSP pathway to generate a reporter system that enables early detection of neurons stressed by various environmental and physical insults occurring well before the manifestation of symptoms. With a primary focus on environmental exposure models, we show that reporter-labeled cells that otherwise elude identification at early times postinsult indeed exhibit altered morphological and functional properties.

Results

Validation of the HSE-RFP Reporter for Detection of HSF1 Activation. Using the cellular stress-inducible mechanisms of HSF1, we developed a reporter system (HSE-RFP) in which HSF1 binds to a Heat Shock-response Element (HSE) containing the mouse *Hsp70* promoter to transcribe a Red Fluorescent Protein (RFP) reporter (Fig. 1*A*). We first tested the specific detection of HSF1 activation by this HSE-RFP reporter system by in vivo gene

Significance

We have developed a fluorescence reporter system in mice capable of exposing neurons that survive prenatal damage but remain vulnerable in postnatal life. This reporter system can be exploited to study molecular and cellular mechanisms involved in neuronal response to stress and pathogenesis of various tissues and organs during development as well as in adulthood.

Author contributions: M.T., P.R., and K.H.-T. designed research; M.T., M.S., Y.-W.C., S.I., and K.H.-T. performed research; S.G.W. and J.D.K. contributed new reagents/analytic tools; M.T., S.G.W., J.D.K., P.R., and K.H.-T. analyzed data; and M.T., S.G.W., J.D.K., P.R., and K.H.-T. wrote the paper.

Reviewers: M.F.M., Albert Einstein College of Medicine; and B.Y., Mediterranean Institute of Neuroscience-Insern 98 (France).

The authors declare no conflict of interest.

See Commentary on page 2109.

[1]M.T. and M.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: khtorii@cnmc.org or pasko.rakic@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620641114/-/DCSupplemental.

transfer using in utero electroporation (IUE). Embryonic cortices of wild-type (CD-1) mice were electroporated at E14 with the pHSE-RFP plasmid or the pmutHSE-RFP plasmid [in which point mutations were introduced in the HSE gene to disrupt HSF1 binding to HSE (Fig. 1*A*)], together with the caHSF1 expression plasmid (pCAGIG-caHSF1), which expresses a constitutively active form of HSF1 [caHSF1 = HSF1ΔRDT (11)] and Green Fluorescent Protein (GFP) under the ubiquitous CAG promoter. In 24 h, we observed strong RFP expression with the HSE-RFP reporter (Fig. 1 *B*, *D*, and *D'*) in neural progenitor cells but not with the mutHSE-RFP reporter (Fig. 1 *B* and *C*). Similar experiments without HSF1 (coelectroporated with an empty vector) or with other forms of HSF1 [the full-length wild-type and loss-of-function mutants (R71A and R71E)] (11) also did not induce reporter expression (Fig. 1 *B* and *E*). The reporter was also confirmed to specifically detect endogenous Hsf1 activation induced by exposure to prenatal environmental stressors such as ethanol (EtOH). These results validate the specificity of the HSE-RFP reporter for detection of cells in which the HSF1-mediated stress pathway is activated.

Generation of the HSE-RFP Reporter Transgenic Mice. We next generated a transgenic mouse model of our reporter for broad application of the HSE-RFP reporter system in various organs and tissues at different ages. From the several founder lines, we obtained three that showed the RFP expression induced by caHSF1 expressed in the brain by IUE. Of note, we found that the HSE-RFP reporter transferred by IUE (Fig. 1) shows higher sensitivity in detecting HSF1 activity than in these transgenic lines (% of RFP^+ cells in caHSF1-expressing cells: IUE, 38.7 ± 5.8; Tg#B9, 9.24 ± 1.89; Tg#B11, 8.08 ± 1.77; Tg#B49, 7.10 ± 2.15; $n \geq 4$ mice). Thus, the IUE-mediated reporter assay has an advantage in detecting cells with any level of HSF1 activation, whereas the transgenic reporter mice are useful in specifically detecting cells exhibiting high levels of the HSF1-mediated stress response. Line #B9, which showed the most robust RFP expression, was used in subsequent studies. As in the IUE-mediated reporter assay (Fig. 1), little RFP reporter expression was induced in these mice by the HSF1 full-length wild-type and loss-of-function mutants (R71A and R71E) transferred by IUE.

In Vitro Detection of Cells Affected by Environmental Factors. Next, we tested the applicability of the HSE-RFP reporter for in vitro experiments using primary neuronal cultures as a model system. Primary neuronal cultures were prepared from the embryonic cerebral cortex of the HSE-RFP reporter mice at E14. To assess reporter specificity and sensitivity, neurons were exposed to either

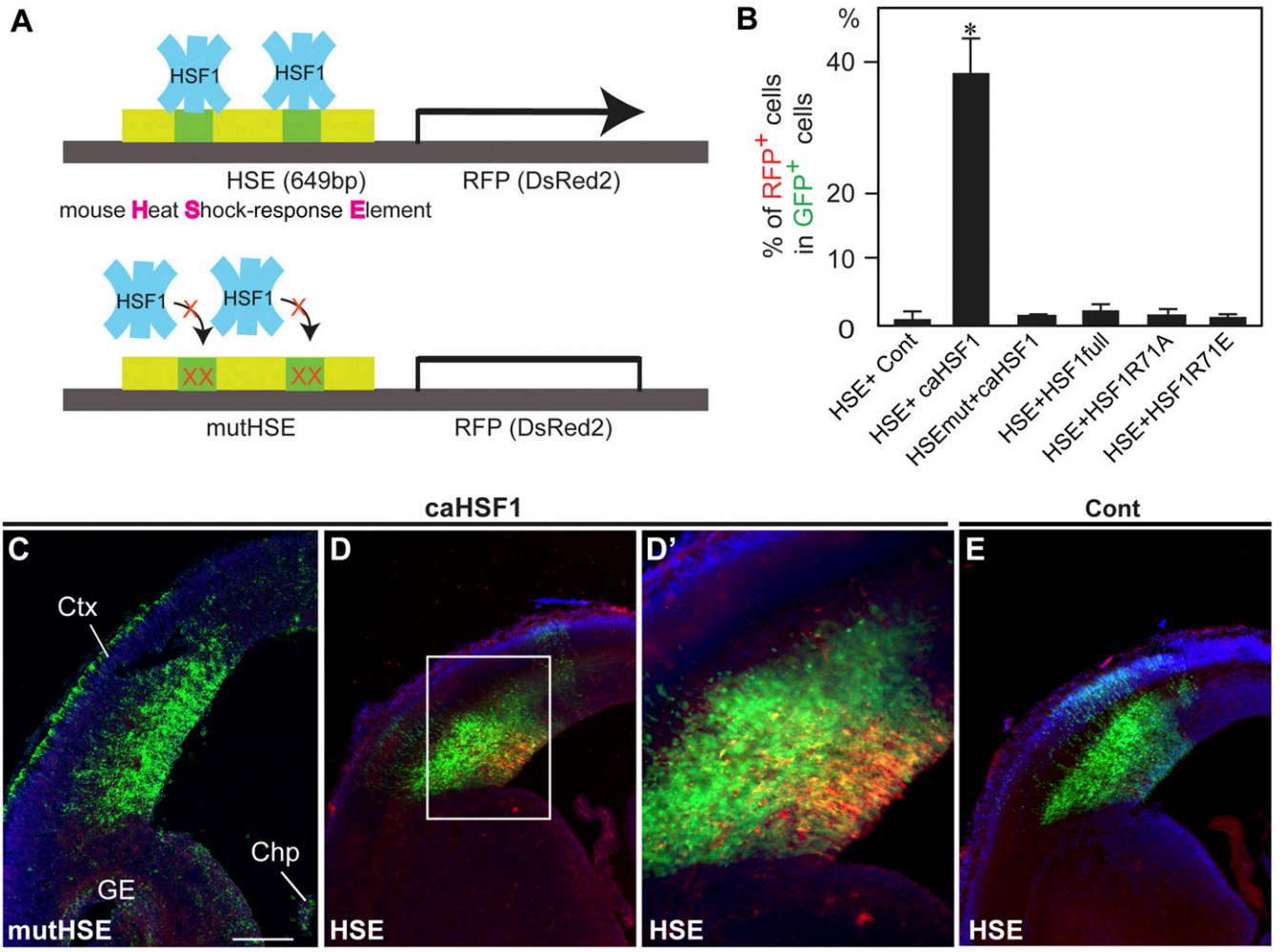

Fig. 1. The reporter construct for the detection of the HSF1 activation. (*A*) Design of the HSE and mutHSE reporter plasmids. (*B*) Percentages of RFP^+ cells in total electroporated (GFP^+) cells in the cerebral cortex 1 d postelectroporation with the indicated plasmids at E14 ($n \geq 4$ per condition). One-way ANOVA, $F(5, 31) = 124.13$ ($P < 0.001$). *$P < 0.01$ by post hoc Tukey's tests between HSE+caHSF1 and each of the other five groups. (*C*–*E*) Representative images showing RFP reporter expression in the cortex 1 d postelectroporation of the indicated plasmids at E14. *D'* shows the higher magnification view of the boxed area in *D*. Chp, choroid plexus; Ctx, cerebral cortex; GE, ganglionic eminence. (Scale bar, 0.1 mm.)

EtOH at several different concentrations [from 0 (PBS only control) to 150 mM] or to heat shock (42 °C for 1 h following incubation at 37 °C). FACS analysis 12 h postexposure revealed that many neurons expressed the reporter at as low as 5 mM of EtOH. Further, the percentage of reporter$^+$ neurons increased with increasing concentrations of EtOH (Fig. S1*A*). The reporter$^+$ neurons showed higher levels of reactive oxygen species (ROS) production (Fig. S1 *B* and *C*), confirming that these neurons were under oxidative stress. The reporter expression was detectable 4 d after exposure (DAE) to EtOH (Fig. S1 *D* and *F*). Similarly, reporter expression was detectable from as early as 8 h post-heat shock exposure in live neurons until at least 4 DAE (Fig. S1 *D* and *F*). In both the EtOH treatment and heat shock exposure, the RFP reporter-expressing cells did not overlap with markers of cell death throughout the observation period. Interestingly, HSE-RFP reporter-positive and -negative neurons in the same dish exhibited different morphologies at 4 DAE (Fig. S1 *G* and *H*). This may reflect differential stress responses by distinct neuronal subtypes or may reflect different levels of stress among individual cells. In both EtOH and heat shock exposure, the reporter expression was no longer detectable at 14 DAE (Fig. S1 *E* and *F*). Given that the total number of neurons was similar between cultures with and without stress, it is likely that neurons that expressed the reporter at 4 DAE survive but lose the reporter expression by 14 DAE. Overall, the data obtained from in vitro experiments corroborate the data from in vivo experiments, demonstrating the reliability of the reporter system in both approaches.

Early in Vivo Detection of Cells Affected by Environmental Insults Using HSE-RFP Reporter Mice. To evaluate the potential of the reporter mice for broad application to early detection of cellular damage, we first examined whether they can detect endogenous Hsf1 activation previously reported to be induced in the embryonic brain by exposure to prenatal environmental stressors (4). The HSE-RFP transgenic embryos were exposed to 2 g/kg EtOH in PBS or PBS only (control) by i.p. injection to the dam once daily from E14 to E16. Reporter expression was induced broadly throughout the bodies of a subset of animals, which was detectable even after birth (until at least P4) in live animals using an epifluorescence stereomicroscope (Fig. 2*A*). Reporter$^+$ cells were detected as early as 3 h after the second injection of EtOH at E15, in multiple organs and tissues including the brain, liver, heart, and eye, but not in PBS-exposed mice (Fig. 2 *B* and *C* and Fig. S2). FACS analysis of the cells dissociated from EtOH-exposed E15 embryonic cerebral cortices revealed that the reporter$^+$ cells include a large proportion of Nestin$^+$ neural progenitor cells and fewer Tuj1$^+$ neurons (Fig. 2*D*).

We then tested the scalability of HSE-RFP reporter transgenic mice by exposing embryos to various environmental stressors, including chemical agents and physiological stimuli known to increase susceptibility to late-onset psychiatric disorders (Table 1). Similar to EtOH, the activation of Hsf1-mediated stress response was detected not only in the brain but throughout the body of the reporter transgenic embryos, following maternal exposure to hyperglycemia, sodium arsenite, pentylenetetrazol (PTZ)-induced maternal seizure (4), lipopolysaccharide (LPS)-induced maternal inflammation, and suramin (a P2 receptor antagonist) (Figs. S3 and S4). In contrast, reporter expression was not induced by nicotine and valproic acid even at sublethal levels (Table 1), consistent with the known specificity of Hsf1 activation by various types of stimuli (12). We also found that the reporter mice are able to detect the impact of postnatal stressors such as EtOH, asphyxia, and X-ray exposure on brain cells (Fig. S3*I* and Table 1). The frequency of reporter-positive embryos/pups varied depending on the type of stimuli used, likely due to distinct differences in both the type and level of insult (Table 1). Regardless of the type of stimuli, reporter expression in the brain was induced in both neural progenitor cells and glial cells (Fig. S3).

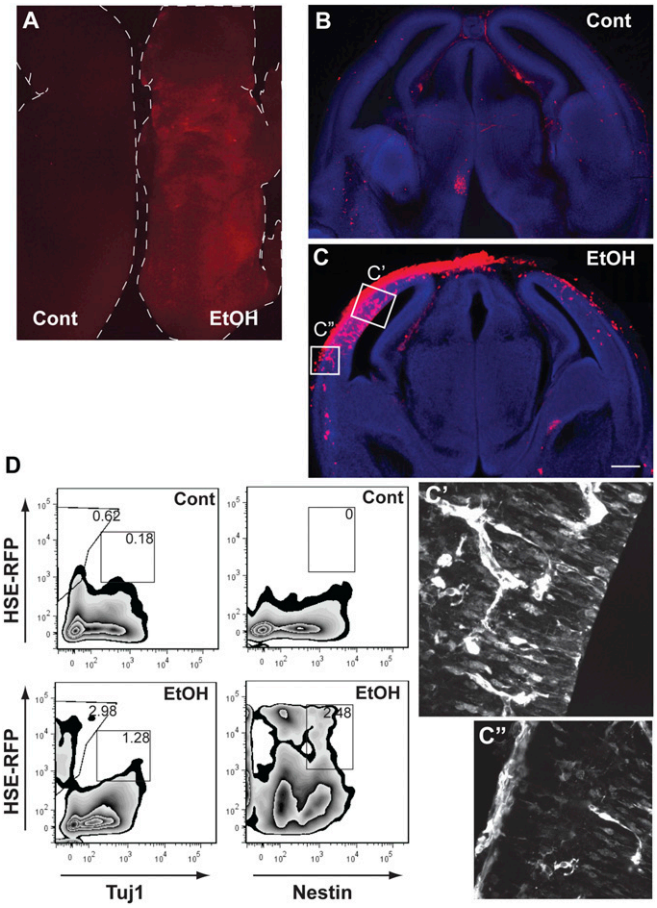

Fig. 2. In vivo detection of cellular damages with the HSE-RFP transgenic mouse. (*A*–*C*) The HSE-RFP transgenic mice were exposed to EtOH (pups on the right in *A* and *C*–*C″*) or PBS (pups on the left in *A* and *B*) daily at E14–16. Images show the expression of the HSE-RFP reporter in the body of live pups at P0 (*A*) and in the coronal section of the cerebral cortex at E15 (*B* and *C*). *C′* and *C″* are the higher magnification views of the squared regions in *B* around the ventricular and marginal zones, respectively. The reporter expression is observed in the blood vessels, neural progenitor cells, and neurons. [Scale bar, 0.1 (*B* and *C*) and 0.01 (*C′* and *C″*) mm.] (*D*) Flow cytometric analysis of the cells dissociated from E15 embryonic cortices 3 h after the last exposure to EtOH or PBS in utero. The RFP$^+$ cells include Nestin$^+$ neural progenitor cells and fewer Tuj1$^+$ immature neurons.

Most importantly, our data demonstrated that the reporter$^+$ cells indeed have differential physiological features from their reporter$^-$ counterparts. An example of this is the effects of embryos exposed to suramin, in which reporter$^+$ cells in the cerebral cortex showed, at least temporarily, differential migration patterns from those of surrounding reporter$^-$ cells (Fig. S4). Delayed migration of the reporter$^+$ cells was also observed in the embryonic cortex exposed to EtOH. These results demonstrate the power of our reporter system to detect cells in vivo that exhibit aberrant cellular phenotypes elicited by various types of environmental stressors both at early and later stages of brain development.

Long-Term Tracing of the Descendants of the Cells Affected by Environmental Stress. An intriguing question arises as to whether the cells originating from progenitor cells affected by prenatal environmental stress inherit epigenetic changes as well as their subsequent vulnerability, which may eventually cause malfunction of various organs and tissues in the adult. For example, epigenetic changes in the progeny neurons inherited from neural progenitor cells have been previously reported to affect adult behavior (1). To

Table 1. Reporter expression by prenatal/postnatal environmental factors

Stressor	Exposure condition > observation		Exposure stage	Reporter$^+$ frequency
Heat	42°C (1 h)	>0.5 DAE	Primary culture	12/12
EtOH	50 mM	>0.5 DAE	Primary culture	16/16
Sodium arsenite	50 μM	>0.5 DAE	Primary culture	5/5
PBS (control)	50 mM	>0.5 DAE	Primary culture	0/7
EtOH	2 g/kgw (E14–16)	>E16	Embryonic	10/15
Suramin	62.5 mg/kgw (E14–16)	>E16	Embryonic	5/62
Maternal inflammation	100 mg/kgw (LPS, E17)	>E18	Embryonic	3/9
Maternal hyperglycemia	2 mg/kgw (glucose, E14–16)	>E16	Embryonic	3/12
Maternal seizure	59 mg/kgw (PTZ, E14)	>E15	Embryonic	2/6
Sodium arsenite	10 mg/kgw (E14)	>E15	Embryonic	8/8
Valproic acid	300 mg/kgw (E14–16)	>E16	Embryonic	0/17
Nicotine	25 mg/kgw (E14–16)	>E16	Embryonic	0/15
PBS (control)	Multiple conditions		Embryonic/postnatal	1/461
EtOH	2 g/kgw (P0 and P1)	>P2	Postnatal	4/14
Asphyxia	10 min at P0 (= E18 C-section)	>P1	Postnatal	4/10
X-ray	16 Gray (adult)	>4 d postexposure	Postnatal	2/4

The regimen for each stress exposure was determined based on the conditions that have shown effects on brain development in previous studies. The frequency of the observation of reporter expressions was determined based on the number of dishes (in vitro experiments) or animals (in vivo experiments). More detailed conditions are shown in Table S1. kgw, kilogram weight.

develop a tool for addressing these questions, we modified our HSE-RFP reporter system to enable tracing of the descendant cells with the expression of GFP, using the Flippase-FRT recombination system (13). Using the codon-optimized version of Flippase (FLPo) (14), we generated a plasmid that includes an HSE-FLPo cassette flanked by two LoxP sites (Fig. S5*A*). When this plasmid is transferred into the FRT-GFP/CAG-Cre-ER transgenic mouse (15, 16), FLPo is expressed upon exposure to stress, inducing GFP expression via FRT recombination. Subsequent administration of 4-Hydroxytamoxifen (4-OHT) activates Cre, which excises the floxed-HSE-FLPo cassette, thereby disabling further cell labeling (Fig. S5*A*). Thus, the period of HSE-dependent detection of Hsf1 activation can be controlled, while previously labeled cells can be traced by sustained GFP expression (Fig. S5*A*).

We tested this advanced reporter system by transferring the plasmids in the brain of FRT-GFP/CAG-Cre-ER mice using IUE at E15. pCAG-RFP was coelectroporated to mark the electroporated cells. After IUE, EtOH (or PBS control) was injected (i.p.) twice at E16 and E17. At P14, long after prenatal exposure to EtOH, we found that up to 41% of RFP$^+$ neuronal descendants of the electroporated neural progenitor cells express GFP in the cortex of EtOH-exposed animals but not in controls (Fig. S5*B*, n = 4 each). The GFP$^+$ cells were still detectable at P45 in EtOH-exposed animals. As expected, a single injection of 4-OHT 6 h after IUE and before the first EtOH injection largely disabled the activation of this reporter system (Fig. S5*B*). These results demonstrate that this modified reporter system successfully traces the descendants of the cells in which Flippase-FRT recombination has once occurred by Hsf1 activation. This system will enable comprehensive analyses to test whether there are any differences in epigenetic traits between reporter$^+$ and reporter$^-$ neurons.

Discussion

Cellular damage can be caused by many types of endogenous or exogenous injuries of varying severity and duration. Early and precise detection of cellular damage in tissues and organs caused by intentional or inadvertent exposure to harmful agents remains an unmet goal needed to facilitate advancements in both basic and clinical research. The availability of tools such as those described herein offers the possibility of identifying prognostic biomarkers with the potential to facilitate identification of effective treatments for conditions resulting from cellular injury.

In the present study, we used the HSF1–HSP signaling pathway, an evolutionally conserved cytoprotective mechanism (8–10) to develop a fluorescence reporter system for early and permanent detection of cells affected by environmental and physical insults. This HSE-based reporter may help identify subtle cellular injuries triggered by genetic, environmental, or physical factors through in vitro and in vivo analyses. Reporter$^+$ cells are found in limited tissue regions and in varied patterns among embryos upon exposure to environmental insults (Figs. S2 and S3). This heterogeneity may involve probabilistic activation of HSF1–HSP signaling in addition to potential influences of reporter sensitivity.

Our results show that the reporter$^+$ cells include a large proportion of neural progenitor cells but fewer neurons in the ETOH-exposed embryonic cerebral cortex (Fig. 2*D*). This is consistent with previous reports that neural differentiation is associated with a decreased induction of the heat shock response (8, 17). Although the mechanism underlying this maturation-associated differential regulation remains unclear, attenuated HSF1 DNA-binding activity and less accessibility of the Hsp70 promoter due to histone deacetylase repression have been suggested (17, 18). Because EtOH and many other environmental factors are known to cause oxidative stress (Fig. S1) (19, 20) and oxidation of HSF1 has been suggested to enhance HSF1 DNA-binding activity (21), maturation-dependent oxidative intracellular environment (21) may also affect the differential heat shock response (17). An alternative possibility is that prenatal exposure to EtOH may delay neuronal differentiation and maintain the immature state with higher heat shock response (22, 23).

Although we focused on testing this reporter system in the nervous system, our data suggest that it can also detect potentially damaged cells in other organs such as heart, eye, and liver (Fig. S2). For example, in addition to abnormalities in the central nervous system, patients with fetal alcohol spectrum disorder (FASD) caused by prenatal exposure to alcohol also exhibit characteristic abnormal facial features. Consistently, we observed reporter expression in the facial mesenchyme in alcohol-exposed embryos. Similarly, reporter expression may detect affected cells that contribute to the malformation of peripheral organs by other insults including such symptoms observed in maternal seizures [e.g., cleft lip/palate, congenital heart disease, low birth weight (24–26)] and Fetal Valproate Syndrome [e.g., distinctive facial features, arachnodactyly, and heart abnormalities (27)]. As such, characterization of the reporter$^+$ cells can be exploited to address

various issues regarding cellular response, adaptation, and pathogenetic mechanisms in various tissues and organs during development as well as in adulthood.

One unique feature found in reporter$^+$ neurons was their shorter total process length but increased number of branches compared with reporter$^-$ neurons in the same in vitro cultures in response to both heat shock and EtOH (Fig. S1 *G* and *H*). This inverse association can be due to direct impacts of these stressors on multiple independent mechanisms regulating numbers, length, and branching of neuronal processes (28). Because our observation was made 4 d after 1 h of heat shock or 12 h of EtOH exposure, this phenotype may also include recovering processes from the direct impact of insults. Further analysis is required to clarify the mechanism.

It should be noted that our reporter system detects damaged cells in which the HSF1–HSP signaling pathway is activated in response to numerous but not all types of insults. For example, we did not detect reporter expression by nicotine or valproic acid (Table 1), both of which are also listed as prenatal environmental risk factors for neuropsychiatric disorders (1). Using a similar approach to develop reporter systems based on the mechanisms of other stress pathways, such as ER (29, 30), will significantly advance this research field. Furthermore, combining such reporters with the HSE-RFP reporter will also provide insights into how cells coordinate distinct molecular stress responses.

Materials and Methods

Generation of the HSE-RFP Reporter. Using Vista Genome Browser (pipeline.lbl.gov/cgi-bin/gateway2), we predicted the *Hsp70* promoter region conserved across mammalian species. The sequence of this region was amplified by PCR from a mouse BAC clone that includes the *Hsp70* gene and the surrounding genomic region (BACPAC). By sequencing, we confirmed that the 649-bp fragment obtained [see *Mouse HSE Full Sequence (649 bp)*] contains the target genomic region, which includes two HSF1 binding sites (HSE in Fig. 1). This PCR product was inserted into the BamHI site in the multiple cloning site of the pDsRed2-1 plasmid (Clontech) to generate the pHSE-RFP reporter plasmid. The mutant HSE [mutHSE, see *Mouse mutHSE Full Sequence (649 bp)*], which contains 14 point mutations to disable HSF1 binding, was generated using the Quickchange site-directed mutagenesis kit (Stratagene).

Mouse HSE Full Sequence (649 bp). The following is the mouse HSE full sequence (649 bp): CAGCTTCACCCACAGGGACCCCGAAGTTGCGTCGCCTCCGCAACAGTGTCAATAGCAGCACCAGCACTTCCCCACACCCTCCCCCTCAGGAATCCGTACTCTCTAGCGAACCCCAGAAACCTCTGGAGAGTTCTGGACAAGGGCGGAACCCACAACTCCGATTACTCAAGGGAGGCGGGGAAGCTCCACCAGACGCGAAACTGCTGGAAGATTCCTGGCCCCAAGGCCTCCTCCGGCTCGCTGATTGGCCCAGCGGAGAGTGGGCGGGGCCGGTGAAGACTCCTTAAAGGCGCAGGGCGGCGAGCAGGGCACCAGACGCTGACAGCTACTCAGAATCAAATCTGGTTCCATCCAGAGACAAGCGAAGACAAGAGAAGCAGAGCGAGCGGCGCGTTCCCGATCCTCGGCCAGGACCAGCCTTCCCCAGAGCATCCACGCCGCGGAGCGCAACCTTCCCAGGAGCATCCCTGCCGCGGAGCGCAACTTTCCCCGGAGCATCCACGCCGCGGAGCGCAGCCTTCCAGAAGCAGAGCGCGGCGCCATGGCCAAGAACACGGCGATCGGCATCGACCTGGGCACCACCTACTCGTGCGTGGGCGTGTTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGC.

Mouse mutHSE Full Sequence (649 bp). The following is the mouse mutHSE full sequence (649 bp): CAGCTTCACCCACAGGGACCCCGAAGTTGCGTCGCCTCCGCAACAGTGTCAATAGCAGCACCAGCACTTCCCCACACCCTCCCCCTCAGGAATCCTTACTCTTTATCGAACCTCATAAACCTTTGTAGAGTTTTGGACAAGGGCGGAACCCACAACTCCGATTACTCAAGGGAGGCGGGGAAGCTCCACCAGATGCTAAACTGTTGTAAGATTTCTTGCCCCAAGGCCTCCTCCGGCTCGCTGATTGGCCCAGC-GGAGAGTGGGCGGGGCCGGTGAAGACTCCTTAAAGGCGCAGGGCGGCGAGCAGGGCACCAGACGCTGACAGCTACTCAGAATCAAATCTGGTTCCATCCAGAGACAAGCGAAGACAAGAGAAGCAGAGCGAGCGGCGCGTTCCCGATCCTCGGCCAGGACCAGCCTTCCCCAGAGCATCCACGCCGCGGAGCGCAACCTTCCCAGGAGCATCCCTGCCGCGGAGCGCAACTTTCCCCGGAGCATCCACGCCGCGGAGCGCAGCCTTCCAGAAGCAGAGCGCGGCGCCATGGCCAAGAACACGGCGATCGGCATCGACCTGGGCACCACCTACTCGTGCGTGGGCGTGTTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGC.

Animals. All animals were handled according to protocols approved by the Institutional Animal Care and Use Committees of the Children's National Medical Center, the VA Connecticut Healthcare System, and Yale University. For generation of the HSE-RFP transgenic mice, the HSE-RFP fragment was excised and purified according to the standard protocol for microinjection. Microinjection into the C57BL/6J X SJL/J strain was performed by Yale animal genomics service. The founder lines were screened by PCR genotyping for RFP and confirmed by RFP fluorescence in the neonates from alcohol-treated dams under a dissecting microscope equipped with epifluorescence. From the obtained three founder lines (B9, B11, and B49), we selected the strongest (most sensitive) reporter line, B9, for the study. The embryos and pups from wild-type females crossed with HSE-RFP homozygous transgenic males were used. For the routine genotyping, oligonucleotide PCR primers (forward, 5′-AAGGTGTACGTGAAGCACCC-3′; reverse, 5′-CCCATGGTCTTCTTCTGCAT-3′) were used for the amplification of the 250 bp partial sequence of the DsRed2 gene with Hotstar taq DNA polymerase kit (Qiagen). FRT-GFP and CAG-CRE-ER mice were purchased from Jackson laboratory and were crossed to obtain double transgenic mice. 4-OHT (Sigma) diluted in corn oil at 20 mg/mL was administered to a subset of the double transgenic mice at 20 mg/kg body weight through i.p. injections. The same solution without 4-OHT was injected as the vehicle-only control. CD-1 mice were purchased from Jackson laboratory.

IUE. IUE was performed as previously described (31, 32). All control experiments were performed using empty vectors at the same concentrations. Electroporated DNA constructs are pHSE-RFP and pmutHSE-RFP (1.0 μg/μL); pCAGIG-caHSF1, pCAGIG-HSF1 full, pCAGIG-HSF1R71A, and pCAGIG-HSF1R71E (0.5 μg/μL); pCAG-GFP and pCAG-RFP (0.5–2 μg/μL) (32, 33); and pHSE-FLPo (2 μg/μL). pHSE-FLPo was generated by inserting a synthesized oligonucleotide cassette of loxP-HSE-FLPo-polyA-loxP into pUC57 (Addgene).

Primary Culture of Embryonic Cortical Neurons. Cells were dissociated from the cerebral cortex of the transgenic mice at E17 and passaged on laminin-coated plates in DMEM (Life Technologies) containing N2 and B27 supplements (Life Technologies). Stress exposure in vitro is described below.

Exposure to Prenatal/Postnatal Stressors. Experimental paradigms, including the stress intensity/dose, duration, and the time of observation for individual experiments, are described in *Results* as well as shown in Table 1 (detailed in Table S1). The conditions for prenatal EtOH exposure, in vitro EtOH exposure, and PTZ-induced maternal seizures were previously described (4, 34). For in vivo exposure models, each chemical substrate was administered intraperitoneally once or multiple times once daily for the indicated duration to the pregnant mice (prenatal exposure models) or the pups (postnatal exposure models). For in vitro models, each substrate was added in culture medium for the indicated duration. All chemical substrates were dissolved in PBS, and PBS only was used as controls. For in vitro heat shock exposure, neurons were exposed to heat shock at 42 °C for 1 h following incubation at 37 °C. For X-ray irradiation and asphyxia, see below.

X-Ray Irradiation. Mice were transported to the Therapeutic Radiology Department at the Yale University School of Medical in accordance with VA and Yale transportation guidelines. Once there, mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). Mice were then irradiated with a surface dose of 16 Gray confined to the cranium. The rest of the body was shielded by a lead apron. The radiotherapy machine (HRT 239, Siemens) was operated at 250 kV, 15 mA with a 0.5 mm CU and 1.0 mm A1 filter.

Asphyxia. Pups were subjected to intrauterine asphyxia; at E18, uterine horns were removed from anesthetized pregnant mice via cesarean section and kept in warm sterile PBS for 10 min at 37 °C. The pups were then removed, stimulated for breathing by removal of the delivery fluid and tactile stimulation of the oral region, and left to recover in an incubator at 37 °C. The recovery from asphyxia was assessed by observing the gasping, movement, and skin color of the pups. Then the pups were returned to the litter of the foster mom (CD-1).

Flow Cytometric Analysis. The cortex was dissected into small pieces in cold PBS and was incubated with TripLE Select (Invitrogen) at 37 °C for 10 min. After the dissociation of cells, immunostaining was performed with PBS containing 0.5% BSA and 0.1% NaN3. LIVE/DEAD Fixable Dead Cell Stain Kit was used for the staining of dead cells. Using Perm & Fix (Invitrogen), the cells were labeled with RFP-biotin (Abcam), Nestin-PerCP (SCBT), or Tuj1-FITC (eBioscience) (all used at 1:500) and Streptavidin-PE (eBioscience) (1:2,000). Antibody reaction was done for 30 min on ice. Recording and analysis were performed using BD LSRII with Green laser, DIVA software, and FlowJo (TreeStar). A total of 100,000 cells were recorded per sample.

Immunohistochemistry, Immunocytochemistry, and ROS Detection. Immunohistochemistry was performed following the methods previously described (31). Pretreatment with 2N HCl was performed for Bromodeoxyuridine

(BrdU) staining. The staining was amplified using biotin-conjugated secondary antibodies, VECTASTAIN ABC system (Vector), and TSA Plus system (PerkinElmer). Immunocytochemistry was performed following a standard protocol. Briefly, cells were fixed with 4% (wt/vol) paraformaldehyde for 10 min at room temperature and then washed with PBS three times. After blocking in PBS containing 2% (wt/vol) BSA for 1 h at room temperature, the cells were incubated with primary antibodies at 4 °C overnight. After washing with PBS three times, the cells were incubated with secondary antibodies conjugated with Alexa Fluor 488 or 555 for 1 h at room temperature. We used the following primary antibodies: monoclonal mouse anti-BrdU (1:100, BD Biosciences), anti-Tuj1 (1:500–1:1,000, Abcam), anti-MAP2 (1:500, EMD Millipore), polyclonal chicken anti-GFAP (1:1,000, Abcam), rabbit anti-Sox2 (1:1,000, Abcam), and anti–RFP-biotin (1:1,000, Abcam). ROS was detected using CM-H_2DCFDA (ThermoFisher) following the manufacturer's protocol.

Imaging and Data Analysis. Tissue sections and cells were imaged using a Zeiss LSM510 confocal microscope. The imaging of postnatal pups was performed with a Zeiss Discovery V8 stereomicroscope. All quantitative data are presented as mean ± SEM unless otherwise noted. Statistical tests were performed to assess the significance of the results from multiple samples and/or experiments (at least $n \geq 4$ for each experimental condition).

ACKNOWLEDGMENTS. We thank Dr. Alexander Son for critical reading of the manuscript. This work was supported by National Institute of Health Grants R21AA024882 (to K.H.-T. and M.T.), R01AA025215 (to K.H.-T.), R01DA023999 and R01EY002593 (to P.R.), the Alcoholic Beverage Medical Research Foundation/The Foundation for Alcohol Research and Clinical and Translational Science Institute at Children's National (K.H.-T.); the Brain & Behavior Research Foundation, Scott-Gentle Foundation, and the Kavli Institute for Neuroscience at Yale (K.H.-T., M.T., and P.R.); Avery Translational Research Career Development Program Award (M.T.); Japan Society for the Promotion of Science KAKENHI Grants 24890181 and 25462227 (to M.S.); the Medical and Rehabilitation and Development Research Services of Department of Veterans Affairs (S.G.W.); and the VA Spinal Cord Injury Translational Collaborative Consortium for Regenerative Medicine, National Multiple Sclerosis Society Grant RG2135, and Connecticut's Stem Cell Research Program 12-SCB-Yale-05 (to J.D.K.).

1. Ishii S, Hashimoto-Torii K (2015) Impact of prenatal environmental stress on cortical development. *Front Cell Neurosci* 9:207.
2. Thompson BL, Levitt P, Stanwood GD (2009) Prenatal exposure to drugs: Effects on brain development and implications for policy and education. *Nat Rev Neurosci* 10(4): 303–312.
3. Ben-Ari Y (2008) Neuro-archaeology: Pre-symptomatic architecture and signature of neurological disorders. *Trends Neurosci* 31(12):626–636.
4. Hashimoto-Torii K, et al. (2014) Roles of heat shock factor 1 in neuronal response to fetal environmental risks and its relevance to brain disorders. *Neuron* 82(3):560–572.
5. Davidoff GN, Roth EJ, Richards JS (1992) Cognitive deficits in spinal cord injury: Epidemiology and outcome. *Arch Phys Med Rehabil* 73(3):275–284.
6. Roth E, et al. (1989) A controlled study of neuropsychological deficits in acute spinal cord injury patients. *Paraplegia* 27(6):480–489.
7. Chang YW, Waxman SG (2010) Minocycline attenuates mechanical allodynia and central sensitization following peripheral second-degree burn injury. *J Pain* 11(11): 1146–1154.
8. Morimoto RI (1998) Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* 12(24):3788–3796.
9. Lindquist S (1986) The heat-shock response. *Annu Rev Biochem* 55:1151–1191.
10. Akerfelt M, Morimoto RI, Sistonen L (2010) Heat shock factors: Integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* 11(8):545–555.
11. Inouye S, et al. (2007) Heat shock transcription factor 1 opens chromatin structure of interleukin-6 promoter to facilitate binding of an activator or a repressor. *J Biol Chem* 282(45):33210–33217.
12. El Fatimy R, et al. (2014) Heat shock factor 2 is a stress-responsive mediator of neuronal migration defects in models of fetal alcohol syndrome. *EMBO Mol Med* 6(8): 1043–1061.
13. Turan S, Kuehle J, Schambach A, Baum C, Bode J (2010) Multiplexing RMCE: Versatile extensions of the Flp-recombinase-mediated cassette-exchange technology. *J Mol Biol* 402(1):52–69.
14. Raymond CS, Soriano P (2007) High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. *PLoS One* 2(1):e162.
15. Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: A tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244(2):305–318.
16. Sousa VH, Miyoshi G, Hjerling-Leffler J, Karayannis T, Fishell G (2009) Characterization of Nkx6-2-derived neocortical interneuron lineages. *Cereb Cortex* 19(Suppl 1):i1–i10.
17. Yang J, Oza J, Bridges K, Chen KY, Liu AY (2008) Neural differentiation and the attenuated heat shock response. *Brain Res* 1203:39–50.
18. Gómez AV, et al. (2015) Characterizing HSF1 binding and post-translational modifications of hsp70 promoter in cultured cortical neurons: Implications in the heat-shock response. *PLoS One* 10(6):e0129329.
19. Al-Gubory KH (2014) Environmental pollutants and lifestyle factors induce oxidative stress and poor prenatal development. *Reprod Biomed Online* 29(1):17–31.
20. Heyer DB, Meredith RM (2016) Environmental toxicology: Sensitive periods of development and neurodevelopmental disorders. *Neurotoxicology* 58:23–41.
21. Voellmy R (2004) On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress Chaperones* 9(2):122–133.
22. Zhou FC, et al. (2011) Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcohol Clin Exp Res* 35(4):735–746.
23. Santillano DR, et al. (2005) Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. *BMC Neurosci* 6:59.
24. Chen YH, Chiou HY, Lin HC, Lin HL (2009) Affect of seizures during gestation on pregnancy outcomes in women with epilepsy. *Arch Neurol* 66(8):979–984.
25. Speidel BD, Meadow SR (1972) Maternal epilepsy and abnormalities of the fetus and newborn. *Lancet* 2(7782):839–843.
26. Stumpf DA, Frost M (1978) Seizures, anticonvulsants, and pregnancy. *Am J Dis Child* 132(8):746–748.
27. Hersh JH (2003) *Fetal Valproate Syndrome. NORD Guide to Rare Disorders* (Lippincott Williams & Wilkins, Philadelphia, PA), pp 192–193.
28. Scott EK, Luo L (2001) How do dendrites take their shape? *Nat Neurosci* 4(4):359–365.
29. Iwawaki T, Akai R, Kohno K, Miura M (2004) A transgenic mouse model for monitoring endoplasmic reticulum stress. *Nat Med* 10(1):98–102.
30. Mao C, Dong D, Little E, Luo S, Lee AS (2004) Transgenic mouse model for monitoring endoplasmic reticulum stress in vivo. *Nat Med* 10(10):1013–1014, author reply 1014.
31. Hashimoto-Torii K, et al. (2008) Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex. *Neuron* 60(2):273–284.
32. Torii M, Hashimoto-Torii K, Levitt P, Rakic P (2009) Integration of neuronal clones in the radial cortical columns by EphA and ephrin-A signalling. *Nature* 461(7263): 524–528.
33. Hashimoto-Torii K, et al. (2003) Differential activities of Sonic hedgehog mediated by Gli transcription factors define distinct neuronal subtypes in the dorsal thalamus. *Mech Dev* 120(10):1097–1111.
34. Hashimoto-Torii K, Kawasawa YI, Kuhn A, Rakic P (2011) Combined transcriptome analysis of fetal human and mouse cerebral cortex exposed to alcohol. *Proc Natl Acad Sci USA* 108(10):4212–4217.