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Two Weeks of Variable Stress Increases Gamma-H2AX Levels in the Mouse Bed Nucleus of the Stria Terminalis

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Abstract

Recent reports demonstrate that DNA damage is induced, and rapidly repaired, in circuits activated by experience. Moreover, stress hormones are known to slow DNA repair, suggesting that prolonged stress may result in persistent DNA damage. Prolonged stress is known to negatively impact physical and mental health; however, DNA damage as a factor in stress pathology has only begun to be explored. Histone H2A-X phosphorylated at serine 139 (γ H2AX) is a marker of DNA double-strand breaks (DSB), a type of damage that may lead to cell death if unrepaired. We hypothesized that a 14-day period of variable stress exposure sufficient to alter anxiety-like behavior in male C57BL/6J mice would produce an increase in γ H2AX levels in the bed nucleus of the stria terminalis (BNST), a region implicated in anxiety and stress regulation. We observed that 14 days of variable stress, but not a single stress exposure, was associated with increased levels of γ H2AX 24 h after termination of the stress paradigm. Further investigation found that phosphorylation levels of a pair of kinases associated with the DNA damage response, glycogen synthase kinase 3 β (GSK3 β) and p38 mitogen-activated protein kinase (MAPK) were also elevated following variable stress. Our results suggest that unrepaired DNA DSBs and/or repetitive attempted repair may represent an important component of the allostatic load that stress places on the brain.

Keywords

stress; anxiety; bed nucleus of the stria terminalis; gamma-H2AX; glycogen synthase kinase 3-beta

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INTRODUCTION

Neurons are prone to DNA damage due to their post-mitotic state, longevity, and high metabolic rate. DNA DSBs are particularly detrimental and may contribute to neurodegeneration (Madabhushi et al., 2014) or apoptosis (Jackson, 2002), if unrepaired. During nervous system development, apoptosis from failed DNA repair can be offset by replacement from germinal zones. Though this may be an option in certain brain regions, such as the subgranular zone of the dentate gyrus, replacement is not a viable option for post-mitotic neurons in much of the mature brain. Thus, rapid and accurate repair is critical to neuronal survival.

Until recently, it was thought that the normal mature brain was not subject to DNA DSB damage since histone H2AX phosphorylated at serine 139 (γ H2AX), an early indicator of DSB formation (Rogakou et al., 1998) was not readily detectable. However, recent studies showed that neuronal DNA DSBs are evident in cortical and hippocampal neurons activated by novel experiences (Suberbielle et al., 2013; Madabhushi et al., 2015). Experience-associated DNA DSBs suggest that even normal neuronal activity may produce DNA damage. However, such damage is subject to rapid repair as the γ H2AX rapidly returned to baseline levels shortly after the experience (Suberbielle et al., 2013). Neuronal activation brought about by stress may produce different outcomes as stress hormones slow DNA repair (Flint et al., 2007) and promote the accumulation of DNA damage (Hara et al., 2011). Very few studies have examined neuronal DSB levels after stress, and those that have are limited to a single time point after stress (Hara et al., 2013; He et al., 2016). These studies demonstrate an increase in γ H2AX following stress; however, it remains unclear whether the increase represents damage accumulated during stress exposure or unrepaired damage generated by the final stress experience.

Previous work indicates that stress-associated release of epinephrine facilitates the increase in DSBs following repeated restraint stress exposure (Hara et al., 2013). Interestingly, alternative pathways associated with DSB-related pathologies have not been investigated. p38 MAPK phosphorylation of GSK3 β at serine 389 (S³⁸⁹) was recently characterized as a GSK3 β repressor that is independent of the traditionally studied GSK3 β serine 9 (S⁹) phosphorylation site (Thornton et al., 2008). Additional work has demonstrated the GSK3 β S³⁸⁹ phosphorylation is induced by DSB-inducing agents and colocalizes with nuclear γ H2AX in lymphocytes (Thornton et al., 2016) and brain (Thornton et al., 2017). Notably, GSK3 β S³⁸⁹ phosphorylation was demonstrated to be critical for neuronal survival in hippocampal neurons (Thornton et al., 2017), indicating that repression of nuclear GSK3 β by p38 MAPK may be a critical step in promotion of cell survival after DSB. The present study was undertaken to investigate the role of p38 MAPK-mediated S³⁸⁹ phosphorylation of GSK3 β and γ H2AX expression, as a marker for DSBs, following acute and variable stress exposure in the BNST.

EXPERIMENTAL PROCEDURES

Subjects

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) ($n = 65$) were obtained at six weeks of age and acclimated for 7 days in an Association for Assessment and Accreditation of Lab Animal Care approved facility (light 0700 h–1900 h) with *ad libitum* access to food and water. All procedures were approved by the University of Vermont Animal Care and Use Committee.

Variable stress paradigm

To maintain consistency with our previous research (Hare et al., 2012) and the methods of others that have investigated DSBs using a variable stress procedure (He et al., 2016), mice assigned to the 14-day variable stress condition were individually housed following the first stressor, while control animals remained group housed for the duration of the experiment. To prevent habituation, stressors were administered in a pseudorandom sequence in which all stressors were performed before any stressor was repeated. The stressors have been described in detail elsewhere (Hare et al., 2012). Stressors used were forced swim (5 min, 20–25 °C), oscillation (30 min), pedestal (30 min), foot shock (2 shocks, 1.0 mA, 5 s each), and restraint (60 min). A single stressor was administered per day. The variable stress procedure terminated with forced swim exposure. All animals were weighed daily.

Acute stress

In each case, the acute stressor administered was forced swim as described above. Forced swim was chosen to maintain consistency with the final stressor administered in the variable stress procedure.

Acoustic startle

Acoustic startle was used to balance animals into stressed ($n = 8$) and control ($n = 8$) conditions prior to manipulation, and again to assess anxiety following variable stress. The acoustic startle test was conducted as described previously (Fox et al., 2008). Briefly, animals were presented with 20-ms noise bursts, 10 each at 95, 100 and 105 dB (60-s intertrial interval), in a pseudorandom fashion. The average startle amplitude over each test was used to calculate a percent change between the tests.

Western blot analysis

For all experiments, unanesthetized mice were decapitated and tissue was rapidly harvested (within 3 min) 24 h after the final stressor (1200 h to 1300 h). Additional animals were sacrificed 30 min and 90 min after an acute forced swim exposure to assess the immediate effect of stress on γ H2AX and GSK β S³⁸⁹ phosphorylation. Coronal slices (2 mm) were obtained using a 1-mm brain matrix (Stoelting, Wood Dale, IL, USA). Individual brain regions were microdissected from the slice with a 1-mm tissue punch (Stoelting, Wood Dale, IL) using the following approximate anteroposterior, mediolateral and dorsoventral Bregma coordinates (Paxinos and Franklin, 2004): amygdala (−1.06, 2.75, −4.5), BNST (0.26, 1.0, −4.25), hippocampus (−2.06, 1.0, −2.0) and medial prefrontal cortex (mPFC) (1.34, 0.25,

–3.0). Punched tissue was transferred to centrifuge tubes on dry ice and stored at –80 °C until analyzed. Western blot analysis was performed using crude whole-cell lysates as described previously (Lluri et al., 2008) except the tissue was homogenized in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA, USA). All blots in a given analysis were exposed on the same X-ray film (Biomax MR, Kodak, Rochester, NY, USA) to ensure the same exposure time and linear range conformation. Semi-quantitative densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Brains were rapidly dissected, coronal blocks with exposed BNST generated, and submersion fixed in 10% buffered formalin (Fisher Scientific) for 4 h prior to paraffin embedding. Sections (8 µm) were mounted onto gelatin-coated slides, paraffin was removed by standard xylene and ethanol exposure, and immunohistochemistry was performed as previously described (Jaworski et al., 2006). After three washes with phosphate-buffered saline (PBS), slides were steamed in DAKO target retrieval solution for 15 min and then cooled to room temperature for 30 min (Agilent Technologies, Santa Clara, CA, USA). After three more PBS washes, tissue was blocked (Dulbecco's Modified Eagle Medium with 5% fetal bovine serum, 0.1% glycine, 0.1% lysine, Triton 0.2%) for 30 min and then incubated overnight at 4 °C with primary antibodies in blocking buffer. Tissue was washed three times in PBS before incubation with species-specific secondary antibodies in blocking buffer for one hour. Following three PBS washes, tissue was coverslipped with Citifluor containing DAPI (Electron Microscopy Sciences, Hatfield, PA, USA), examined with a Nikon E800 microscope (Micro Video Instruments, Avon, MA, USA) and images were captured with a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). The percent of cells displaying DNA DSBs was quantified by three individuals blinded to treatment by counting the number γ H2AX-positive cells relative to all DAPI-labeled cells. Antibodies used for immunohistochemistry identified a single immunoreactive species on western blot analysis, suggesting antibody specificity. Antibody specificity was further confirmed by comparable stress-mediated regulation detected by western blot analysis and immunohistochemistry. Lastly, no immunoreactivity was observed when primary antibody was excluded.

Antibodies

For western blot analysis, rabbit anti-human γ H2AX (Ser139) (1:1000, #9718S, lot 10), rabbit anti-human GSK3 β (1:15,000, #9315, lot 12), rabbit anti-human p38 MAPK (1:1000, #9212, lot 11) and rabbit anti-human phospho-p38 MAPK (Thr180/Tyr182) (1:1000, #9215, lot 7) were obtained from Cell Signaling (Danvers, MA). Rabbit anti-mouse phospho-GSK3 β (Ser389) (1:3000, #07-2275, lot 2272702) was obtained from Millipore (Billerica, MA, USA). Blots were normalized using mouse anti-chicken α -tubulin (1:50,000, #T6199, Sigma, St. Louis, MO). For western blots, goat anti-rabbit IgG (H + L) (#111-035-144) and goat anti-mouse IgG (H + L) (#115-035-146) horseradish peroxidase-conjugated secondary antibodies were utilized (1:3000, Jackson ImmunoResearch, West Grove, PA, USA). For immunohistochemistry, rabbit anti-mouse phospho-GSK3 β (Ser389) (1:400, Millipore) and

mouse antihuman γ H2AX (Ser139) (1:500, Millipore #05-636, lot 2476967, the same lot as used in (Thornton et al., 2016)) antibodies were utilized. For single γ H2AX labeling, Cy3-conjugated donkey anti-mouse IgG (H + L) (#715-165-150) was used, while for dual γ H2AX and phospho-GSK3 β co-localization, DyLight 488-conjugated donkey anti-mouse IgG (H + L) (#715-485-151) and Cy3-conjugated donkey anti-rabbit IgG (H + L) (#711-165-152) secondary antibodies were utilized (1:500, Jackson ImmunoResearch).

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Sample distributions met normality and variance assumptions; thus, parametric statistics were employed. Acoustic startle was analyzed by independent sample *t*-test. Body weight and western blot data were analyzed by a two-way ANOVA (group $\times$ day, region $\times$ group, or stress duration $\times$ group) and significant interactions were followed by post hoc tests with the Bonferroni correction. The relationship between observed protein levels in each sample was calculated using Pearson's correlation coefficient. Results were analyzed by SPSS software version 22 (IBM; Armonk, NY, USA) with α set at 0.05.

RESULTS

Variable stress increases startle amplitude and reduces weight gain

To assess the impact of stress on DNA DSBs, mice were subjected to 14 days of variable stress. The effect of stress on body weight gain was assessed as an index of stress efficacy prior to tissue harvest (Fig. 1A). A group by day repeated measures ANOVA produced significant main effects for day ($F_{(13,182)} = 6.85$, $p < 0.0001$) and group ($F_{(1,14)} = 8.35$, $p < 0.05$) along with a significant interaction ($F_{(13,182)} = 4.00$, $p < 0.0001$). Control mice gained weight at a greater rate than stressed mice such that by day 8, control mice weighed more than stressed mice ($t_{(14)} = 3.64$, $p < 0.01$). To further assess the efficacy of the stress procedure, acoustic startle was assessed prior to and subsequent to variable stress (Fig. 1B). Acoustic startle is a sensitive measure of anxiety, with greater startle amplitude indicative of greater anxiety (Walker et al., 2003). Startle amplitude was elevated in stressed mice relative to control mice ($t_{(14)} = 2.20$, $p < 0.05$).

Elevated γ H2AX and associated signaling is evident in the stressed brain

To assess the regulation of γ H2AX, a protein induced in response to DNA DSBs, following variate stress, western blot analysis was performed on multiple stress-associated brain regions (i.e., amygdala, BNST, hippocampus and mPFC) (Fig. 2). A significant group effect was observed ($F_{(1,24)} = 9.36$, $p < 0.01$), indicating the presence of increased DNA DSBs following stress in the brain areas examined (Fig. 2). We next sought to investigate γ H2AX-associated proteins by assessing p38 MAPK and GSK3 β , two kinases which have been linked to DNA damage response (Thornton et al., 2008, 2016; Wood et al., 2009) (Fig. 3). Variable stress produced an increase in levels of both phosphorylated p38 MAPK (p-p38 MAPK; Group $F_{(1,24)} = 31.80$, $p < 0.0001$; Fig. 3A) and total MAPK (t-p38 MAPK, Group $F_{(1,24)} = 57.99$, $p < 0.0001$; Fig. 3B). The increased p-p38 MAPK and t-p38 MAPK expression observed in widespread brain regions following stress suggests an overall activational effect of stress on p38 MAPK signaling that may be due, in part, to the demands

of DNA repair. This hypothesis is supported by the significant positive correlation observed between γ H2AX and p-p38 MAPK levels ($r_{(30)} = 0.57$, $p < 0.001$). The protective effect of increased p38 MAPK activity following stimuli that induce DNA damage (Tan et al., 2014; Wang et al., 2014) may be through inhibition of GSK3 β (Yang et al., 2011; Thornton et al., 2016) via p38 MAPK-mediated phosphorylation of GSK3 β at S³⁸⁹ exclusive of the traditionally studied Akt-mediated S⁹ phosphorylation site (Thornton et al., 2008). GSK3 β S³⁸⁹ phosphorylation (p-S³⁸⁹, Group $F_{(1,24)} = 20.19$, $p < 0.001$; Fig. 3C) and total GSK3 β expression (Group $F_{(1,24)} = 4.56$, $p < 0.05$; Fig. 3D) were significantly increased following stress. Similar to p-p38 MAPK, GSK3 β p-S³⁸⁹ positively correlated with γ H2AX levels ($r_{(30)} = 0.50$, $p < 0.01$).

BNST γ H2AX levels are elevated 24 h after variable stress, but not 24 h after a single stressor

To determine whether increased γ H2AX represents an accumulation of damage, indicative of delayed repair kinetics, γ H2AX expression in the BNST was examined 24 h after stress exposure. Animals were subjected to either an acute stressor (i.e., forced swim) or chronic variable stress terminating with forced swim (Fig. 4). The BNST was chosen because of its role in anxiety-like behavior (Walker et al., 2003; Kim et al., 2013). In line with this, we observed elevated anxiety-like behavior in stressed animals. A two-way ANOVA revealed a critical interaction, demonstrating that the effect of variable stress was different than that of acute stress ($F_{(1,16)} = 5.07$, $p < 0.05$). No change in γ H2AX level was observed in the BNST 24 h after acute forced swim (acute stress *vs* control, $t_{(8)} = 0.04$, $p > 0.05$), yet increased γ H2AX was again observed following the variable stress procedure (stress *vs* control, $t_{(8)} = 3.21$, $p < 0.01$), suggesting that DNA DSBs are accumulating over the variable stress period.

To further confirm increased DNA DSBs following stress, the percent of cells displaying nuclear γ H2AX foci in the dorsal BNST was quantitated in control mice and mice subjected to variable stress (Fig. 5). Immunohistochemical analysis appeared to be more sensitive to changes in γ H2AX levels than western blot analysis. Stressed animals clearly demonstrated more γ H2AX-positive nuclei in the dorsal BNST than controls ($t_{(6)} = 8.30$, $p < 0.001$, Fig. 5A). Qualitatively, GSK3 β p-S³⁸⁹ was found to co-localize with γ H2AX-positive nuclei, further linking GSK3 β S³⁸⁹ phosphorylation and stress-mediated accumulation of DNA DSBs (Fig. 5B).

BNST γ H2AX and GSK3 β p-S³⁸⁹ levels are unchanged 30 and 90 min after an acute stress exposure

DSB levels typically peak within 30 min following application of damage-inducing agents such as ionizing radiation (Redon et al., 2009). To assess whether an initial stress exposure was sufficient to produce DNA DSB in the BNST, animals were subjected to a single forced swim exposure and sacrificed 30 min (Fig. 6A) or 90 min (Fig. 6B) later. Western blot analysis revealed no change in γ H2AX or GSK3 β p-S³⁸⁹ levels at either time point ($p > 0.10$), indicating that the elevation in γ H2AX and GSK3 β p-S³⁸⁹ levels observed after variable stress is likely a unique consequence of repeated and/or prolonged stress exposure.

DISCUSSION

Our data demonstrate that stress produces an accumulation of γ H2AX, a marker of DNA DSBs. These findings support previous work suggesting that experience may produce robust DNA damage (Suberbielle et al., 2013; Madabhushi et al., 2015). However, contrary to previous reports demonstrating rapid repair following an acute stress experience, our findings demonstrate that multiple stressors generate persistent DNA damage. Indeed, we observed increased γ H2AX 24 h after a final stressor in animals subjected to a 14-day variable stress procedure. This is an important observation as the constant need to repair damaged DNA could represent a significant energetic demand. As such, DNA damage may represent an important component of the daily “wear-and-tear” or, allostatic load, that repeated stress places on an organism. Given the association between DNA damage and neurodegenerative diseases (Madabhushi et al., 2014), these findings are concerning.

Previous reports have demonstrated that DNA DSBs are present in the cerebral cortex and hippocampus following neuronal activation induced by diverse experiences; however, repair occurred within a period of hours (Suberbielle et al., 2013; Madabhushi et al., 2015). Consistent with these reports, we found that elevated DNA DSBs were not present 24 h after acute stress. However, increased γ H2AX levels were detectable 24 h after the final stress exposure of the variable stress paradigm, suggesting that DNA damage accumulates during chronic stress. It is notable that we were unable to observe significant effects of acute stress on DNA DSBs in the BNST. The BNST would likely be recruited during the forced swim experience, as acute swim in mice bred on a CD1 background has been shown to produce a robust increase in BNST activation (Gaszner et al., 2012). Notably, chronic stress has been shown to produce an increase in BNST fosB expression in the same mouse line (Kormos et al., 2016; Farkas et al., 2017). Increased fosB levels are typically interpreted as demonstrating sustained activation as might be expected during chronic stress (Nestler et al., 2001). Thus, sufficient activation of the BNST to produce DNA DSBs may require repeated or prolonged exposure to stressors or stress hormones. In support of this, our results show that repeated exposure to variable stress, sufficient to produce an anxiety-like response, is also sufficient to produce persistent DNA damage that is corroborated by increases in p38 MAPK activity and GSK3 β repression through S³⁸⁹ phosphorylation.

There are now multiple examples of stress exposure producing DNA DSBs in various brain regions. Notably, a chronic unpredictable mild stress model increased DSBs at two weeks in the hippocampus, and produced a further increase at four weeks that was blocked by antidepressant treatment that also rescued behavioral deficits (He et al., 2016). Homotypic restraint stress exposure for 4 h per day over a two-week period increased DNA DSBs in the frontal cortex, an effect that was blocked by administration of a β 2-adrenergic antagonist (Hara et al., 2013). In the study by Hara et al. (2013), animals were sacrificed immediately following the final stressor so it is unclear if the increase in DSBs represents an accumulation of damage or is a result of the final stress exposure. However, the stress response is known to habituate during homotypic stress experience. This would suggest that the DSBs were accumulating across repeating stress exposures, even if reduced in magnitude. Finally, stress exposure appears to also increase markers of oxidative DNA damage (Maluach et al., 2017). Together, these findings indicate that DNA damage is a

common facet of stress exposure that deserves further investigation into its causative role in anxiety and depression given its expression in limbic regions after stress.

We observed increases in DNA damage-associated kinase levels after variable stress. p38 MAPK activity plays a role in a number of processes associated with stress pathology, including synaptic destabilization (Collingridge et al., 2010), and is known to play a role in the acute behavioral effects of stress (Bruchas et al., 2007). However, numerous reports suggest that the increased p38 MAPK activity observed following stimuli that induce DNA damage may actually be protective (Tan et al., 2014; Wang et al., 2014). GSK3 β is a kinase that regulates a diverse array of cell properties including structure, gene expression, and apoptosis (Kaidanovich-Beilin and Woodgett, 2011). Our data are the first to demonstrate *in vivo* regulation of GSK3 β S³⁸⁹ phosphorylation by stress. Nuclear GSK3 β activation in response to DNA damage is known to engage proapoptotic signaling (Watcharasit et al., 2002), and inhibition has been shown to promote DNA repair in hippocampal culture (Yang et al., 2011). p38 MAPK inhibition of GSK3 β through S³⁸⁹ phosphorylation has been shown to be independent of S⁹ phosphorylation, predominantly nuclear, and induced by DNA damage (Thornton et al., 2008, 2016). Given the data reported here, nuclear GSK3 β S³⁸⁹ phosphorylation by p38 MAPK is likely a defensive adaptation, to inhibit GSK3 β and promote DNA repair (Thornton et al., 2016). Indeed, our recent report demonstrates that mice that cannot inhibit nuclear GSK3 β via S³⁸⁹ phosphorylation exhibit an augmented fear response that is associated with neurodegradation in limbic brain regions (Thornton et al., 2017).

Unrepaired DNA damage may result in cell death, and accumulated DNA damage is hypothesized to underlie numerous neurodegenerative pathologies (Madabhushi et al., 2014). The data reported here suggest that accumulating DNA damage may play an important role in stress pathology, and point to a pair of kinases that may play a protective role when such damage is evident. Continued work to understand the timing of DNA damage induction and repair, as well as the causative role of DNA damage in stress pathology, is necessary to better understand the implications of the DNA damage produced by stress exposure.

Abbreviations

BNST	bed nucleus of the stria terminalis
DSBs	double-strand breaks
GSK3β	glycogen synthase kinase 3 β
MAPK	mitogen-activated protein kinase
mPFC	medial prefrontal cortex
PBS	phosphate-buffered saline
γH2AX	Histone H2A-X phosphorylated at serine 139

References

- Bruchas MR, Land BB, Aita M, Xu M, Barot SK, Li S, Chavkin C. Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. *J Neurosci*. 2007; 27:11614–11623. [PubMed: 17959804]
- Collingridge GL, Peineau S, Howland JG, Wang YT. Long-term depression in the CNS. *Nat Rev Neurosci*. 2010; 11:459–473. [PubMed: 20559335]
- Farkas J, Kovacs LA, Gaspar L, Nafz A, Gaszner T, Ujvari B, Kormos V, Csernus V, Hashimoto H, Reglodi D, Gaszner B. Construct and face validity of a new model for the three-hit theory of depression using PACAP mutant mice on CD1 background. *Neuroscience*. 2017; 354:11–29. [PubMed: 28450265]
- Flint MS, Baum A, Chambers WH, Jenkins FJ. Induction of DNA damage, alteration of DNA repair and transcriptional activation by stress hormones. *Psychoneuroendocrinology*. 2007; 32:470–479. [PubMed: 17459596]
- Fox JH, Hammack SE, Falls WA. Exercise is associated with reduction in the anxiogenic effect of mCPP on acoustic startle. *Behav Neurosci*. 2008; 122:943–948. [PubMed: 18729648]
- Gaszner B, Kormos V, Kozicz T, Hashimoto H, Reglodi D, Helyes Z. The behavioral phenotype of pituitary adenylate-cyclase activating polypeptide-deficient mice in anxiety and depression tests is accompanied by blunted c-Fos expression in the bed nucleus of the stria terminalis, central projecting Edinger-Westphal nucleus, ventral lateral septum, and dorsal raphe nucleus. *Neuroscience*. 2012; 202:283–299. [PubMed: 22178610]
- Hara MR, Kovacs JJ, Whalen EJ, Rajagopal S, Strachan RT, Grant W, Towers AJ, Williams B, Lam CM, Xiao K, Shenoy SK, Gregory SG, Ahn S, Duckett DR, Lefkowitz RJ. A stress response pathway regulates DNA damage through beta2-adrenoreceptors and beta-arrestin-1. *Nature*. 2011; 477:349–353. [PubMed: 21857681]
- Hara MR, Sachs BD, Caron MG, Lefkowitz RJ. Pharmacological blockade of a beta(2)AR-beta-arrestin-1 signaling cascade prevents the accumulation of DNA damage in a behavioral stress model. *Cell Cycle*. 2013; 12:219–224. [PubMed: 23287463]
- Hare BD, D'Onfro KC, Hammack SE, Falls WA. Prior stress interferes with the anxiolytic effect of exercise in c57bl/6j mice. *Behav Neurosci*. 2012; 126:850–856. [PubMed: 23181384]
- He ZY, Wang WY, Hu WY, Yang L, Li Y, Zhang WY, Yang YS, Liu SC, Zhang FL, Mei R, Xing D, Xiao ZC, Zhang M. Gamma-H2AX upregulation caused by Wip1 deficiency increases depression-related cellular senescence in hippocampus. *Sci Rep*. 2016; 6:34558. [PubMed: 27686532]
- Jackson SP. Sensing and repairing DNA double-strand breaks. *Carcinogenesis*. 2002; 23:687–696. [PubMed: 12016139]
- Jaworski DM, Soloway P, Caterina J, Falls WA. Tissue inhibitor of metalloproteinase-2 (TIMP-2)-deficient mice display motor deficits. *J Neurobiol*. 2006; 66:82–94. [PubMed: 16216006]
- Kaidanovich-Beilin O, Woodgett JR. GSK-3: functional insights from cell biology and animal models. *Front Mol Neurosci*. 2011; 4:1–25. [PubMed: 21441980]
- Kim SY, Adhikari A, Lee SY, Marshel JH, Kim CK, Mallory CS, Lo M, Pak S, Mattis J, Lim BK, Malenka RC, Warden MR, Neve R, Tye KM, Deisseroth K. Diverging neural pathways assemble a behavioural state from separable features in anxiety. *Nature*. 2013; 496:219–223. [PubMed: 23515158]
- Kormos V, Gaspar L, Kovacs LA, Farkas J, Gaszner T, Csernus V, Balogh A, Hashimoto H, Reglodi D, Helyes Z, Gaszner B. Reduced response to chronic mild stress in PACAP mutant mice is associated with blunted FosB expression in limbic forebrain and brainstem centers. *Neuroscience*. 2016; 330:335–358. [PubMed: 27282087]
- Lluri G, Langlois GD, Soloway PD, Jaworski DM. Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates myogenesis and beta1 integrin expression in vitro. *Exp Cell Res*. 2008; 314:11–24. [PubMed: 17678891]
- Madabhushi R, Gao F, Pfenning AR, Pan L, Yamakawa S, Seo J, Rueda R, Phan TX, Yamakawa H, Pao PC, Stott RT, Gjoneska E, Nott A, Cho S, Kellis M, Tsai LH. Activity-induced DNA breaks govern the expression of neuronal early-response genes. *Cell*. 2015; 161:1592–1605. [PubMed: 26052046]

- Madabhushi R, Pan L, Tsai LH. DNA damage and its links to neurodegeneration. *Neuron*. 2014; 83:266–282. [PubMed: 25033177]
- Maluach AM, Misquitta KA, Prevot TD, Fee C, Sibille E, Banasr M, Andreazza AC. Increased neuronal DNA/RNA oxidation in the frontal cortex of mice subjected to unpredictable chronic mild stress. *Chronic Stress (Thousand Oaks)*. 2017; 1:1–11.
- Nestler EJ, Barrot M, Self DW. DeltaFosB: a sustained molecular switch for addiction. *Proc Natl Acad Sci USA*. 2001; 98:11042–11046. [PubMed: 11572966]
- Paxinos, G., Franklin, KBJ. *The mouse brain in stereotaxic coordinates*. Elsevier Academic Press; 2004.
- Redon CE, Dickey JS, Bonner WM, Sedelnikova OA. Gamma-H2AX as a biomarker of DNA damage induced by ionizing radiation in human peripheral blood lymphocytes and artificial skin. *Adv Space Res*. 2009; 43:1171–1178. [PubMed: 20046946]
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 1998; 273:5858–5868. [PubMed: 9488723]
- Suberbielle E, Sanchez PE, Kravitz AV, Wang X, Ho K, Eilertson K, Devidze N, Kreitzer AC, Mucke L. Physiologic brain activity causes DNA double-strand breaks in neurons, with exacerbation by amyloid-beta. *Nat Neurosci*. 2013; 16:613–621. [PubMed: 23525040]
- Tan W, Yu HG, Luo HS. Inhibition of the p38 MAPK pathway sensitizes human gastric cells to doxorubicin treatment in vitro and in vivo. *Mol Med Rep*. 2014; 10:3275–3281. [PubMed: 25270341]
- Thornton TM, Delgado P, Chen L, Salas B, Kremmentsov D, Fernandez M, Vernia S, Davis RJ, Heimann R, Teuscher C, Krangel MS, Ramiro AR, Rincon M. Inactivation of nuclear GSK3beta by Ser(389) phosphorylation promotes lymphocyte fitness during DNA double-strand break response. *Nature Commun*. 2016; 7:1–12.
- Thornton, TM., Hare, B., Colie, S., Pendlebury, WW., Nebreda, AR., Falls, W., Jaworski, DM., Rincon, M. Failure to inactivate nuclear GSK3beta by Ser389-phosphorylation leads to focal neuronal death and prolonged fear response. *Neuropsychopharmacology*. 2017.
- Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B, Rincon M. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. *Science*. 2008; 320:667–670. [PubMed: 18451303]
- Walker DL, Toufexis DJ, Davis M. Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur J Pharmacol*. 2003; 463:199–216. [PubMed: 12600711]
- Wang X, Wang XL, Chen HL, Wu D, Chen JX, Wang XX, Li RL, He JH, Mo L, Cen X, Wei YQ, Jiang W. Ghrelin inhibits doxorubicin cardiotoxicity by inhibiting excessive autophagy through AMPK and p38-MAPK. *Biochem Pharmacol*. 2014; 88:334–350. [PubMed: 24522112]
- Watcharasit P, Bijur GN, Zmijewski JW, Song L, Zmijewska A, Chen X, Johnson GVW, Jope RS. Direct, activating interaction between glycogen synthase kinase-3 β and p53 after DNA damage. *Proc Natl Acad Sci USA*. 2002; 99:7951–7955. [PubMed: 12048243]
- Wood CD, Thornton TM, Sabio G, Davis RA, Rincon M. Nuclear localization of p38 MAPK in response to DNA damage. *Int J Biol Sci*. 2009; 5:428–437. [PubMed: 19564926]
- Yang ES, Nowsheen S, Wang T, Thotala DK, Xia F. Glycogen synthase kinase 3beta inhibition enhances repair of DNA double-strand breaks in irradiated hippocampal neurons. *Neuro Oncol*. 2011; 13:459–470. [PubMed: 21398658]

Fig. 1.

(A) Normalized body weight comparison between mice subjected to variable stress and unstressed controls. (B) Normalized acoustic startle response. Startle tests were conducted 24 h prior to and 24 h subsequent to variable stress exposure. $n = 8/\text{group}$, $*p < 0.05$.

Fig. 2. γ H2AX expression, normalized to tubulin, 24 h after the final stress exposure of a 14-day variable stress paradigm. Western blot analysis revealed that stress increased γ H2AX levels across brain regions. $n = 4/\text{group}$. $**p < 0.01$. Amygdala (Amyg), bed nucleus of the stria terminalis (BNST), hippocampus (Hippo), medial prefrontal cortex (mPFC).

Fig. 3.

DNA damage-associated kinase expression, normalized to tubulin, 24 h after the final stress exposure of a 14-day variable stress paradigm. (A) Stress resulted in a broad elevation in phosphorylated p38 MAPK (p-p38MAPK) expression. (B) Levels of total p38 MAPK (t-p38MAPK) were also increased following stress. (C) Elevated levels of GSK3 β serine 389 (p-S³⁸⁹) phosphorylation were evident following stress. (D) Stress-associated elevations in total GSK3 β were also apparent. $n = 4/\text{group}$, Statistical comparisons detailed in text. Amygdala (Amyg), bed nucleus of the stria terminalis (BNST), hippocampus (Hippo), medial prefrontal cortex (mPFC).

Fig. 4.

Bed nucleus of the stria terminalis (BNST) γ H2AX expression, normalized to tubulin, 24 h after single (acute) or a 14-day variable stress exposure. A single forced swim stress was insufficient to increase γ H2AX expression 24 h after the exposure. In contrast, variable stress for 14 days, terminating with swim stress, resulted in elevated levels of γ H2AX 24 h after the final stressor. $n = 5/\text{group}$ ** $p < 0.01$ versus respective control.

Fig. 5.

Immunohistochemical analysis of γ H2AX expression following variable stress. (A) Percent of cells with γ H2AX foci was quantitated in the dorsal BNST. Immunohistochemistry demonstrates that variable stress significantly increased the percent of cells, indicated by DAPI nuclear staining (blue), with detectable γ H2AX foci (red). $n = 4/\text{group}$, *** $p < 0.001$. (B) γ H2AX (green) is highly co-localized with GSK3 β p-S³⁸⁹ immunoreactivity (red). Scale bars = 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Short-term effects of stress exposure on γ H2AX and GSK3 β p-S³⁸⁹ levels. Changes in γ H2AX and GSK3 β p-S³⁸⁹ levels were not observed (A) 30 min or (B) 90 min after a single swim stress exposure. $n = 5-6$ /group.