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Behavioral Stress-induced Activation of FoxO3a in the Cerebral Cortex of Mice

Wenjun Zhou, Ligong Chen, Sufen Yang, Fuzeng Li, and Xiaohua Li

Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract

Background—The transcription factor FoxO3a is highly expressed in brain, but little is known about the response of FoxO3a to behavioral stress and its impact in the associated behavioral changes.

Methods—We tested the response of brain FoxO3a in the learned helplessness (LH) paradigm and tested signaling pathways that mediate the response of FoxO3a.

Results—A single session of inescapable shocks (IES) in mice reduced FoxO3a phosphorylation at the Akt-regulating serine/threonine residues and induced prolonged nuclear accumulation of FoxO3a in the cerebral cortex, both indicate activation of FoxO3a in brain. The response of FoxO3a is accompanied by a transient inactivation of Akt and a prolonged activation of glycogen synthase kinase-3 β (GSK3 β). Noticeably, FoxO3a formed a protein complex with GSK3 β in the cerebral cortex, and the interaction between the two proteins was stronger in IES-treated mice. Inhibition of GSK3 was able to abolish IES-induced LH behavior, disrupt IES-induced GSK3 β -FoxO3a interaction, and reduce nuclear FoxO3a accumulation. *In vitro* approaches further revealed that the interaction between GSK3 β and FoxO3a was strongest when both were active, FoxO3a was phosphorylated by recombinant GSK3 β , and GSK3 inhibitors effectively reduced FoxO3a transcriptional activity. Importantly, IES-induced LH behavior was markedly diminished in FoxO3a-deficient mice that have minimal FoxO3a expression and reduced levels of FoxO3a-inducible genes.

Conclusions—FoxO3a is activated in response to IES by interacting with GSK3 β , and inhibition of GSK3 β or reducing FoxO3a expression promotes resistance to stress-induced behavioral disturbance by disrupting this signaling mechanism.

Keywords

FoxO3a; GSK3 β ; Akt; stress; learned helplessness; signal transduction

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Correspondence: Xiaohua Li, MD, PhD, 1720 7th Avenue South, Sparks Center 1001A, Birmingham, AL 35294, Phone: (205) 934-1169, Fax: (205) 934-2500, xili@uab.edu.

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CONFLICT OF INTEREST

The authors report no biomedical financial interests or potential conflicts of interest.

INTRODUCTION

FoxO3a is a subtype of mammalian FoxO transcription factors (1,2) that are identified as orthologs of the *C. elegans* DAF16 (3). FoxO3a is active when it is localized in the nucleus (4-6), a process tightly regulated by posttranslational modification. Activation of Akt by trophic signals phosphorylates FoxO3a at Thr32, Ser253, and Ser315 residues, which promotes sequestration of FoxO3a from the nucleus to the cytosol by the chaperone protein 14-3-3, therefore inactivates FoxO3a (7-11). Besides responding to trophic signals, environmental and physiological stresses, such as oxidative stress, UV irradiation, and food restriction, can activate FoxO3a, a response regulated by Akt-independent mechanisms, such as Jun N-terminal Kinase (JNK), acetyltransferase CBP and p300, and sirtuin deacetylases (12-18).

FoxO3a is highly expressed and widely distributed in adult brain (2,4,19,20). Destructive brain insults, such as ischemia and epileptic seizures, have been shown to increase the level of active FoxO3a that acts to eliminate damaged neurons by apoptosis (21-23). However, it is less known if brain FoxO3a is only active during the extreme apoptotic insults or FoxO3a has other functions in response to abnormal brain activity, such as behavioral stress.

Behavioral stress often induces mood-related behavioral disturbance in vulnerable individuals, such as depression (24,25), as a result of disturbed neurotransmission, brain gene expression, and neuroplasticity (26,27). We and others previously reported that neurotrophins phosphorylate and inactivate FoxO3a in neuronal cells (28,29). Enhancing serotonin neurotransmission in animal brain also strongly phosphorylates and inactivates brain FoxO3a (30), a result in agreement with findings in *C. elegans* that activation of serotonin receptors led to inhibition of DAF-16 transcriptional activity (31). Furthermore, both the monoamine reuptake inhibitor antidepressant imipramine and the mood stabilizer lithium suppress FoxO3a activity in mouse brain via different mechanisms of action (30,32). In accordance with these findings, mice with FoxO3a-deficiency have higher resistance to stress-induced despair behavior in the forced swim and tail suspension tests (30).

We therefore hypothesize that brain FoxO3a may be overactive in response to behavioral stress. In this study, we investigated the response of mouse brain FoxO3a to inescapable foot shocks (IES) in the learned helplessness (LH) paradigm, and examined the underlying mechanisms mediating the response of FoxO3a and the behavioral impact of FoxO3a.

MATERIALS AND METHODS

In addition to the brief descriptions of methods below, detailed Materials and Methods can be found online in Supplemental Information.

Animals

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the experimental protocol using mice. Adult (10-12 wk old) male mice were used for all experiments. GSK3 inhibitor BIP-135 (33,34) or saline was infused into the right cerebral ventricle of mice via a cannula once daily. Behavioral stress was induced by repeated inescapable foot shocks (IES) (35,36). Escape latency and failure were recorded as described (37), and social interaction was tested with a modified protocol (38).

Cells

Human SH-SY5Y neuroblastoma cells and embryo kidney (HEK)-293 cells were used for adenovirus infection and DNA plasmid transfection of FoxO3a and GSK3 β DNA constructs.

Bioassays

For brain protein assays, mice were sacrificed on day-1, day-3 and day-8 after IES (Fig. S1A in the Supplement). Proteins from homogenate and nuclear/cytosolic extracts of mouse cerebral cortex were prepared as described (30,39). Proteins were immunoblotted with antibodies to phosphorylated or total FoxO3a, Akt, GSK3 β , GSK3 α , and JNK-1. Immunoprecipitation was performed using anti-FoxO3a and anti-GSK3 β antibodies (40).

Bioluminescence resonance energy transfer (*BRET*) assay (41) was performed in HEK cells transiently co-transfected with RLuc-FoxO3a and YFP-GSK3 β or their mutants as described (40).

FoxO3a phosphorylation by GSK3 β was performed *in vitro* using immunoprecipitated HA-tagged wild type FoxO3a and recombinant human GSK3 β in the presence of 32 P-ATP.

FoxO3a transcriptional activity was measured by luciferase assay in HEK cells co-transfected with FoxO3a and 6xDBE-firefly/renilla dual-luciferase plasmids (32).

Gene expression of FoxO3a, Bim, p27, and GADD-45 in the cerebral cortex of mouse brain were measured by quantitative Real-time PCR (qRT-PCR) using the TaqMan Universal PCR system (Applied Biosystems).

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analyses were conducted using SigmaStat 10.0. All data were checked for assumptions of normal distribution and homogeneity of variances in study samples, and any unexplained outlier value greater than ± 2 standard deviations from the mean of the group was excluded. For a two-group comparison, statistical analysis was performed using unpaired Student's t-test. For comparison of more than three experimental groups, one-way or two-way analysis of variance (ANOVA) was used to test for significant differences ($p \leq 0.05$) between groups. Any significant difference detected by ANOVA was followed by post hoc or inter-group comparisons using Tukey's, Holm-Sidak, or Tamhane's test.

RESULTS

To induce behavioral stress, adult (10-12 wk old) male wild type C57BL/6 mice were subjected to a single session of IES that includes 180 repeated inescapable foot shocks delivered at unpredictable shock durations and intervals. Control mice were exposed to the foot shock apparatus for the same length of time without receiving IES. When behaviors were tested on post-IES day-1, IES-treated mice exhibited significantly longer escape latency than control mice and higher numbers of escape failure ($77.6 \pm 7.1\%$ of IES-treated mice and $5.4 \pm 3.6\%$ of control mice failed more than 15 of the 30 escape trials) (Fig. S1B in the Supplement). In addition, IES-treated mice exhibited poorer social interaction when compared to control mice (Fig. S1C in the Supplement). When the duration of LH behavior after a single session of IES was tested along a 15-day period of time, the elevated escape latency peaked on post-IES day-1, gradually reduced to approximately 50% of maximal but significantly higher than control level between day-3 and day-8, and remained at a residual longer escape latency even during day-11 and day-15 (Fig. S1D in the Supplement).

The response of FoxO3a to IES was measured in the cerebral cortex that is a major stress-responding brain area (42-44). Although the levels of phospho-T32-FoxO3a and phospho-S253-FoxO3a (representing the Akt-regulated inactive state of FoxO3a (8)) were not different between IES-treated and control mice on post-IES day-1 (Fig. 1A), both were significantly lower in IES-treated mice than control mice on post-IES day-3. On post-IES

day-8, there was no significant difference on phospho-T32-FoxO3a between IES-treated and control mice, but phospho-S253-FoxO3a showed a trend of reduction ($p=0.057$) in IES-treated mice.

Since active FoxO3a locates in the nucleus, the levels of nuclear and cytosolic FoxO3a in the cerebral cortex were measured. On post-IES day-1, IES did not cause significant change of nuclear or cytosolic FoxO3a, whereas on post-IES day-3, nuclear FoxO3a in IES-treated mice elevated to a significantly higher level than in control mice, and this was accompanied by a small but significant reduction of cytosolic FoxO3a (Fig. 1B). Surprisingly, the nuclear FoxO3a remained at a significantly higher level in IES-treated mice than control mice on post-IES day-8, suggesting that IES causes a prolonged activation of FoxO3a in the cerebral cortex.

Since Akt-dependent FoxO3a phosphorylation was decreased following IES treatment, we measured the levels of phospho-T308-Akt and phospho-S473-Akt that represent active Akt to determine if Akt activity is decreased by IES. No significant difference of phospho-T308-Akt or phospho-S473-Akt between IES-treated and control mice was found on post-IES day-1, but on post-IES day-3, the levels of both phospho-T308-Akt and phospho-S473-Akt reduced significantly in IES-treated mice without a change of total Akt (Fig. 2). The reduction, however, was no longer significant on post-IES day-8. Therefore, IES caused a transient inactivation of Akt, which is in accordance with the time of reduction in Akt-dependent FoxO3a phosphorylation. However, the transient reduction of Akt activity does not concur with the prolonged robust nuclear accumulation of FoxO3a, suggesting that other regulatory mechanisms may be involved.

We previously reported (37) that the sensitivity of developing IES-induced LH behavior is markedly enhanced in GSK3 knock-in mice (45) that express constitutively active GSK3, a protein kinase that is also negatively regulated by Akt (46). We therefore measured the inhibitory serine phosphorylation and the total level of GSK3 β and GSK3 α , the two isoforms of GSK3. On post-IES day-1, there was no significant change of phospho-S9-GSK3 β in the cerebral cortex when IES-treated mice were compared to control mice (Fig. 3A). However, the level of phospho-S9-GSK3 β was significantly lower in IES-treated mice than in control mice on post-IES day-3, but the level of total GSK3 β did not change. Different from Akt phosphorylation, phospho-S9-GSK3 β remained significantly lower in IES-treated than in control mice on post-IES day-8, and there was a trend of increase in total GSK3 β ($p=0.064$), both suggest a prolonged active state of GSK3 β , which has a similar time course as the prolonged elevation of nuclear FoxO3a. In contrast, there was no significant change of phospho-Ser21-GSK3 α or total GSK3 α at the tested post-IES days (Fig. 3B).

JNK-1 was reported to activate FoxO3a during oxidative and physical stresses (12-14), but no significant changes of phosphorylated or total JNK-1 was found in the cerebral cortex after IES (Table S1 in the Supplement).

With the prominent effect of IES inducing a concomitant activation of GSK3 β and FoxO3a, we next tested if there is an interaction between GSK3 β and FoxO3a in mouse brain when they respond to IES. Interestingly, not only that GSK3 β and FoxO3a from the cerebral cortex co-immunoprecipitated with each other, but the amount of co-immunoprecipitated proteins was significantly higher in IES-treated mice than in control mice on both post-IES day-3 and day-8 (Fig. 4A). To further test the GSK3 β -FoxO3a interaction during IES, control and IES-treated mice were infused with the GSK3 inhibitor BIP-135 (0.08 nmol, i.c.v.). A single BIP-135 infusion 2 hr prior to behavior tests on post-IES day-1 significantly reduced LH behavior and normalized social interaction (Fig. 4B). Remarkably, once daily BIP-135 infusion on post-IES day-1, day-2, and day-3 largely reduced the interaction

between GSK3 β and FoxO3a (Fig. 4C) and completely occluded IES-induced increase in nuclear FoxO3a (Fig. 4D) on post-IES day-3.

To support a GSK3 β -dependent regulation of FoxO3a, we performed additional experiments *in vitro*. Using the distance-based BRET assay that detects protein interaction (41), we tested if the activation state of GSK3 β and FoxO3a affects their interaction. In this assay, a fixed concentration (0.5 μ g DNA) of RLuc-FoxO3a-WT or RLuc-FoxO3a-TM (the triple mutant that is resistant to Akt-regulated phosphorylation (8)) was co-transfected with different concentrations of YFP, YFP-GSK3 β -WT, or YFP-S9A-GSK3 β (Mutant GSK3 β that is unable to be phosphorylated at the inhibitory Ser-9 residue (47,48)) in HEK cells. YFP alone did not interact with FoxO3a, but YFP-GSK3 β -WT and FoxO3a-WT had a low level of interaction in a DNA concentration-dependent manner, reaching maximal interaction at 1.5 μ g YFP-GSK3 β -WT (Fig. 5A). Interestingly, the interaction was stronger when GSK3 β -WT was co-transfected with the active FoxO3a-TM or when the active S9A-GSK3 β was co-transfected with FoxO3a-WT, and the interaction was strongest when the active S9A-GSK3 β was co-transfected with the active FoxO3a-TM. This interaction pattern was indicated by a left shift of maximal effective concentration of YFP-GSK3 β and a significant difference in the maximal interaction at 1.5 μ g YFP-GSK3 β . Furthermore, the interaction between S9A-GSK3 β and FoxO3a-TM was significantly reduced by three GSK3 inhibitors, BIP-135, Bio, and SB216763 (Fig. 5B). To test if activation states of GSK3 β and FoxO3a affect their protein complex formation in cells, SH-SY5Y cells were infected with the adenovirus-carried HA-tagged FoxO3a-WT or FoxO3a-TM. When FoxO3a was immunoprecipitated, endogenous GSK3 β was found to co-immunoprecipitate strongly with FoxO3a-TM, but less with FoxO3a-WT (Fig. 5C). Vice versa, when SH-SY5Y cells were infected with the adenovirus-carried HA-tagged GSK3 β -WT or S9A-GSK3 β , only S9A-GSK3 β , but not GSK3 β -WT, co-immunoprecipitated with endogenous FoxO3a.

To test if GSK3 β phosphorylates FoxO3a, GSK3 β kinase assay was performed using immunoprecipitated HA-FoxO3a as the candidate substrate, which was indeed phosphorylated by recombinant GSK3 β *in vitro* (Fig. 5D). To also test if GSK3 β affects FoxO3a transcriptional activity, HEK cells transfected with FoxO3a-WT and the canonical 6xDBE-Luc were treated with GSK3 inhibitors BIP-135, Bio, or SB216763 under a serum-free condition (to reduce FoxO3a phosphorylation and increase its activity) followed by measuring luciferase activity (32). All three GSK3 inhibitors significantly reduced FoxO3a-induced luciferase activity (Fig. 5E).

To further understand the role of FoxO3a in IES-induced behavior, we tested LH behavior in FoxO3a-deficient mice that do not express functional FoxO3a mRNA and protein (30,49). When LH behavior was tested on post-IES day-1, control FoxO3a-deficient mice displayed similar escape latency as control wild type mice. However, IES-induced escape latency in FoxO3a-deficient mice was significantly lower than in wild type mice (Fig. 6A), and the escape failure only occurred in 12.5 $\pm$ 9.2% of IES-treated FoxO3a-deficient mice, which was significantly lower than IES-treated wild type mice (77.6 $\pm$ 7.1%). Also, when the escape latency was tested along the 15 days of post-IES period, in remarkable contrast to wild type mice, the mildly elevated escape latency in IES-treated FoxO3a-deficient mice rapidly returned to basal level on post-IES day-3, and there was no further observable LH behavior throughout the 15-day test (Fig. 6B). The IES-resistant behavior in FoxO3a-deficient mice was unlikely due to insensitivity to pain because these mice had similar sensitivity as wild type mice in the hot plate test.

Since FoxO3a-deficient mice are deprived of FoxO3a prior to IES, the observed behavioral effect could be the result of either resistance to stress or to developing LH behavior. We therefore measured phosphorylation of Akt and GSK3 β that were reduced in response to IES

in wild type mice on post-IES day-3. In FoxO3a-deficient mice, IES treatment was able to significantly reduce phospho-T308-Akt, phospho-S473-Akt, and phospho-Ser9-GSK3 β (Fig. 6C), which is a similar effect as found in wild type mice (Fig. 2 and Fig. 3), supporting a role of FoxO3a in developing LH behavior.

To test if altered level of FoxO3a in the cerebral cortex is associated with change of gene expression, the levels of several FoxO-regulated genes, Bim, p27, and GADD-45 (29,50-53) in the cerebral cortex were measured by qRT-PCR. In wild type mice, neither of these genes was changed significantly on the post-IES day-3, but IES caused a small albeit significant increase of P27 on post-IES day-8 (Fig. S2A in the Supplement). In FoxO3a-deficient mice that essentially had no FoxO3a expression, both Bim and p27 were significantly reduced by 37%, and GADD-45 was reduced by 27% (Fig. S2B in the Supplement).

DISCUSSION

Findings of this study strongly support the hypothesis that FoxO3a is activated by behavioral stress, indicated by IES-induced reduction of the inhibitory phosphorylation of FoxO3a and elevation of nuclear FoxO3a in the cerebral cortex. Since the cerebral cortex is a major brain area within the stress-regulating neurocircuit (42,43,54), and FoxO3a is highly expressed in the cerebral cortex (Allen Brain Atlas) (55), the study mainly tested the response of FoxO3a in this brain area. The significant activation of FoxO3a observed from the entire cerebral cortex suggests that the effect of IES to FoxO3a is robust, although it is possible that the increased activity of FoxO3a preferentially occurs in a sub-region of the cerebral cortex, such as the prefrontal cortex, which remains to be determined in detail. Among the FoxO subtypes expressed in brain, FoxO3a has the widest brain expression, whereas FoxO1 is mostly expressed in the hippocampus and striatum, and FoxO6 is mostly expressed during developmental stage with a nucleus-predominant distribution (2,4,19,20). In our previous studies (29,30,32,56), we demonstrated prominent regulation of FoxO3a by neurotrophins, serotonin, and psychotropics, and an antidepressant-like phenotype of FoxO3a-deficiency mice. Therefore, this study focused on studying FoxO3a, whereas it may also be important in the future to determine the similarity and difference of FoxO subtypes in response to stress.

Although our initial measurement of FoxO3a activity on post-IES day-1 did not reveal a significant change, it does not exclude an early response of FoxO3a to IES. IES can cause acute biochemical changes of the brain, such as increasing serotonin (57). Serotonin has been shown to increase FoxO3a phosphorylation by activating brain Akt (30), which could mask IES-responsive signals that lead to activation of FoxO3a at an early post-IES period. Despite being an acute stress, IES-induced biological changes in brain may last for several days (58,59), such as differentially changing the levels of neurotrophins (58), down-regulating signal transducers activators of transcription 3 (Stat3)-interacting protein (StIP1), up-regulating the suppressor of cytokine signaling 3 (Socs3) (60), and elevating the immediate early gene nerve growth factor-inducible gene A (NGFI-A) (59). The robust increase in FoxO3a activity on post-IES day-3 and day-8 highly suggests that there are FoxO3a-regulating machineries responsible for the prolonged activation of FoxO3a, and this study focused on identifying the underlying mechanism.

Since Akt is a major regulator of FoxO3a (7-9,11,61,62), and Akt-dependent FoxO3a phosphorylation decreased following IES treatment, we speculated that IES may inactivate Akt in the cerebral cortex, which was indicated by the reduction of Akt phosphorylation in IES-treated mice on post-IES day-3. Since it occurred at the same time point as the change of FoxO3a, it could be responsible for the initial reduction of FoxO3a phosphorylation and nuclear localization observed on post-IES day-3. However, the lack of significant reduction

of Akt activity on post-IES day-8 suggests that additional stress-responsive mechanisms other than Akt may be involved in maintaining the prolonged nuclear FoxO3a accumulation.

JNK was reported to activate FoxO3a during oxidative and physical stresses (12-14), but significant changes of total and phosphorylated active JNK-1 were not found in the cerebral cortex after IES. The negative finding suggests that activation of FoxO3a by behavioral stress may represent a very different pathological process from the better understood activation of FoxO3a by oxidative stress (15).

We measured phosphorylation of GSK3 in this study because we previously found (37) that the sensitivity of developing IES-induced LH behavior is markedly enhanced in mice that express constitutively active GSK3 that is resistant to phosphorylation at the N-terminal serine (45). Although the study found a robust dephosphorylation of GSK3 β by IES, but less effect on GSK3 α in the cerebral cortex, we do not exclude a response of GSK3 α to IES since GSK3 phosphorylation is measured in only one brain area for the purpose of this study, and phosphorylation at the N-terminal serine is not the only mechanism of regulating GSK3 activity. As the phosphorylation effect of IES appeared most significant on GSK3 β in the cerebral cortex, we chose to focus this current study on this GSK3 isoform, whereas the role of GSK3 α on FoxO3a activity remains to be studied in future.

GSK3 β is negatively regulated by Akt at serine-9 residue (46). The reduced phospho-S9-GSK3 β on post-IES day-3 has the same time point of change as seen with Akt, suggesting that activation of GSK3 β is at least partially the result of IES-induced inactivation of Akt. However, the reduced phospho-S9-GSK3 β and the trend of increase in total GSK3 β were prolonged to post-IES day-8 when Akt activity has recovered. The uncoupling between Akt and GSK3 β may be due to a selective dephosphorylation mechanism that affects GSK3 β , but spares dephosphorylation of Akt. For example, protein phosphatase-1 inhibitor-2 was reported to differentially regulate the KCl-mediated dephosphorylation of GSK3 β and Akt (63). Although the actual mechanism remains to be determined, the similar time course of the prolonged activation state of GSK3 β and FoxO3a let us hypothesize that GSK3 β could be an upstream regulator of FoxO3a in response to IES (Fig. S3 in the Supplement).

GSK3 β is a protein kinase that regulates many transcription factors either by phosphorylation or direct interaction (64,65), but regulation of FoxO3a by GSK3 β in mammalian brain has never been reported. We previously reported that chronic lithium treatment robustly inactivates FoxO3a in mouse brain (32). Since lithium is a GSK3 inhibitor both *in vitro* and *in vivo* (66-68), it raises the possibility that the effect of lithium on FoxO3a is via its inhibition of GSK3. A recent study with RNAi screening for kinase and phosphatase regulators of dFoxO in *Drosophila* S2 cells identified GSK3 β as one of the regulators of dFoxO (69). Our sequence search also revealed enriched GSK3 consensus phosphorylation sites (S/TXXXS/T) throughout FoxO3a protein sequence, suggesting that FoxO3a is a candidate phosphorylation substrate of GSK3. Results of this study provided convincing evidence that GSK3 β is a direct regulator of FoxO3a in response to IES by direct interaction and phosphorylation. Our data appears to suggest that the non-Akt-phosphorylated FoxO3a is a better substrate for GSK3 β , whereas dephosphorylated active GSK3 β has better access to FoxO3a, both processes can be facilitated by inactivating Akt (Fig. S3 in the Supplement). However, once GSK3 β and FoxO3a form a protein complex, GSK3 β becomes the driving force to maintain nuclear FoxO3a activity independently from Akt regulation.

While there is prolonged activation of FoxO3a after IES, It is also noticeable that both FoxO3a deficiency and GSK3 inhibitor reduced LH behavior on post-IES day-1. This effect may partly due to disrupting the baseline GSK3 β -FoxO3a complex, which is present at a

lower level in control mice. The behavioral effect in FoxO3a-deficient mice was not due to a pre-existing stress-resistant phenotype because IES is able to induce dephosphorylation of Akt and GSK3 β in FoxO3a-deficient mice despite of no observable LH behavior. In addition, GSK3 inhibitor administered after IES delivery was able to reduce LH behavior. Therefore, our data suggests a behavioral-biochemical model in that GSK3 β -FoxO3a complex is required for stress to induce behavioral disturbance. Meanwhile, stress triggers signal transduction mechanisms to build up a stronger GSK3 β -FoxO3a complex that may contribute to prolonged behavioral disturbance as well as induce gene effect. The significance of interrupting the GSK3 β -FoxO3a signaling pathway is therefore not to prevent stress, but to occlude stress-induced abnormal behaviors, which may serve as an effective treatment in stress-induced behavioral disturbance, such as depression.

As a transcription factor, activation of FoxO3a by IES is expected to induce gene expression. This study, however, only detected a small increase of p27 at post-IES day-8. The small change of p27 may be the initial gene response to IES-activated FoxO3a, but this result does not exclude the possibility that FoxO3a may regulate other neuronal genes that remain to be identified. On the other hand, prominent reduction of Bim and p27 were detected in FoxO3a-deficient mice, which is likely the result of lifetime FoxO3a deficiency. Bim is a proapoptotic gene, and reduced expression may have subtle neuroprotective effect (29,70,71). p27 is a cell cycle regulator (72,73), and in the cerebral cortex, p27 was found to promote neuronal differentiation and migration (74,75). Although not the focus of this study, it could be interesting to further determine if deletion of Bim or p27 or other FoxO3a-targeting genes has a direct effect in reducing IES-induced behavioral disturbance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

IES-induced activation of FoxO3a in the cerebral cortex. The cerebral cortex from control (CTL) and IES-treated mice were dissected on post-IES day-1 (n=6/group), day-3 (n=14/group), and day-8 (n=8/group). (A) Protein homogenates were immunoblotted for phospho-T32-FoxO3a, phospho-S253-FoxO3a, and total FoxO3a. (B) Nuclear and cytosolic proteins were immunoblotted separately for total FoxO3a. Histone and β -tubulin were immunoblotted as quality control of nuclear/cytosolic extraction. Data is expressed as % control (no IES). Mean $\pm$ SEM, * p <0.05 in Student's t-test when IES-treated mice were compared to control mice.

Figure 2.

IES inactivates Akt in the cerebral cortex. Protein homogenates from the cerebral cortex of control (CTL) and IES-treated mice were immunoblotted for phospho-T308-Akt, phospho-S473-Akt, and total Akt. Data is expressed as % control (no IES). Mean $\pm$ SEM, $n=6-14$ /group, * $p<0.05$ in Student's t-test when IES-treated mice were compared to control mice.

Figure 3.

IES decreases the inhibitory serine phosphorylation of GSK3 β in the cerebral cortex. Protein homogenates from the cerebral cortex of control (CTL) and IES-treated mice were immunoblotted for (A) phospho-S9-GSK3 β and total GSK3 β , (B) phospho-S21-GSK3 α and total GSK3 α . Data is expressed as % control (no IES). Mean $\pm$ SEM, n=6-14/group, *p<0.05 in Student's t-test when IES-treated mice were compared to control mice.

Figure 4.

GSK3 β interacts and regulates FoxO3a in mouse cerebral cortex. (A) The cerebral cortex from control (C) and IES (S)-treated mice were dissected on post-IES day-3 and day-8. Protein homogenates were immunoprecipitated with anti-FoxO3a or anti-GSK3 β , and immunocomplex and homogenates (input) were immunoblotted for FoxO3a and GSK3 β . Data is expressed as the ratio of the protein in the immunocomplex (Co-IP) to total lysate. Mean $\pm$ SEM, $n=4-6$ /group, $*p<0.05$ in Student's t -test when IES-treated mice were compared to control mice. (B-D) Control and IES-treated mice received daily i.c.v. infusion of saline or the GSK3 inhibitor BIP-135 (0.08 nmol). (B) LH behavior and social interaction were tested on post-IES day-1, 2 hr after GSK3 inhibitor treatment. Mean $\pm$ SEM, $n=6$ /group, $*p<0.05$ in Student's t -test. (C) After 3 doses of BIP-135 on post-IES day-1, 2, and 3, protein homogenates from the cerebral cortex were immunoprecipitated for FoxO3a and the immunocomplexes were immunoblotted for FoxO3a and GSK3 β . Data is expressed as the ratio of the protein in the immunocomplex (Co-IP) to total lysate. Mean $\pm$ SEM, $n=6$ /group, $*p<0.05$ in two-way ANOVA with post-hoc analysis. (D) After BIP-135 on post-IES day-1,

2, and 3, nuclear proteins from the cerebral cortex were immunoblotted for FoxO3a. Data is expressed as % control (no IES). Mean $\pm$ SEM, n=6/group, *p<0.05 in Student's t-test.

Figure 5.

Interaction and regulation of FoxO3a *in vitro* by GSK3β. (A) BRET assay in HEK cells transfected with a fixed concentration (0.5 μg DNA) of RLuc-FoxO3a-WT or RLuc-FoxO3a-TM and different concentrations of YFP, YFP-GSK3β-WT, or YFP-S9A-GSK3β. Right panel shows statistical analysis from 1.5 μg of YFP or GSK3β transfection. Data is expressed as BRET ratio. Mean ± SEM, n=3/group, *p<0.05 in two-way ANOVA. (B) HEK cells expressing S9A-GSK3β and FoxO3a-TM were treated with GSK3 inhibitors BIP-135, Bio, and SB216763 (10 μM) for 2 or 4 hr before BRET assay. Mean ± SEM, n=6/group, *p<0.05 in Student's t-test when GSK3 inhibitor-treated cells were compared to no inhibitor treatment. (C) SH-SY5Y cells infected with the adenovirus-carried HA-tagged FoxO3a-WT or FoxO3a-TM were immunoprecipitated with anti-FoxO3a, and immunocomplexes were immunoblotted for endogenous GSK3β. SH-SY5Y cells infected with the adenovirus-carried HA-tagged GSK3β-WT or the S9A-GSK3β were immunoprecipitated with anti-GSK3β, and immunocomplexes were immunoblotted for endogenous FoxO3a. (D) Protein lysate from SH-SY5Y cells infected with adeno-HA-FoxO3a were immunoprecipitated with anti-HA-FoxO3a. The immunocomplex was then incubated *in vitro* with recombinant GSK3β in the presence of ³²P-ATP. HA and FoxO3a in the cell lysates and immunocomplex were detected by immunoblots, and phosphorylated FoxO3a were detected by a phosphoimager. (E) HEK cells were transfected with FoxO3a-WT and 6xDBE-luciferase, and treated with the GSK3 inhibitors BIP-135, Bio, or SB216763 (10 μM) for 2 or 4 hr, followed by measuring 6xDBE-luciferase activity. Data is expressed as relative luciferase activity. Mean ± SEM, n=4/group, *p<0.05 in one-way ANOVA.

Figure 6.

Effects of FoxO3a-deficiency in LH behavior. (A) Control (CTL) and IES-treated wild type and FoxO3a-deficient (F3a-def) mice were tested for LH behavior on post-IES day-1. Mean $\pm$ SEM, $n=8-10$ /group. $*p<0.05$ in two-way ANOVA. (B) LH behavior in wild type and FoxO3a-deficient mice were tested on post-IES day-1, 3, 5, 8, 11, and 15. Mean $\pm$ SEM, $n=6$ /group, $*p<0.05$ among groups in repeated ANOVA. (C) Wild type and FoxO3a-deficient mice were tested for sensitivity to hot plate. Mean $\pm$ SEM, $n=5$ /group. (D) The cerebral cortex of control (CTL) and IES-treated FoxO3a-deficient mice were dissected on post-IES day-3, and protein homogenates were immunoblotted for phospho-T308-Akt, phospho-S473-Akt, and phospho-S9-GSK3 β . Data is expressed as % control (no IES). Mean $\pm$ SEM, $n=4$ /group, $*p<0.05$ in Student's t-test.