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RESEARCH ARTICLE

GSK-3β MAY BE A FACTOR FOR RESTRAINT STRESS-INDUCED DEPRESSION-RELATED BEHAVIORS AND SPATIAL MEMORY IMPAIRMENT INDEPENDENTLY OF TAU PHOSPHORYLATION

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ABSTRACT

Restraint stress significantly prolonged immobility time in forced swim and tail suspension tests for mice, indicating that restraint stress causes depression-related behaviors. Moreover, restraint stress significantly prolonged the retention latency in the water maze test, indicating that restraint stress causes spatial memory impairment. Phosphorylation of glycogen synthase kinase 3β (GSK-3β) in the hippocampus and hypothalamus from mice with restraint stress was significantly reduced as compared with that for mice without restraint stress, indicating that GSK-3β is activated by restraint stress. In contrast, tau phosphorylation was not affected both in the hippocampus and hypothalamus from mice with restraint stress. Taken together, the results of the present study suggest that GSK-3β is a pivotal factor for stress-induced depression and dementia, independently of tau phosphorylation.

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INTRODUCTION

Glycogen synthase kinase 3 (GSK-3), a serine/threonine protein kinase, regulates a wide-range of biological processes such as cell differentiation, metabolism, immunity, and cell survival (Maurer *et al*., 2014). GSK-3 includes two isoforms, α and β, and of them GSK-3β is most abundantly expressed in neurons (Leroy and Brion, 1999). Accumulating evidence has shown that GSK-3β plays a significant role in serotonergic transmission-mediated signaling networks relevant to mental illnesses such as mood disorder, psychosis, and depression (Li and Jope, 2010; Latapy *et al*., 2012; Polter *et al*., 2012). Moreover, GSK-3β is implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (Takashima, 2006; Laurett*iet al*., 2020). GSK-3β phosphorylates tau protein, causing aggregation of tau and formation of neurofibrillary tangles (NFT) (Amaral *et al*., 2021). A consistent pathological finding is that NFT is found in the brain of patients with dementia including Alzheimer's disease (Gomez-Isla *et al*., 1997; Gomez-Isla *et al*., 2008). Notably, soluble phosphorylated forms of tau could also exert the synaptotoxic effect by propagating between neurons across neuronal networks (Dujardin *et al*., 2014; Tai *et al*., 2012). Intriguingly, it is suggested that depression is related to dementia (Byers and Yaffe, 2011). Little, however, is known about the underlying mechanism. To address this point, we carried out forced swim and tail suspension tests and water maze test in mice with restraint stress, followed by Western blotting in the hippocampus and hypothalamus from the mouse brains.

The results of the present study demonstrate that restraint stress causes depression-related behaviors and spatial memory impairment and that GSK-3β phosphorylation at Ser9 was significantly reduced both in the hippocampus and hypothalamus from mice with restraint stress without affecting tau phosphorylation at Ser202/Thr205 and Ser396. This indicates that stress may cause depression and dementia by activating GSK-3β, independently of tau phosphorylation.

MATERIALS AND METHODS

Animal care: All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Restraint stressed mice: Male C57BL/6J mice were obtained at 8 week of age from Japan SLC Inc. (Shizuoka. Japan). Mice were restricted in a plastic cylinder 11.5 cm in height and 2.7 cm in diameter for 3 h once a day in the morning, and this operation was performed for consecutive three days. Control mice without restraint stress were bred in a normal cage till behavioral tests.

Forced swim test: Forced swim test was performed on day 7 after restraint stress by the method previously described (Porsolt*et al*., 1978). Briefly, mice were placed in a plastic cylinder 25 cm in height and 10 cm in diameter filled with water at 23 °C to 15-cm height for 6 min, and the floating time without moving their hindlimbs and swimming, observed during the last 4 min, was measured as an immobility time.

Tail suspension test: Mice, different from ones used for forced swim test, were tested using a tail suspension apparatus on day 7 after restraint stress by the method as previously described (Crowley *et al*., 2004). Briefly, mice were suspended by their tails with tape in a box. Immobility was defined as the absence of initiated movements and included passive swaying, and immobility duration was measured in the maximal 6-min test time.

Water maze test: Water maze test was carried out from one day after forced swim test in non-stressed control and stressed mice, and live video was recorded for each trial using a standard tracking system. A circular plastic water tank 90 cm in diameter and 36 cm in deep was used for a water maze test. The entire inside of the pool was painted black, and the pool was filled up to 20 cm from the bottom with water containing India ink at 22 °C. A platform (11 cm in diameter) painted black was placed into water, the top submerged 0.5 cm below water surface. The pool was put in a test room where there were several marks that mice are able to see from the pool. The position of the marks remained unchanged throughout testing. A platform was located in the constant position, i.e., in the middle of one quadrant, equidistant from the center and edge of the pool. Mice facing the wall of the pool were placed into water at one of 5 positions selected at random, and time from start to escape onto the platform (acquisition latency) was measured. When succeeded, mice were allowed to stay on the platform for 10 s. When mice failed to find the platform within 90 s, the trial was stopped and mice were put on the platform for 10 s. Two trials were carried out a day, and the second trial began 2 min after the end of the first trial. Mice received the task for consecutive 8 days, and the mean latency from consecutive 2 days was calculated. Path length and time-of-arrival to the platform for each mice were measured and swim speed was calculated. Seven days later, the platform was removed and the retention latency (time from the start to arrival to the place where the platform had been set, 30 s in maximum) was measured. In a different set of experiments, the number of passes over the missing platform was counted during 90 s.

Western blotting: After water maze test the hippocampus and hypothalamus were isolated from the mouse brain, and lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis using a TGX gel (BioRad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes. After blocking with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin, blotting membranes were reacted with antibodies (1:1000 dilution) against phospho-Ser9-GSK-3β (pS9)(Cell Signaling Technology), GSK-3β (Cell Signaling Technology), phospho-Ser202/Thr205-tau (pS202/T205)(Thermo Fisher Scientific, Waltham, MA, USA), phospho-Ser396-tau (pS396)(Cell Signaling Technology), and tau (Cell Signaling Technology), followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Signal density was measured with an ImageQuant software (GE Healthcare).

Statistical analysis: Statistical analysis was carried out using Dunnett's test.

RESULTS

Restraint stress causes depression-related behaviors: We initially examined the effect of restraint stress on depression-related behaviors in the forced swim and tail suspension tests. In the forced swim test, the immobility time for mice with restraint stress was significantly longer than that for non-stressed control mice (Figure 1).

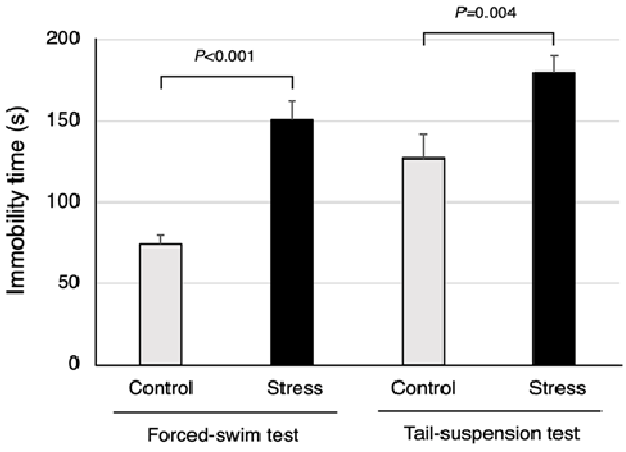

Figure 1. Restraint stress causes depression-related behaviors. Forced swim and tail suspension tests were carried out in non-stressed control (Control) and stressed mice (Stress), and immobility time was measured. In the graph, each column represents the mean (± SEM) immobility time (n=8 independent mice). P values, Dunnett's test.

In the tail suspension test, the immobility time for mice with restraint stress was also significantly prolonged as compared with that for non-stressed control mice (Figure 1). Collectively, these results indicate that restraint stress causes depression-related behaviors.

Restraint stress causes spatial memory impairment: We subsequently examined the effect of restraint stress on spatial learning and memory in the water maze test. There was no significant difference in the acquisition latency between for non-stressed control and stressed mice (data not shown). In contrast, the retention latency for mice with restraint stress was significantly longer than that for control mice (Figure 2A), indicating that restraint stress causes spatial memory impairment. In support of this note, the number of passes over missing platform for mice with restraint stress was significantly decreased as compared with that for non-stressed

control mice (Figure 2B).No significant difference in the swim speed was found between non-stressed control and stressed mice (Figure 2C). This implies that prolongation in the retention latency for mice with restraint stress is not due to motility disturbance. Taken together, these results indicate that restraint stress is a factor for memory disturbance.

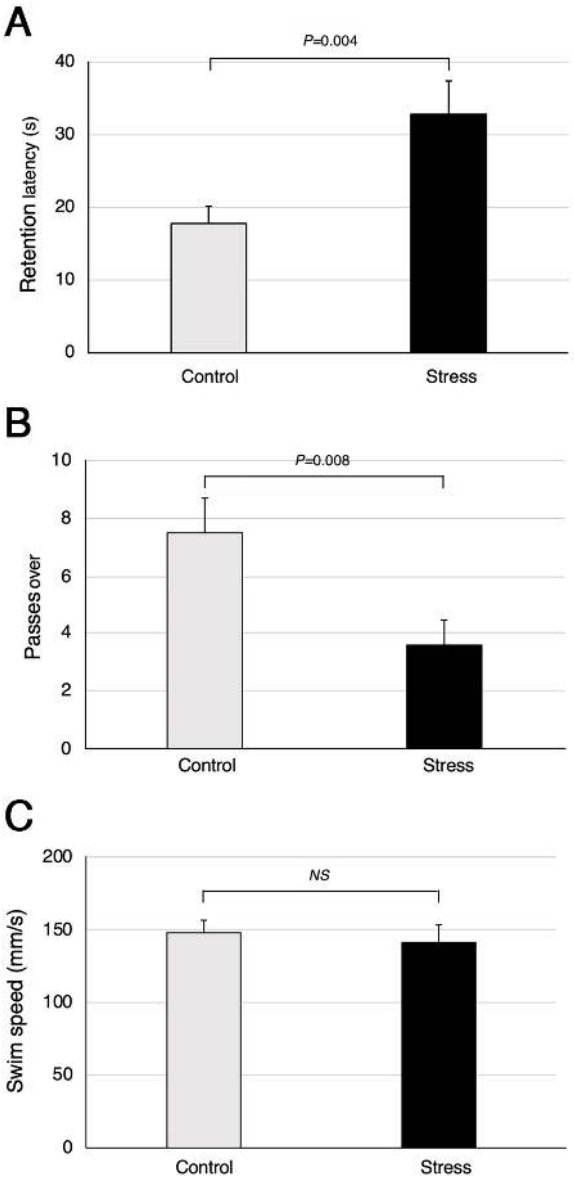

Figure 2. Restraint stress causes spatial memory impairment. Water maze test was carried out from one day after forced swim test in non-stressed control (Control) and stressed mice (Stress). (A) Each column represents the mean (± SEM) retention latency (n=8 independent mice). (B) Each column represents the mean (± SEM) number of passes over missing platform (n=8 independent mice). (C) Each column represents the mean (± SEM) swim speed (n=8 independent mice). P values, Dunnett's test. NS, not significant.

Restraint stress activates GSK-3β in the hippocampus and hypothalamus: We finally examine the effect of restraint stress on the GSK-3β activity. Phosphorylation of GSK-3β at Ser9 both in the hippocampus and hypothalamus from mice with restraint stress was significantly reduced as compared with that for control mice (Figure 3). This indicates that restraint stress activates GSK-3β both in the hippocampus and hypothalamus.

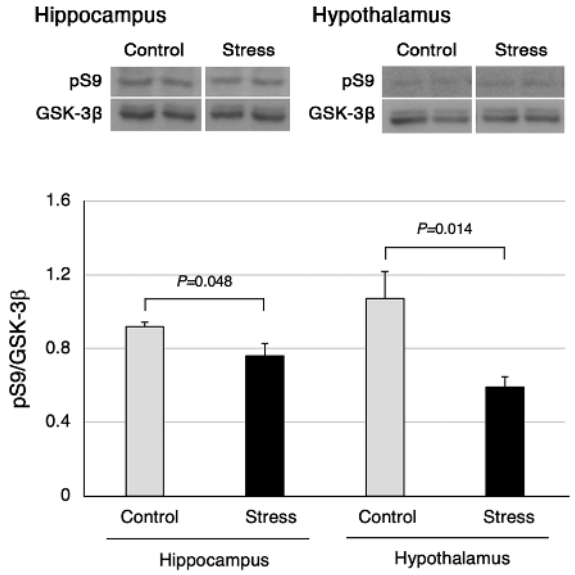

Figure 3. Restraint stress activates GSK-3β.After water maze test, the hippocampus and hypothalamus were isolated from non-stressed control (Control) and stressed mice (Stress) and lysed, followed by Western blotting. In the graph, each column represents the mean (± SEM) ratio: signal intensity for pS9 relative to that for total GSK-3β (n=8 independent experiments). P values, Dunnett's test

GSK-3β is well-recognized to phosphorylate tau, to produce NFT. Unexpectedly, there was no significant difference in the tau phosphorylation at Ser202/Thr205 and Ser396 in the hippocampus and hypothalamus between from non-stressed control and stressed mice (Figure 4). This interprets that restraint stress does not activate GSK-3β sufficiently to phosphorlatetau. Overall, these results indicate that restraint stress causes depression-related behaviors and spatial memory impairment by activating GSK-3β, regardless of tau phosphorylation.

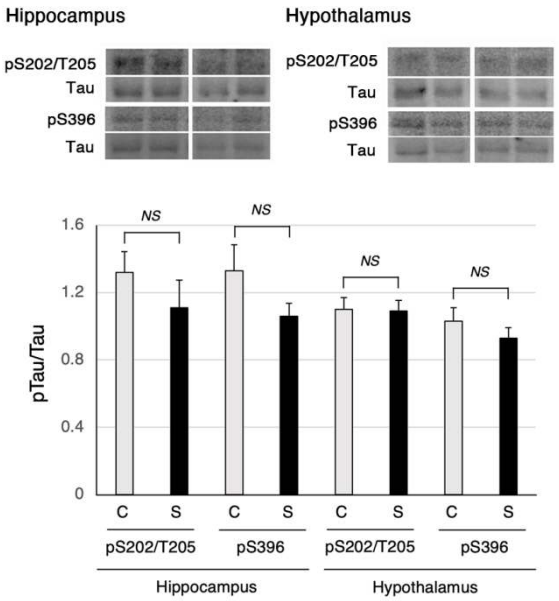

Figure 4. Restraint stress does not affect tau phosphorylation. After water maze test, the hippocampus and hypothalamus were isolated from non-stressed control (C) and stressed mice (S) and lysed, followed by Western blotting. In the graph, each column represents the mean (± SEM) ratio: signal intensity for pS202/T205 or pS396 relative to that for total tau (n=8 independent experiments). NS, not significant; Dunnett's test.

DISCUSSION

A growing body of evidence has pointed to the link between depression and dementia, based upon the notion that depressive symptoms often occur among patients with dementia, that depression may be a reaction to early cognitive deficits, and that depression is capable of impairing cognitive function (Byers and Yaffe, 2011). In the present study, restraint stress induces both depression-like behaviors and spatial memory impairment in mice. This provides evidence that depression is associated with dementia, and vice versa. GSK-3β is originally in the active form. When phosphorylated at Ser9, GSK-3β is inactivated, but otherwise when phosphorylated at Tyr216, GSK-3β activation is enhanced (Forde and Dale, 2007). GSK-3β is recognized to play a significant role both in the pathogenesis of mental illness including depression (Li and Jope, 2010; Latapy *et al*., 2012; Polter *et al*., 2012) and neurodegenerative diseases including Alzheimer's dementia (Gomez-Isla *et al*., 1997; Gomez-Isla *et al*., 2008). In the present study, phosphorylation of GSK-3β at Ser9 in the hippocampus and hypothalamus from mice with restraint stress was significantly reduced as compared with that for non-stressed control mice. This indicates that stress activates GSK-3β by reducing phosphorylation at Ser9, which could cause depression and dementia.

Tau is a target of GSK-3β. Aging, inflammation, and stress activate GSK-3β, which triggers tau phosphorylation, responsible for mild cognitive impairment (MCI), a preliminary group of Alzheimer's disease. When added under the condition of MCI, amyloid β further enhances GSK-3β activation and accelerates tau phosphorylation, leading to progression into Alzheimer's disease from MCI (Ballatore *et al*., 2007; Hurtado *et al*., 2010; Nishizaki,2017). Aggregation of hyperphosphorylated tau causes tauopathies associated with dementia such as frontotemporal dementia and parkinsonism linked to chromosome 17, progressive supranuclear palsy, Pick's disease, and corticobasal degeneration as well as Alzheimer's disease. In the present study, tau phosphorylation at Ser202/Thr205 and Ser396, in spite of restraint stress-induced activation of GSK-3β, was not enhanced in the hippocampus and hypothalamus from mice with restraint stress. This implies that restraint stress does not activate GSK-3β sufficiently to phosphorylate tau. This also suggests that GSK-3β could cause depression and dementia by the unknown mechanism independent of tau phosphorylation. Of particular interest is the finding that early stage activation of GSK-3β accelerates depletion of neural stem cell pool, leading to suppression of adult hippocampal neurogenesis and memory disturbance (Liu*et al*., 2020). This may account for additional action of GSK-3β responsible for the pathogenesis of dementia. To elucidate how GSK-3β causes depression and dementia, further studies need to be carried out. Now the life of people is considerably limited under the COVID-19 pandemic, and this would become extremely crucial stress on all the people before they notice. Accumulating studies show that people with dementia worsened neuropsychiatric symptoms such as depression, apathy, delusions, anxiety, irritability, and agitation since the outbreak of COVID-19 (Wei *et al*., 2022) or that patients with Alzheimer's disease and mild cognitive impairment worsened neuropsychiatric symptoms with agitation, apathy and aberrant motor activity in during 5 weeks of lockdown (Lara *et al*., 2020).COVID-19 lockdown is just like restraint stress shown in the present study. Depression and dementia, therefore, must increase during and after the COVID-19 pandemic still in healthy people. To prevent this, intensive and extensive early care would be a significant issue required.

Conclusion

The results of the present study suggest that GSK-3β is a pivotal factor for stress-induced depression and dementia, independently of tau phosphorylation.

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