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学 位 論 文 題 名

Involvement of CaMKIV in neurogenic effect with chronic fluoxetine treatment

(慢性Fluoxetine投与による神経細胞新生におけるCaMKIVの関与)

学位論文内容の要旨

[Background and Objective]

Selective serotonin reuptake inhibitors, such as fluoxetine (FLX), are the most commonly prescribed drugs for the treatment of depression and anxiety. Chronic (>2-4 weeks) but not acute treatment with fluoxetine increases neurogenesis of subgranular zone (SGZ) of adult hippocampus in not only rodents but also non-human primate, and this time course parallels with clinical antidepressant responses. Behavioral studies suggest that antidepressant treatments exert their therapeutic effects via both neurogenesis-dependent and -independent pathways.

Ca^{2+}/calmodulin dependent protein kinase IV (CaMKIV) is a multifunctional, serine-threonine protein kinase that is activated in the presence of increased intracellular calcium. The tissue distribution of CaMKIV is restricted primarily to discrete region of brain, T-lymphocytes, and post meiotic germ cells. In the brain, CaMKIV is expressed in the parietal cortex, cerebellum and hippocampus. CaMKIV requires Ca^{2+}/calmodulin to initiate kinase activity and consequently phosphorylated by Ca^{2+}/calmodulin dependent protein kinase kinase (CaMKK) on Thr^{200} in human CaMKIV (Thr^{196} in the mouse) is necessary for maximal CaMKIV activity. CaMKIV is detected predominantly in the nuclei of neurons and plays a role in the activity-dependent phosphorylation of cAMP-response element binding protein (CREB). As the most characterized transcription factor, CREB regulates the expression of genes involved in neurogenesis, and chronic antidepressant treatments increase CREB activity within the hippocampus.

Given that CaMKIV participates in phosphorylation of nuclear CREB after chronic FLX treatment and the pivotal role of CREB in neurogenesis, we hypothesize that CaMKIV might regulate some aspects of adult neurogenesis with chronic FLX treatment. Experiments were therefore designed to assess the effects of chronic FLX treatment on cell proliferation and survival in SGZ of adult hippocampus between CaMKIV knockout (KO) mice and their wild type (WT) littermates. Meanwhile, the expression of CREB and phosphorylation of CREB was detected by RT-PCR and Western blotting. Furthermore, the behavioral effects of fluoxetine in KO and WT mice were examined in novelty suppressed feeding test (NSF test), which reflects neurogeneis-dependent actions of chronic fluoxetine.

[Materials and Methods]

Animal, agent and schedule

Wild type (WT) male C57BL/6 mice and CaMKIV knockout (KO) male mice, 8～12 weeks old, were group-housed 3-4 per cage and maintained in air-conditioned rooms at 22 ± 1 ℃ and a 12 h light/dark (6:00/18:00) cycle with free access to food and water. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals and approved by Hokkaido University School of Medicine Animal Care and Use Committee. Fluoxetine was dissolved in distilled water and delivered by gastric gavage at a volume of 18mg/kg once a day.

For experiment measuring proliferation, mice were injected with fluoxetine for 2 weeks and bromodeoxyuridine (BrdU) was singly injected intraperitoneally after last fluoxeitine administration. The mice were sacrificed after 4 weeks of BrdU injection. For experiment measuring survival, mice was singly injected with BrdU and followed by 4 weeks fluoxetine treatment. The mice were sacrificed on the second day of last fluoxetine injection. For novelty suppressed feeding test, mice were injected with fluoxetine for 3 weeks. After 24h of last injection, behavior test was done and mice were sacrificed on second day: hippocampus were quickly excised and prepared for RT-PCR and Western bloting.

Experiment-1

Adult neurogenesis (cell proliferation and survival) was investigated by immunohistochemistry.

Experiment-2

Expression and phosphorylation of CaMKIV and CREB were investigated by RT-PCR or Western blotting, respectively.

Experiment-3

Novelty suppressed feeding test, a neurogenesis-dependent behavior test, was used to investigate the behavioral consequence.

[Results]

Result-1

For cell proliferation, chronic FLX significant increased the BrdU-positive cells in WT (vehicle: 659.3 ± 97.78, n = 8; *vs* fluoxetine: 3101 ± 1000, n = 6; $p < 0.01$) but not in KO mice (vehicle: 632.3 ± 99.77, n = 8; *vs* fluoxetine: 820.5 ± 354.2, n = 6). Baseline of BrdU-positive cell number in vehicle-treated WT and KO mice were similar. For cell survival, fluoxetine significantly increased the BrdU-positive cells in WT (vehicle: 693.9 ± 80.94, n = 10; *vs* fluoxetine: 1716 ± 177.6, n = 11; $p < 0.001$) and in KO mice (vehicle: 648.0 ± 70.26, n = 7; *vs* fluoxetine: 1238 ± 243.6, n = 7; $p < 0.05$). Baseline of BrdU-positive cell number in vehicle-treated WT and KO mice were similar.

Result-2

Chronic FLX treatment has no effect on mRNA expression on CaMKIV and CREB. Phosphorylation of CaMKIV was increased in WT mice ($t = 2.522$, $df = 8$; $p < 0.05$). FLX increased the phosphorylation of CREB in WT but not KO mice ($p < 0.05$)

Result-3

Chronic FLX treatment significantly shortened the latency to feed in NSF test both in WT ($p < 0.01$) and KO mice ($p < 0.05$). There was no effect on the home cage feeding.

[Discussion]

Over the past decade, studies have shown that psychosocial stress impaired adult hippocampal neurogenesis and antidepressant treatment reversed the effect of stress. Meanwhile, the anti-depressive behavioral effect of fluoxetine in some animal models is dependent on hippocampal neurogenesis. To elucidate the pivotal components that participate in adult neurogenesis will contribute to our better understanding of depression and the development of new drugs. In present study, CaMKIV knockout impaired the FLX-induced cell proliferation, but not cell survival; meanwhile, the baseline of BrdU-positive cells between vehicle-treated WT and KO was not influenced. Previous *in vitro* researches showed that CaMKIV/CREB was involved in proliferation of neural stem/progenitor cell, and CaMKIV was shown having pro-survival effect in culturing isolated cerebellar granule cells; meanwhile, the phosphorylation of CREB was impaired in KO mice. These results confirmed the role of CaMKIV/CREB in cell proliferation *in vivo*, and indicated that CaMKIV are not involved in physiological or antidepressant induced cell survival. Finally, the behavior performance in NSF test showed that FLX decreased latency to feed in both types, paralleling with the result of cell survival, which suggested that cell survival might contribute to the performance in this behavior model.

[Conclusion]

Present data show that CaMKIV involved in neurogenic effect of chronic FLX treatment, and the downstream transcriptional factor CREB play an important role for this phenomenon. The role of CaMKIV in other depressive models needs to be further studied, which will deepen our understanding of depression.

学位論文審査の要旨

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Depression is a common mental disorder and among the leading causes of disability. To date, the etiology and pathogenesis of depression is not fully understood. Adult hippocampal neurogenesis has been a hot point recently because researches have shown that some antidepressant actions are neurogenesis-dependent. Therefore, efforts are focusing to see pivotal factors that are involved in adult neurogenesis. In this research, the role of Ca^{2+}/calmodulin dependent protein kinase IV (CaMKIV) in adult neurogenesis was investigated; meanwhile, the possible mechanism and behavioral consequence were detected.

Prof. Yuji Morimoto firstly asked whether adult hippocampal neurogenesis correlated to clinical symptoms of depression and what the relationship of the behavioral results and clinical depression was. The applicant answered that it was very difficult to mimic the core symptoms of depression in animal models. Researchers use the animal's behavioral performance in a certain animal model to define the depression. For example, in forced swimming test, immobility is considered as a behavioral correlate of negative mood, representing a kind of hopelessness. Evidence suggesting the role of neurogenesis in the mood includes that ablation of neurogenesis will lead to an increased HPA axis response to an acute stress. It is possible that young neurons may contribute to hippocampal-dependent negative feedback of HPA axis. Then Prof. Morimoto asked applicant to describe the function of adult neurogenesis. The answer was that there was some evidence that it was important for learning. For example, studies have demonstrated that learning is associated with increased neuronal survival. On the other hands, hippocampal neurogenesis may be important to memory. A number of computational models of the mammalian hippocampus suggest that the continued addition of new neurons may be required for allowing continual storage or indexing

of new information, without losing the ability to recall older memories.

Prof. Hiroyoshi Fujita asked the applicant to introduce about novelty-suppressed feeding test and the other depressive models. He answered novelty-suppressed feeding (NSF) measured a rodent's aversion to eating in a novel environment. The test is sensitive to chronic antidepressant treatment but insensitive to acute antidepressants. Recently, this test is suggested to be hippocampal neurogenesis-dependent. In their experiment, he showed that behavioral effect of chronic fluoxetine was intact in both KO and Wild type mice. He imaged that the surviving cells were finally integrated into the existed circuits to function. It is still not clear whether CaMKIV is involved in other depressive animal models and their group is now working on it. Another question from Prof. Fujita was what the relationship of the results of CREB and behavioral test was. The applicant answered that CREB was a pivotal transcriptional factor that participates in neurogenesis. Activity of CREB could be regulated by antidepressants which consequently initiate transcription of downstream factors. Despite research showed that over-expression of CREB in dentate gyrus of hippocampus could result in antidepressant effect in two depressive models, learned helplessness and forced swimming test, it could not link the expression of CREB a behavior performance directly.

Finally, I asked what the possible mechanism that activates CaMKIV with antidepressant fluoxetine treatment is. The applicant answered that previous researches showed that Ca^{2+}/calmodulin initiated CaMKIV activity and consequently phosphorylated by Ca^{2+}/calmodulin dependent protein kinase kinase (CaMKK) was necessary for maximal CaMKIV activity. Calcium fluxes may be altered by neurotransmitters of G protein-coupled receptors that activate phospholipase C, in turn inducing a rise in intracellular calcium by stimulation of release from internal stores. Antidepressant treatment might possibly increase concentration of calcium in the neurons by this pathway and consequently activate CaMKIV. Another question was how to explain the unchanged cell survival. The applicant answered that CREB was shown to be involved in many aspects of neurogenesis. These effects, however, are suggested to be dependent on different neurogenic zones and stages of postnatal brain development. With chronic antidepressant treatment, CaMKIV and MAPK pathways have been shown to activate CREB. Meanwhile, it has also been shown that downstream of MAPK, such as RSK1/2, could not only activate CREB but also participate in cell survival through inactivating proapoptotic protein Bad. Therefore, it was suggested that other pathways might explain the unchanged cell survival and CaMKIV/CREB is more important for cell proliferation with antidepressant treatment.

This paper is the first research showing the role of CaMKIV in adult hippocampal neurogenesis with chronic antidepressant treatment. The research is well designed and performed. The results are very interesting that provide us new angle of view upon the neurobiological mechanism of antidepressant and we are looking forward to further researches.

All investigators are highly complimentary about this research. Together with other prerequisite for graduation, we are in consensus that the candidate is qualified for doctor degree.