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Caffeic Acid Phenethyl Ester Ameliorates Depression- and Anxiety-like Behaviors of Mice Exposed to Chronic Mild Stress

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Abstract

Depression and anxiety like symptoms appeared in mice when they were kept in cages and sequentially subjected to leaning, drenching, and rotation within 1-2 days for 3 weeks (chronic mild stress: CMS). The depression-like symptom was evaluated by performing the tail suspension test; and the anxiety-like symptom, by the elevated plus-maze test and light-dark box test. Caffeic Acid Phenethyl Ester (CAPE), a component of propolis, showed a preventive effect against both depression- and anxiety-like symptoms when administered during the stress loading, and CAPE also displayed a therapeutic effect against both symptoms when administered after the stress loading. Furthermore, CAPE restored the CMS-induced decrease in the level of the phosphorylated forms of extracellular signal-regulated protein kinases (ERK) 1/2 and cAMP-response element binding protein (CREB) in the hippocampus to a normal level. These results suggest that CAPE is a promising tool for therapy of mood disorders through activation of the hippocampal ERK1/2-CREB signaling cascade.

Keywords: Caffeic Acid Phenethyl Ester (CAPE); Chronic Mild Stress (CMS); Propolis; Depression; Anxiety; Tail Suspension Test (TST); Elevated Plus-Maze Test (EPMT); Light-Dark Box Test (LDT)

Depression and anxiety frequently coexist [1,2] and are sometimes preceded by stressful life events [3, 4]. Despite the high occurrence of and significant disability resulting from such disorders, the pathophysiology of stress-related mood disorders is not fully understood. Recent investigations have demonstrated that impaired function of Brain-Derived Neurotrophic Factor (BDNF), a member of the neurotrophin family of neurotrophic factors, is positively correlated with depression [5] and anxiety-related personality traits [6]. BDNF plays roles in the maintenance of neuronal function and plasticity during development and adulthood [7,8]. This factor is highly expressed in the hippocampus, and a growing body of evidence indicates that hippocampal BDNF is involved in the etiology and treatment of stress-related mood disorders including depression and anxiety [6,9]. For example, exposure to a stressor inhibits neurogenesis in the dentate gyrus [10,11] and causes a reduction in hippocampal volume [12,13]. Importantly, such exposure also reduces the level of BDNF in the hippocampus [14]. These findings imply that a stress-induced reduction in neurotrophic support causes hippocampal damage [9,15], suggesting that substances that mimic intracellular signals of neurotrophins including BDNF may be promising candidates for therapy.

Caffeic Acid Phenethyl Ester (CAPE) is a component of propolis, which is a substance taken from the hives of honeybees; and it exhibits many biological activities including anti-oxidative, anti-inflammatory, anti-tumor, and anti-viral ones [16]. The effects of CAPE have been recently expanded to the central nervous system; i.e., the infarct volume and degree of neurological deficit induced by artery occlusion become smaller in CAPE-administered animals [17-19], and CAPE prevents cultured cerebellar granule neurons against glutamate-induced neurotoxicity [20]. Also, earlier we found

that CAPE enhances the recovery of locomotor function and reduces lesion size in the injured spinal cord while suppressing the mRNA expression of pro-inflammatory cytokines and inflammatory enzymes [21]. Furthermore, we recently found that CAPE increased the level of phosphorylated forms of ERK1/2 and cAMP-response element-binding protein (CREB) in cultured central neurons (Soga et al., unpublished results), suggesting BDNF-like biological activity of CAPE. From these observations, CAPE would be expected to have ameliorative activity toward stress-related mood disorders including depression and anxiety, as in the case of BDNF.

We used a murine model of Chronic Mild Stress (CMS) in this present study, because Willner [22] described that this model has good predictive validity (behavioral changes are reversed by chronic treatment with a wide variety of antidepressants), face validity (almost all demonstrable symptoms of depression have been demonstrated), and construct validity (CMS causes a generalized decrease in responsiveness to rewards, comparable to anhedonia, the core symptom of the melancholic subtype of major depressive disorder).

In this study, we examined the effects of CAPE on depression-like and anxiety-like symptoms and the influence of CAPE on the signal transduction pathways of BDNF by using a CMS animal model.

Materials and Methods

Animals

Seven-week-old male ddY mice (Japan SLC, Hamamatsu, Japan), weighing 35-40 g, were used. The mice were housed under conditions of constant temperature (23±2ºC), humidity (55±10 %), and a 12-h light/12-h dark cycle with food and water freely available. All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals of Gifu Pharmaceutical University.

Drug treatment

CAPE (see Figure 1) was provided by Api Co. Ltd. (Gifu, Japan). Fluvoxamine was also used as a currently prescribed antidepressant to check the validity or availability of the experimental systems used in this study including CMS-loading systems or behavioral tests. The drugs were dissolved in phosphate-buffered saline (PBS) and administered orally to mice by use of a stomach tube. Control animals received vehicle (PBS) without drug. The volume of solution administered was 0.25 mL/mouse.

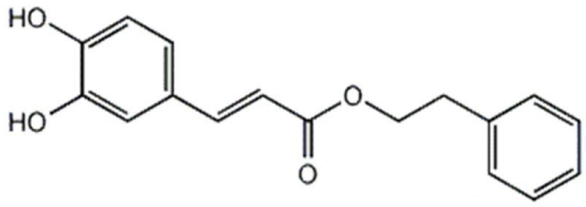

Figure 1: Chemical structure of CAPE

Stress-induced depression-like and anxiety-like model mice

Chronic mild stress (CMS) was applied to the mice according to a previously published method [23] with a slight modification [24]. As shown in Figure 2, mice were exposed to CMS, which consisted of 3 different and sequential stress situations: inclining their cage by 20 degrees from the horizontal (CMS1), keeping them on chip bedding fully wetted with water (CMS2), and shaking their cages at 180 rpm by use of a rotatory shaker (CMS3). CMS1, CMS2, and CMS3 were sequentially applied for 48, 24, and 24 h, respectively, with a 24-h interval between each CMS. This set of stress-loading was repeated 3 times over a 20-day period.

In order to evaluate the protective activity against the depression- and anxiety-like symptoms, we administered the drug once a day for 21 days starting at the time of the first stress-loading till the end of the stress-loading period (Figure 2A). On the other hand, for the experiments to test the therapeutic activity, the drug was injected once a day for 7 or 14 days starting the next day after the end of the stress-loading (Figure 2B).

Tail suspension test (TST)

TST is a standard assay for the depression state because decreased motivation is a hallmark symptom [25]. In this test, a mouse was suspended by its tail from a hanger attached to a precision linear load cell [24]. Although measurements were taken for 7 min, immobility was calculated by determining the time spent immobile during the last 6 min of the test, because all mice were uniformly active for the first min. Immobility time was scored by a blinded observer. Mice that climbed their tail or fell off the hanger were excluded from the analysis.

Elevated plus-maze test (EPMT)

EPMT is a standard test to measure fear and the anxiety-like state. After treatment, the animals were placed in the center of a 4-arm maze (30 cm × 5 cm/arm) elevated to a height of 50 cm, in which 2 arms were open and 2 were closed [26]. The number of times the animal entered each of the arms and the time spent in each arm were recorded during a 5-min test period. The procedure was conducted in a sound-attenuated room.

Light-dark box test (LDT)

LDT is one of the most widely used tests to measure anxiety-like behavior in mice. This test is based on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploratory behavior in novel environments [27]. The apparatus consists of a dark chamber and a brightly illuminated chamber. Mice are allowed to move freely between the 2 chambers. The number of entries into the bright chamber and the duration of time spent in bright-space were recorded during a 5-min test period.

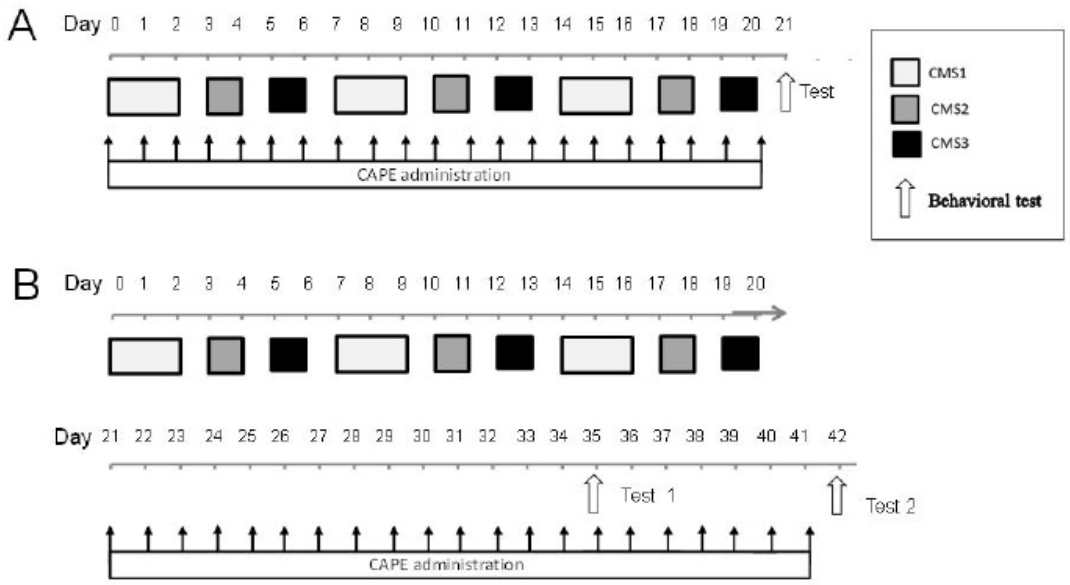

Figure 2: Protocols for stress-loading and drug administration.

A: Protocol for evaluation of protective activity. Mice were orally given the drug once a day for 21 days (indicated by the arrows) during exposure to the 3 types of CMSs (CMS1, CMS2 or CMS3, indicated as boxes colored white, gray, and black, respectively). Then, the behavioral test was performed at 1 day after the end of the stress-loading (open arrow).

B: Protocol for evaluation of therapeutic activity. Mice were subjected to the 3 different CMSs (CMS1, CMS2 or CMS3, illustrated as boxes as defined in "A.") for 20 days. Each drug was orally given once a day for 14 or 21 days starting 1 day after the stress-loading, and the behavioral test was performed at 1 day after the final drug administration (open arrows).

Results

Efficacy of CAPE for treatment of the depression-like symptom

Protective activity against the symptom: The protective activity of CAPE against depression-like symptom was evaluated according to protocol A (Figure 2A). Mice were divided into 8 groups (n = 8/group). Four groups were exposed to the CMSs, and the remaining 4 groups were not. Animals of all groups were orally administered vehicle or CAPE (10, 50 or 250 μmol/kg), and subjected to the TST 1 day after the final administration. The immobility time was longer in the stress group than the non-stress group, but such difference disappeared by CAPE administration (Figure 3). Furthermore, stress-induced prolongation of the immobility time was reduced by CAPE in a dose-dependent manner, demonstrating that CAPE protected against the CMS-induced depression-like symptom.

Therapeutic activity against the symptom: Therapeutic activity of CAPE was evaluated by protocol B (Figure 2B). The immobility time in the TST was longer in the stress group than the non-stress one. Then, the stress group was divided into 3 groups (n = 8/group), ensuring a similar behavioral trait distribution in each group. Each group was then administered vehicle, CAPE (10 μmol/kg) or fluvoxamine (3 μmol/kg) once a day for 2 or 3 weeks and then subjected to the TST. After the 2-week treatment, the immobility time was significantly longer in the stress group than in the non-stress one; and CAPE, but not fluvoxamine, significantly reduced the stress-prolonged immobility time (Figure 4A). After 3 weeks of treatment, both CAPE and fluvoxamine attenuated stress-induced prolongation of the immobility time (Figure 4B). These data suggest a possibility that CAPE might work more rapidly than fluvoxamine. It is well known that some antidepressants take a long time, over 3 weeks, to exert their effectiveness, which is a serious problem of currently prescribed antidepressants.

Efficacy of CAPE for treatment of the anxiety-like symptom

Protective activity against the symptom: Next, the anxiolytic-like activity was evaluated by protocol A (Figure 2A). In the EPMT, the time spent in the open arms was significantly shorter in the stress group than in the non-stress group (Figure 5A). However, the stress group treated with CAPE (50 μmol/kg) spent a longer time in the open arms than the vehicle-treated stress group (Figure 5A). The frequency of entry into all arms was constant (Figure 5B). These results suggested that CAPE ameliorated the symptom, because the locomotor activity was not influenced by the stress loading or drug administration.

In the LDT, the results were almost the same as those obtained with the EPMT. The time spent in the bright chamber was significantly shorter in the stress group than in the non-stress group (Figure 6A). The administration of any dose of CAPE resulted in a loss of this significant difference. The stress group treated with CAPE (50 or 250 μmol/kg) spent longer time in the bright space than the vehicle-treated stress group (Figure 6A). The locomotor activity was not influenced, as judged from the constant entry into the bright chamber (Figure 6B).

Therapeutic activity against the symptom: The anxiolytic-like activity of CAPE was evaluated by performing protocol B (Figure 2B). The time spent in open arms (EPMT) or light space (LDT) was shorter in the stress group than in the non-

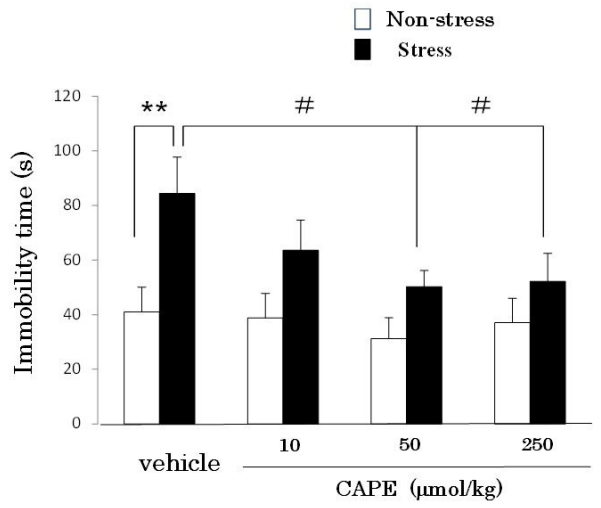

Figure 3: Protective effect against the depression-like symptom.

Mice were daily injected orally with vehicle or CAPE (10, 50 or 250 μmol/kg) for 3 weeks with or without exposure to the CMSs and then subjected to the TST 1 day after the final administration (see protocol A in Figure 2). The significance of the difference between the value of the stress-exposed group and that of the non-stress-exposed group was determined by one-way ANOVA with Tukey's post hoc test as **p<0.01. Among the stress groups, significant differences from the value of the vehicle-treated group were also determined by one-way ANOVA with Tukey's test as #p<0.05.

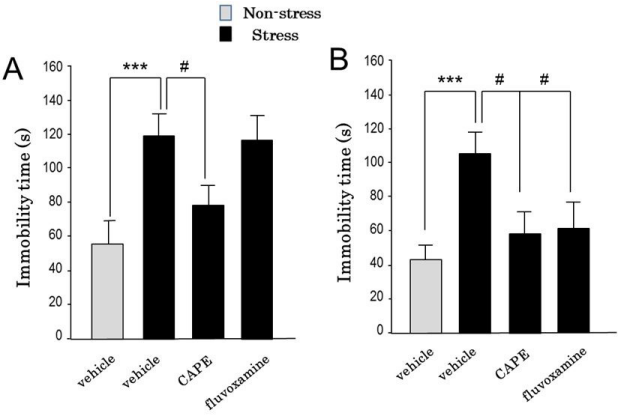

Figure 4: Therapeutic activity for the depression-like symptom.

Vehicle, CAPE (50 μmol/kg) or fluvoxamine (3 μmol/kg) was daily injected orally for 2 (A) or 3 weeks (B) into mice that had been exposed or not to the CMSs (see protocol B in Figure 2). Then, the mice were evaluated for immobility time in the TST 1 day after the final administration. The significance of the difference between the value of the stress-exposed mice and that of the non-stress-exposed mice was determined by one-way ANOVA with Tukey's post hoc test as ***p<0.001. Furthermore, among the CMS-exposed groups, a significant difference from the value of the vehicle-treated mice was similarly determined by one-way ANOVA with Tukey's post hoc test as #P<0.05, ##p<0.01.

stress group. Then, the stress group was divided into 3 groups (n = 8/group), ensuring a similar behavioral trait distribution in each group. Each group was next orally administered vehicle, CAPE or fluvoxamine once a day for 2 or 3 weeks.

In the EPMT or the LDT, the time spent in the open arms or in the bright chamber was significantly shorter, respectively, in the stress group than in the non-stress group 2 or 3 weeks after the end of the CMSs (Figures 7A, B; Figures 8A, B). In both tests, these significant differences disappeared between the stress group administered CAPE or fluvoxamine and the non-stress group. Furthermore, in the EPMT, the significant difference was not found between CAPE- or fluvoxamine-treated stress groups and vehicle-treated stress group after either 2- or 3-week administration (Figures 7A, and 7B). In the LDT, the stress group treated with CAPE spent a significantly longer time in the bright space than the vehicle-treated stress group (Figures 8A, and 8B). The stress group treated with fluvoxamine did so only after the 3-week administration. As the frequency of entry into arms or bright chamber was constant in all experimental groups, the locomotor activity was not influenced (Figures 7C, 7D,8C and 8D).

Influence of CAPE on the levels of pERK1/2 and pCREB in the hippocampus

Mice were treated either by protocol A or B (Figure 2). The hippocampi were dissected out 1 day after the final behavioral test, and used for Western blotting. The ratio of the intensity of the band of phosphorylated (p) extracellular signal-regulated kinases (ERK) 1/2 to that of total ERK1/2 or phosphorylated (p) cAMP-response element-binding protein (pCREB) to that of total CREB was expressed as the fold-increase over the value (taken as "1") for the vehicle-treated non-stress group.

In the mice treated with protocol A, the stress-induced reduction in the ratio of pERK1/2 or pCREB to total ERK1/2 or CREB, respectively, was attenuated by the treatment with 50 or 250 μmol/kg (but not 10 μmol/kg) of CAPE (Figure 9A, C), suggesting that CAPE could protect against the stress-induced reduction in intracellular ERK1/2-CREB signaling. In the mice treated with protocol B, the ratio of pERK1/2/ to ERK1/2 or of pCREB to CRB, which had been reduced by the CMSs, was ameliorated by the treatment with CAPE or fluvoxamine (Figures 9B, 9D). These results suggest that CAPE activated hippocampal ERK1/2 and CREB, and normalized the stress-induced decrease in their levels.

Discussion

Our present results demonstrate that CAPE was active in ameliorating the stress-inducible depression- and anxiety-like symptoms in mice, and it is conspicuous that CAPE restored the stress-reduced levels of pERK1/2 and pCREB in the hippocampus. These observations suggest that CAPE acts on particular brain regions including the hippocampus by generating neurotrophin-like intracellular signaling.

Neurotrophins are a family of neurotrophic factor proteins including BDNF as one of its members. Each neurotrophin binds to a specific Trk family receptor tyrosine kinase [28], which binding causes autophosphorylation of the receptor to trigger signal transduction cascades of pathways involving mitogen-activated protein kinases/ERK1/2, phosphatidylinositol 3-kinase, and phospholipase C-γ[29]. Namely, ERK1/2 is a molecule involved in intracellular signaling pathways evoked by neurotrophic factors such as neurotrophins including BDNF. Activated ERK1/2 leads to activation of the transcriptional factor CREB, which factor regulates the expression of various genes for a variety of neuronal events including neuronal survival, differentiation, and synaptic plasticity [30,31]. Therefore, neurotrophins have been expected to be therapeutically useful for particular neurological disorders. However, the clinical trials using neurotrophins for the treatment of some

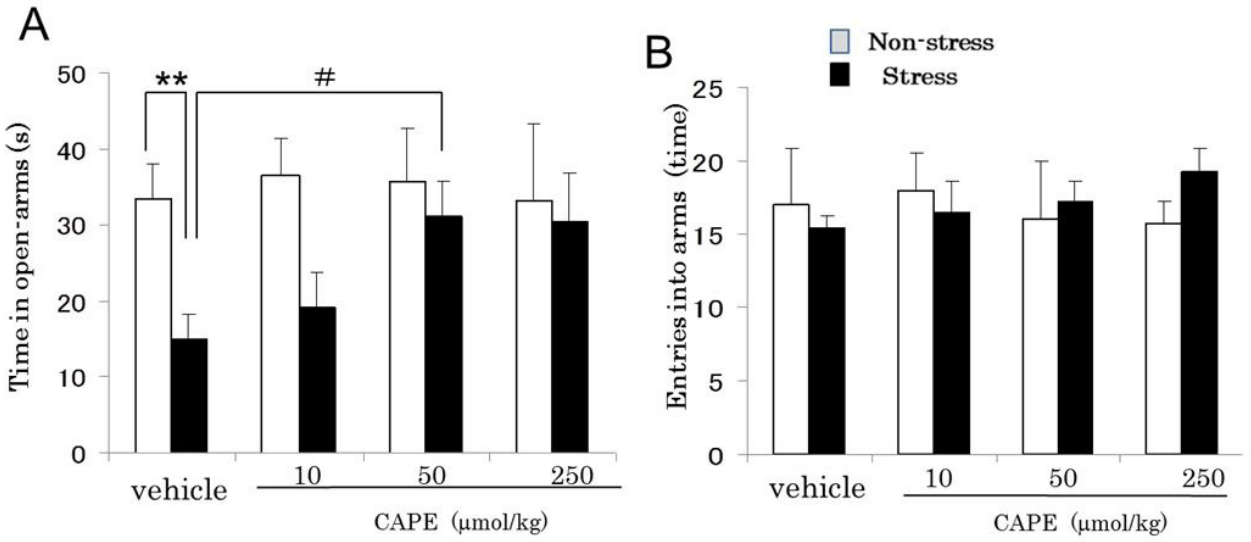

Figure 5: Protective effect against the anxiety-like symptom evaluated by the EPMT.

Mice were daily injected orally with vehicle or CAPE, as described in the legend of Figure 3, for 3 weeks with or without exposure to the CMSs; and they were then subjected to the EPMT 1 day after the final administration (see protocol A in Figure 2). The time spent in open arms (A) and the number of entries into the arms (B) were then evaluated. The significance of the difference between the value of the stress mice and that of the non-stress mice was determined by one-way ANOVA with Tukey's post hoc test as **p<0.01. Among the stress groups, significant differences from the value of the vehicle-treated mice were also determined by one-way ANOVA with Tukey's test as #p<0.05.

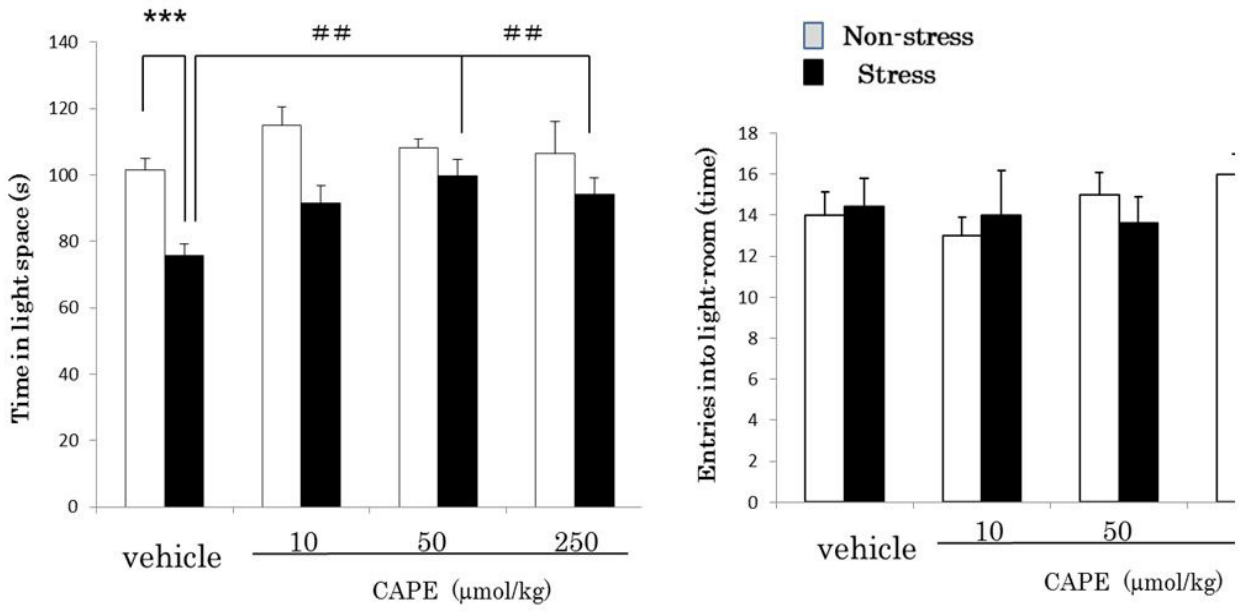

Figure 6: Protective activity against the anxiety-like symptom evaluated by performing the LDT.

Mice were daily injected orally with vehicle or CAPE, as described in the legend of Figure 3, for 3 weeks with or without exposure to the CMSs; and they were then subjected to the LDT 1 day after the final administration (see protocol A in Figure 2). The time spent in the bright chamber (A) and the number of entries into the bright chamber (B) are indicated. The significance of the difference between the value of the stress mice and that of the non-stress mice was determined by one-way ANOVA with Tukey's post hoc test as ***p<0.001. Among the stress groups, significant differences from the value of the vehicle-treated mice were also determined by one-way ANOVA with Tukey's test as ##p<0.01.

neurological disorders have not been successful [32], probably because many technical and pharmacological issues such as instability of the proteins and/or a lack of appropriate delivery systems are problematic. To overcome such drawbacks of neurotrophins, we recently developed 2-decenoic acid ethyl ester (2-DAEE) as a stable and small molecule with neurotrophin-like activity [33]; and we found that it could attenuate stress-induced depression-like [34] and anxiety-like symptoms [35]. These findings prompted us to examine the activity of CAPE, because its neuroprotective activities had been reported earlier [17-19, 21]. The results demonstrated that CAPE behaved like an antidepressant (Figures. 3, 4) and an anxiolytic (Figures. 5-8).

To clarify the underlying action mechanisms of CAPE, we evaluated its effect on the levels of pERK1/2 and pCREB in

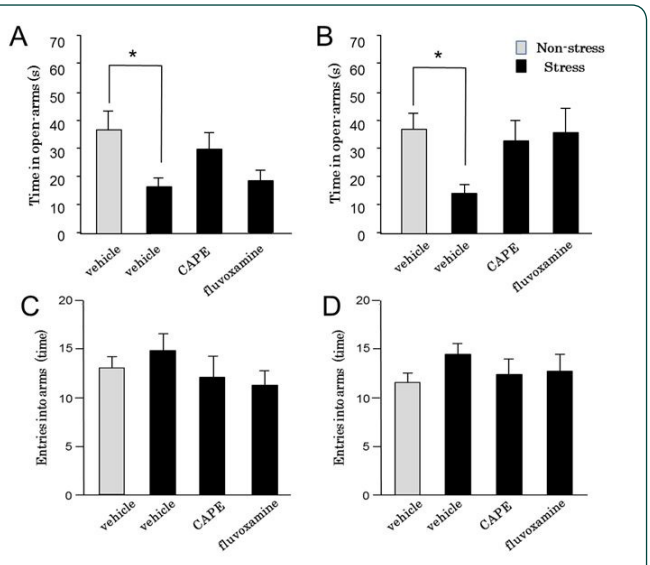

Figure 7: Therapeutic activity against the anxiety-like symptom evaluated by use of the EPMT.
Vehicle, CAPE (50 μmol/kg) or fluvoxamine (3 μmol/kg) was daily injected orally for 2 (A, C) or 3 weeks (B, D) into mice that had been exposed or not to the CMSs (see protocol B in Figure 2). Then, the mice were tested for the time spent in the open arms in the EPMT (A, B) and the number of entries into arms (C, D) 1 day after the final administration. The significance of the difference between the value of the stress mice and that of the non-stress mice was determined by one-way ANOVA with Tukey's post hoc test as *p<0.05. Furthermore, among the stress groups, a significant difference from the value of the vehicle-treated stress mice was examined by one-way ANOVA with Tukey's post hoc test.

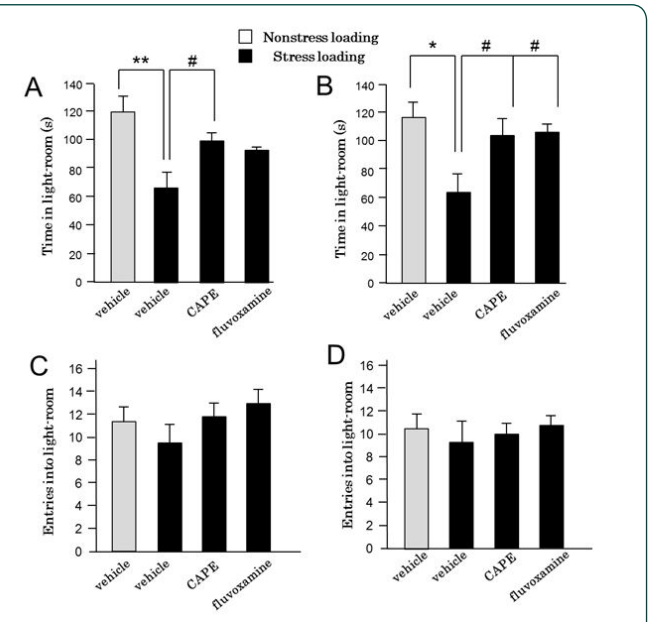

Figure 8: Therapeutic activity against the anxiety-like symptom evaluated by performing the LDT.
Vehicle, CAPE (50 μmol/kg) or fluvoxamine (3 μmol/kg) was daily injected orally for 2 (A, C) or 3 weeks (B, D) into mice that had been exposed or not to the CMSs (see protocol B in Figure 2). Then, the mice were tested for the time spent in the bright chamber in the LDT (A, B) or the number of entries into the bright chamber (C, D) 1 day after the final administration. The significance of the difference between the value of the stress mice and that of the non-stress mice was determined by one-way ANOVA with Tukey's post hoc test as *p<0.05, **p<0.01. Furthermore, among the stress groups, a significant difference from the value of the vehicle-treated stress mice was determined by one-way ANOVA with Tukey's post hoc test as *p<0.05.

the hippocampus of the model mice, and found that CAPE attenuated the stress-induced reduction in these levels, restoring them to normal (Figure 9). These observations suggest that CAPE, having BDNF-like activity, behaved like 2-DAEE. In fact, we found that CAPE facilitated an increase in the level of pERK1/2 in neurons cultured from the mouse hippocampus, thus supporting this possibility (Soga H. et al., unpublished results). In neurons, activated ERK1/2 phosphorylates numerous proteins involved in various cellular processes including long-term potentiation, long-term depression, synaptogenesis, and transcriptional and translational regulation [36, 37]. Namely, the activation of CREB via ERK1/2 may be involved in the action mechanisms of CAPE to up-regulate neuronal functions including synapse plasticity and ameliorate depression- and anxiety-like symptoms.

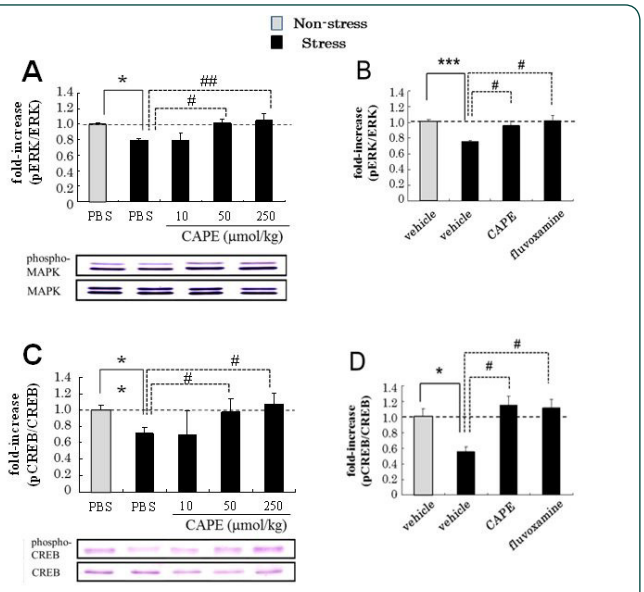

Figure 9: Ameliorative activity against the CMS-induced decrease in the hippocampal pERK1/2 and pCREB levels.
A, C: After daily oral administration of vehicle or CAPE to the mice for 21 days during CMS exposure (see protocol A in Figure 2), the hippocampi were dissected out 1 day after the final administration. Representative images of Western immunoblots are shown.
B, D: Mice were injected with vehicle or CAPE for 14 days after the end of the CMS exposure (see protocol B in Figure 2), and the hippocampi were dissected out 1 day after the end of the administration. The ratio of the intensity of the pERK1/2 (A, B) or pCREB (C, D) band to that of the total ERK1/2 or CREB band was calculated after Western immunoblotting, and the values were expressed as fold-increase over the value of the vehicle-treated non-stress group taken as "1." The significance of differences between the values of the stress and the non-stress mice was determined by one-way ANOVA with Tukey's post hoc test as *P<0.05, **P<0.01 or ***P<0.001. For differences between values of stress groups, the significance was similarly determined by the same statistical treatment as #P<0.05, ##p<0.01.

It has been reported that psychosocial stressors promote inflammation and immune dysfunction [38], which have been implicated in the development of mood disorders [39,40]. Stress-induced macrophage recruitment is evident in specific brain regions and has been implicated in anxiety responses. Namely, monocyte recruitment to the brain in response to social stress contributes to the development of anxiety. CAPE has anti-inflammatory activities, through which it may regulate the anxiety-like state. We reported previously that CAPE enhances the recovery of locomotor function and reduces the lesion

size in the injured spinal cord while suppressing the mRNA expression of the pro-inflammatory cytokine interleukin (IL)-1β and that of inflammatory enzymes such as inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 [21]. It is plausible that CAPE directly inhibits the transcriptional activity of the nuclear factor (NF)-κB transcription factor, because CAPE prevents the binding of NF-κB to DNA [41]. NF-κB is a key mediator of inflammatory responses and is activated by a variety of stimuli including inflammatory cytokines such as interleukin-1β which is released from tissue-resident cells and/or infiltrating monocytes [42, 43]. Although we have not yet examined this possibility, the expression of such inflammatory cytokines and enzymes may be facilitated in the brain of CMS-exposed animals and thus contribute to anxiety-like behavior.

How could CAPE regulate neuronal signals such as phosphorylation of ERK1/2 and CREB? Further experiments to elucidate mechanism of action of CAPE are needed.

Conclusions

CAPE, a component of propolis, was ameliorative to chronic mild stress-induced depression- and anxiety-like symptoms in the CMS mouse model.

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