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Linoleic Acid Derivative DCP-LA Ameliorates Stress-Induced Depression-Related Behavior by Promoting Cell Surface 5-HT$_{1A}$ Receptor Translocation, Stimulating Serotonin Release, and Inactivating GSK-3β

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Abstract Impairment of serotonergic neurotransmission is the major factor responsible for depression and glycogen synthase kinase 3β (GSK-3β) participates in serotonergic transmission-mediated signaling networks relevant to mental illnesses. In the forced-swim test to assess depression-like behavior, the immobility time for mice with restraint stress was significantly longer than that for nonstressed control mice. Postsynaptic cell surface localization of 5-HT$_{1A}$ receptor, but not 5-HT$_{2A}$ receptor, in the hypothalamus for mice with restraint stress was significantly reduced as compared with that for control mice, which highly correlated to prolonged immobility time, i.e., depression-like behavior. The linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) restored restraint stress-induced reduction of cell surface 5-HT$_{1A}$ receptor and improved depression-like behavior in mice with restraint stress. Moreover, DCP-LA stimulated serotonin release from hypothalamic slices and cancelled restraint stress-induced reduction of GSK-3β phosphorylation at Ser9. Taken together, the results of the present study indicate that DCP-LA could ameliorate depression-like behavior by promoting translocation of 5-HT$_{1A}$ receptor to the plasma membrane on postsynaptic cells, stimulating serotonin release, and inactivating GSK-3β.

Keywords DCP-LA · Serotonin · 5-HT$_{1A}$ receptor · GSK-3β · Depression · Antidepressant

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Introduction

Major depression is a recurrent and debilitating mental disorder with a lifetime prevalence of up to 20 % in the general population, among the highest for psychiatric disorders [1]. The standard of care for the last 50 years has focused on monoamine neurotransmitters, and selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) have been widely used for its treatment [2]. The brain responds to stress, and of brain regions the hippocampus, amygdala, and hypothalamus are very sensitive to stress. Dysfunction in these regions might cause depression.

Dysfunction of the serotonergic system is a vulnerability factor for major depressive disorder and other forms of affective illness [3]. So far, at least 14 different serotonin receptors have been identified, and these receptors are divided into seven families denoted, 1, 2, 3, 4, 5, 6, and 7, with subtypes in each family denoted by letters such as A, B, and C [4]. Among them, 5-HT$_{1A}$ receptor is a promising target for development of antidepressants. 5-HT$_{1A}$ receptor, linked to G_i/G_o protein involving inhibition of protein kinase A (PKA)/activation of protein kinase C (PKC), is preferentially localized in the raphe nuclei, frontal cortex, septum, amygdala, hippocampus, and hypothalamus [5, 6]. In the corticolimbic circuits, 5-HT$_{1A}$ receptor is expressed both at presynaptic terminals and postsynaptic cells, and the presynaptic receptor (autoreceptor) reduces the firing rate of neurons, the amount of serotonin released per action potential, and synthesis of the neurotransmitter [7]. Both presynaptic and postsynaptic 5-HT$_{1A}$ receptors are shown to increase under the depressive conditions [8]. Treatments using SSRIs and SNRIs, however, have significant limitations: they can take weeks before showing mood-altering effects, and only one to two out of ten patients shows clinical effects beyond those associated with placebo.

Glycogen synthase kinase 3β (GSK-3β), on the other hand, plays a key role in serotonergic transmission-mediated signaling networks relevant to mental illnesses [9–11]. Systemic inhibition of GSK-3β is effective on mood disorder, psychosis, and depression, and lithium, that is used as an antidepressant, is recognized to inhibit GSK-3β [12].

We have developed the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), with cyclopropane rings instead of *cis*-double bonds, as an anti-dementia drug [13]. DCP-LA selectively activates protein kinase Cε (PKCε) in a diacylglycerol- and Ca^{2+}-independent manner [14, 15] and stimulates α7 ACh receptor translocation to the cell surface in a PKC-dependent manner [16, 17]. DCP-LA also stimulates release of neurotransmitters in a PKC- and α7 ACh receptor-dependent manner [18, 19]. Then, we postulated that DCP-LA might facilitate serotonergic transmission by stimulating 5-HT receptor translocation to the plasma membrane and serotonin release, leading to improvement of depression.

To address this question, we examined the effects of DCP-LA on subcellular distribution of 5-HT_{1A} and 5-HT_{2A} receptors, serotonin release, GSK-3β activity, and depression-related behavior in mice with restraint stress. We show here that DCP-LA could ameliorate depression-like behavior by promoting translocation of 5-HT_{1A} receptor to the postsynaptic cell surface, stimulating serotonin release, and inactivating GSK-3β.

Methods

Animal Care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Analysis for Cellular Distribution of 5-HT Receptors and Phosphorylation of GSK-3β

Rat hippocampal and hypothalamic slices (400 μm in thickness, male Wistar rat, 6 weeks) were incubated in a standard artificial cerebrospinal fluid (ACSF; 117 mM NaCl, 3.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.5 mM glucose) oxygenated with 95 % O_2 and 5 % CO_2 in the presence and absence of DCP-LA (100 nM) for 20 min at 34 °C, together with and without GF109203X (100 nM), or KN-93 (3 μM) from 20 min prior to DCP-LA treatment and botulinum toxin type A (BoTX-A; 0.1 U/ml) from 12 h prior to DCP-LA treatment. In other sets of experiments, the hypothalamus and hippocampus after forced-swim test were isolated from the mouse brain (male C57BL/6 J mouse, 8 weeks). Slices or tissues were homogenized by sonication in an ice-cold mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES; pH 7.5) containing 1 % (*v*/*v*) protease inhibitor cocktail and centrifuged at 800×*g* for 5 min at 4 °C. Some supernatants were used as total lysates for Western blotting using antibodies against phospho-Ser9-GSK-3β (pS9; Cell Signaling, Beverly, MA, USA), phospho-Tyr216-GSK-3β (pY216; BD Biosciences, San Jose, CA, USA), and GSK-3β (Cell Signaling), and the ratio of the signal intensity for pS9 corresponding to that for GSK-3β was calculated. The remaining supernatants were further centrifuged at 11,000×*g* for 15 min at 4 °C, and the collected supernatants were further ultracentrifuged at 100,000×*g* for 60 min at 4 °C. The supernatants and pellets were used as the cytosolic and plasma membrane fractions, respectively, followed by Western blotting using antibodies against 5-HT_{1A} receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 5-HT_{2A} receptor (Calbiochem, San Diego, CA, USA), and the ratio of the signal intensity for each 5-HT receptor in the plasma membrane fraction corresponding to that in the total plasma membrane and cytosolic fractions was calculated. Whether the cytosolic and plasma membrane components were successfully separated was confirmed in the Western blot analysis using antibodies against LDH (Santa Cruz Biotechnology), a cytosolic marker, and cadherin (Sigma, St Louis, MO, USA), a plasma membrane marker.

Analysis for Distribution of 5-HT Receptors in Synaptosomes and Extra-synaptosomes

Rat hypothalamic slices (400 μm in thickness, male Wistar rat, 6 weeks) untreated and treated with DCP-LA (100 nM) for 20 min were homogenized, and the extracts were centrifuged at 1,000×*g* for 10 min at 4 °C. Then, the supernatants were layered on the top of a discontinuous Percoll gradient (0, 3, 10, 15, and 23 % Percoll in sucrose buffer), followed by centrifugation at 31,000×*g* for 5 min at 4 °C. Fractions at the 10–15 and 15–23 % gradient interface were regarded as synaptosomes, and the remaining ones as extra-synaptosomes. Western blotting was carried out in each component using antibodies against 5-HT_{1A} receptor (Santa Cruz Biotechnology), 5-HT_{2A} receptor (Calbiochem), or an anti-β-actin antibody (Sigma). To confirm whether the synaptosome and extra-synaptosome components were successfully separated, Western blotting were carried out using an antibody against the presynaptic marker syntaxin-1 (MBL International, Woburn, MA, USA).

Preparation of Acutely Isolated Hypothalamic Neurons

To remove presynaptic components, rat hypothalamic slices (400 μm in thickness, male Wistar rat, 6 weeks) were treated

with pronase (0.2 mg/ml) and thermolysin (0.2 mg/ml) for 20 min at 31 °C. After washing, cells were mechanically dissociated and plated on poly-L-lysine-coated glass cover slips.

Immunocyochemical Analysis of 5-HT_{1A} Receptor Mobilizations

Acutely isolated neurons were untreated and treated with DCP-LA (100 nM) for 20 min. Then, cells were fixed with 4 % (*w*/*v*) paraformaldehyde, permeabilized with 0.3 % (*v*/*v*) Triton X-100, and blocked with 10 % (*v*/*v*) goat serum at room temperature, followed by immunostaining using antibodies against 5-HT_{1A} receptor (Santa Cruz Biotechnology), and microtubule-associated protein 2 (MAP2; Millipore, Billerica, MA, USA). Fluorescence-labeled cells were visualized with a confocal scanning laser microscope (Axiovert/LSM510; Carl Zeiss, Oberkochen, Germany).

Assay of Serotonin Released

The hypothalamus were isolated from the rat brain (male Wistar rat, 6 weeks) and sliced at 400 μm in thickness. Slices were incubated in the ACSF oxygenated with 95 % O_2 and 5 % CO_2 at room temperature for 1 h followed by at 34 °C for 50 min. Then, slices were transferred to a chamber filled with 1 ml of ACSF oxygenated with 95 % O_2 and 5 % CO_2 containing tetrodotoxin (TTX; 0.5 μM) in the presence and absence of nicotine (1 μM) and DCP-LA together with and without GF109203X (100 nM) or α-bungarotoxin (α-BgTX; 100 nM) at 34 °C for 20 min. After treatment, external solution was collected and serotonin released was labeled with 4-fluoro-7-nitrobenzofurazan (NBD-F). Then, 20 μl of NBD-F-labeled solution was injected onto the column (150× 4.6 mm) and loaded onto the HPLC system. NBD-F was detected at an excitation of wavelength of 350 nm and an emission wavelength of 450 nm using a fluorescence detector.

Cell-Free Protein Phosphatase 1 Assay

The human recombinant protein phosphatase 1 (PP1) was purchased from New England BioLabs Inc. (Ipswich, MA, USA). PP1 activity was assayed by reacting with *p*-nitrophenyl phosphate (pNPP; Sigma-Aldrich, St. Louis, MO, USA) as a substrate under the cell-free conditions by the method previously described [20]. PP1 (1 U/well) was preincubated at 30 °C for 30 min in a reaction medium (50 mM HEPES, 100 mM NaCl, 2 mM dithiothreitol, 0.01 % (*v*/*v*) Brij-35, and 1 mM $MnCl_2$ (pH 7.5) in the presence and absence of calyculin A or DCP-LA. Then, pNPP at a concentration of 5 mM was added to the reaction medium followed by 60-min incubation, and the reaction was terminated by adding 0.1 N NaOH. Dephosphorylated pNPP was quantified at an absorbance of 405 nm with a SpectraMax PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

DCP-LA-Binding Assay to PP1

We synthesized fluorescein-conjugated DCP-LA. PP1 was separated by blue native-polyacrylamide gel electrophoresis (PAGE). Briefly, proteins were dissolved in a sample buffer (50 mM immidazole, 50 mM NaCl, 5 mM 6-aminohexanoic acid, 40 % (*v*/*v*) glycerol, 0.5 % (*w*/*v*) Coomassie G-250, and 1 % (*w*/*v*) digitonin; pH 7.0) and electrophoresed onto a TGX Gel (BioRad, Hercules, CA, USA) in a cathode buffer (50 mM tricine, 7.5 mM imidazole, and 0.02 % (*w*/*v*) Coomassie G-250; pH 7.0) and an anode buffer (25 mM imidazole; pH 7.0). After pretreatment with or without 1 mM nonconjugated DCP-LA at 30 °C for 30 min, gels were reacted with 1 mM fluorescein-conjugated DCP-LA in the presence and absence of 1 mM nonconjugated DCP-LA at 30 °C for 60 min. Fluorescent signals were visualized using FluoroPhoreStar3000 (Anatech, Tokyo, Japan).

Restraint Stressed Mice

Male C57BL/6 J 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 3 days. Control mice without restraint stress were bred in a normal cage till behavioral tests.

Forced-Swim Test

Forced-swim test was carried out 7 days after the end of the restraint stress. 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 during 4-min testing was measured as an immobility time.

DCP-LA, linoleic acid, sulpiride, sertraline, and Li_2CO_3 were dissolved in polyethylene glycol (PEG). These agents or PEG were orally administered to mice without and with restraint stress using a feeding needle once a day from 1 day after restraint stress to the end of the forced-swim test.

Statistical Analysis

Statistical analysis was carried out using Dunnett's test. The linear regression analysis was performed to examine the correlation between immobility time in the forced-swim test and membrane localization index of 5-HT receptors, and the Pearson correlation coefficient (*R*) was determined.

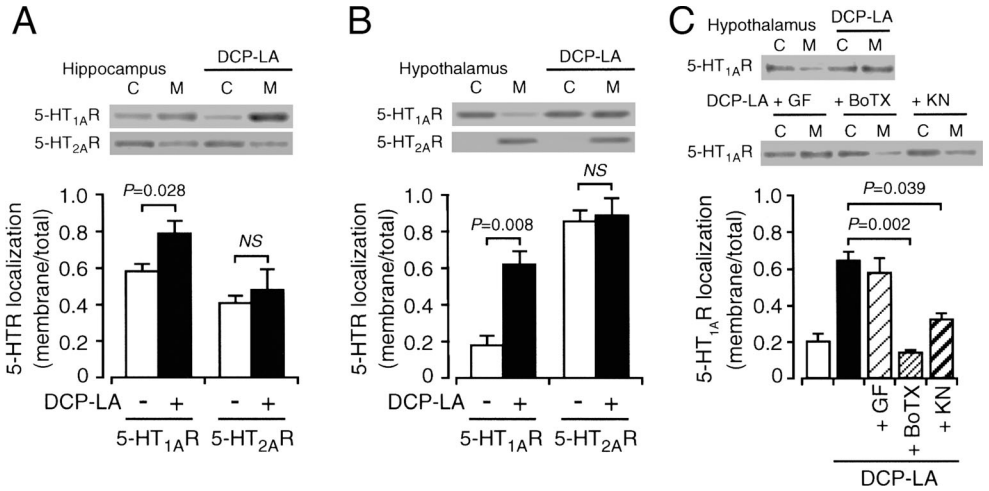

Fig. 1 DCP-LA increases localization of 5-HT_{1A} receptor on the cell surface. Rat hippocampal (**a**) and hypothalamic (**b**) slices were untreated (*negative sign*) and treated (*positive sign*) with DCP-LA (100 nM) for 20 min followed by Western blotting using antibodies against 5-HT_{1A} receptor (*5-HT_{1A}R*) and 5-HT_{2A} receptor (*5-HT_{2A}R*). In a different set of experiments, hypothalamic slices were untreated and treated with DCP-LA (100 nM) in the presence and absence of GF109203X (*GF*; 100 nM), BoTX-A (*BoTX*; 0.1 U/ml), or KN-93 (*KN*; 3 μM) for 20 min followed by Western blotting using a 5-HT_{1A}R antibody (**c**). *C* cytosol, *M* membrane. In the graphs, each column represents the mean (±SEM) signal intensity for 5-HT receptor (*5-HTR*) on the plasma membrane relative to that for total cells (n=4 independent experiments). *P* values, Dunnett's test. *NS* not significant

Results

DCP-LA Increases Cell Surface Localization of 5-HT_{1A} Receptor in the Hypothalamus

We initially examined the effect of DCP-LA on subcellular distribution of 5-HT receptors. We had earlier confirmed that DCP-LA had no effect on expression levels of 5-HT_{1A} and 5-HT_{2A} receptors in rat hippocampal and hypothalamic slices, indicating that DCP-LA does not affect transcription of the 5-HT_{1A} and 5-HT_{2A} receptor genes and translation of their proteins. DCP-LA significantly increased cell surface localization of 5-HT_{1A} receptor in hippocampal and hypothalamic slices (Fig. 1a, b). By contrast, cell surface localization of 5-HT_{2A} receptor both in hippocampal and hypothalamic slices was not affected by DCP-LA (Fig. 1a, b). DCP-LA-induced increase in the cell surface localization of 5-HT_{1A} receptor was abrogated by BoTX-A, an inhibitor of vesicular exocytosis (Fig. 1c). Collectively, these results indicate that DCP-LA stimulates vesicular exocytosis of 5-HT_{1A} receptor, but not 5-

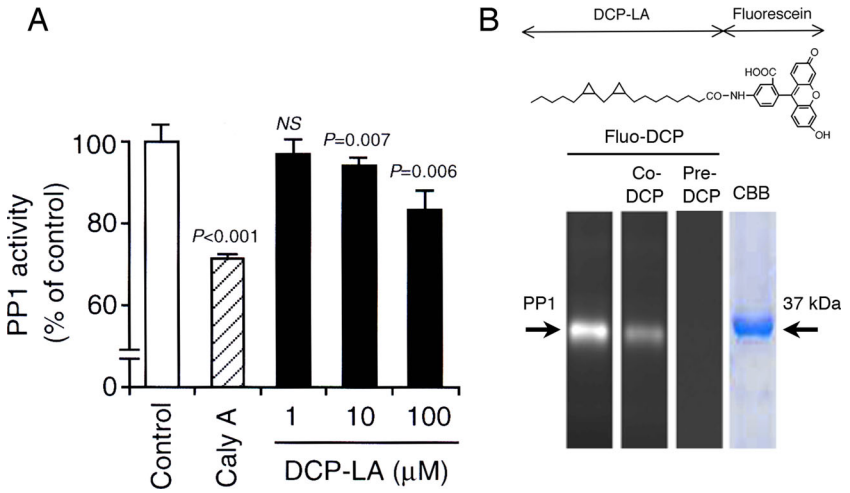

Fig. 2 DCP-LA inhibits PP1 through its direct binding to PP1. **a** PP1 was reacted with pNPP in the presence and absence of calyculin A (*Caly A*; 0.1 nM) or DCP-LA at concentrations as indicated, and dephosphorylated pNPP was quantified as a PP1 activity. In the graph, each value represents the mean (±SEM) percentage of basal PP1 activity (control; n=4 independent experiments). *P* values as compared with control, Dunnett's test. *NS* not significant. **b** Chemical structure for fluorescein-conjugated DCP-LA are shown in the *upper column*. Electrophoresed PP1 was incubated with fluorescein-conjugated DCP-LA (*Fluo-DCP*; 1 mM) in the absence and presence of nonconjugated DCP-LA (*Co-DCP*; 1 mM) or before and after pretreatment with nonconjugated DCP-LA (*pre-DCP*; 1 mM), and fluorescent signals were detected. *CBB* Coomassie brilliant blue staining. Note that similar results were obtained from four independent experiments

HT_{2A} receptor, to increase cell surface localization of the receptor.

The effect of DCP-LA on subcellular distribution of 5-HT_{1A} receptor was significantly prevented by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93, but it was not affected by the PKC inhibitor GF109203X (Fig. 1c). This implies that DCP-LA promotes translocation of 5-HT_{1A} receptor to the plasma membrane in a CaMKII-dependent manner.

Our previous study suggests that DCP-LA might activate CaMKII due to PP1 inhibition [21]. To obtain direct evidence for this, we assayed PP1 activity under the cell-free conditions. Calyculin A (0.1 nM), an inhibitor of PP1, significantly reduced PP1 activity (Fig. 2a), confirming a reliable PP1 assay. DCP-LA also reduced PP1 activity in a concentration-dependent (1–100 μM) manner, with the effect being significant at 10 and 100 μM (Fig. 2a). This indicates that DCP-LA actually suppresses PP1 activity. We subsequently examined interaction of DCP-LA with PP1 using a fluorescein-conjugated DCP-LA. In the blue native-PAGE analysis, a fluorescent signal band was detected at 37 kDa, corresponding to PP1 separated, and the signal band was blurred by cotreatment with nonconjugated DCP-LA as a competitor or abolished by pretreatment with nonconjugated DCP-LA (Fig. 2b). This implies that DCP-LA directly binds to PP1. Taken together, these results indicate that DCP-LA inhibits PP1 through its direct binding to PP1.

DCP-LA Increases Cell Surface Localization of 5-HT_{1A} Receptor on Postsynaptic Cells

To understand further insight into the effect of DCP-LA on subcellular distribution of 5-HT_{1A} receptor, extra-synaptosomal fraction, which reflects postsynaptic components, and synaptosomal fraction, which reflects presynaptic components, were separated from rat hypothalamic slices. In the Western blot analysis, a signal band reactive to an antibody against syntaxin-1, a presynaptic marker, was found only in the synaptosomal fraction (Fig. 3a), confirming successful separation into extra-synaptosomal and synaptosomal fractions. DCP-LA did not increase presence of 5-HT_{1A} or $5HT_{2A}$ receptor in the synaptosomal fraction (Fig. 3b). This interprets that DCP-LA does not stimulate translocation of 5-HT_{1A} or 5-HT_{2A} receptor to the plasma membrane at presynaptic terminals; in other words, DCP-LA increases cell surface localization of 5-HT_{1A} receptor by stimulating translocation of the receptor to the postsynaptic plasma membrane but not the presynaptic membrane.

To obtain further evidence for this, we examined subcellular distribution of 5-HT_{1A} receptor in acutely dissociated rat hypothalamic neurons without presynaptic components. DCP-LA apparently accumulated immunoreactive signals for 5-HT_{1A} receptor along the membrane of cells reactive to

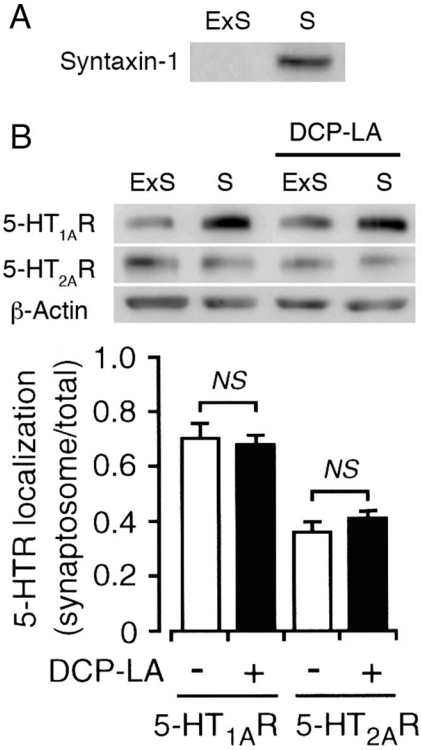

Fig. 3 DCP-LA does not increase presence of 5-HT_{1A} receptor in the synaptosomal fraction. Rat hypothalamic slices were untreated (*negative sign*) and treated (*positive sign*) with DCP-LA (100 nM) for 20 min, and then separated into extra-synaptosomal (*ExS*) and synaptosomal (*S*) fractions. **a** Western blotting using an anti-syntaxin-1 antibody. Note that the immunoreactive signal band is found only in the synaptosomal fraction. **b** Western blotting using antibodies against 5-HT_{1A}R and 5-HT_{2A}R. In the graph, each column represents the mean (±SEM) signal intensity for 5-HTR in the synaptosomal fraction relative to that in total extra-synaptosomal and synaptosomal fractions (n=4 independent experiments). *NS* not significant; Dunnett's test

MAP2, a neuron marker, while only a few spotty immunoreactive signals were found in the absence of DCP-LA (Fig. 4). Collectively, these results indicate that DCP-LA promotes translocation of 5-HT_{1A} receptor to the plasma membrane on postsynaptic cells in the hypothalamus.

DCP-LA Restores Reduced Cell Surface Localization of 5-HT_{1A} Receptor in the Hypothalamus for Depression Model Mice

Cell surface localization of 5-HT_{1A} receptor was not significantly reduced in the hippocampus for mice with restraint stress, a depression model, as compared with that for nonstressed control mice, and DCP-LA recovered reduced localization of 5-HT_{1A} receptor (Fig. 5a). Restraint stress, alternatively, caused significant reduction of cell surface 5-HT_{1A} receptor in the hypothalamus, and amazingly, a drastic increase in the cell surface localization of 5-HT_{1A} receptor was obtained with DCP-LA, leading to full/further recovery (Fig. 5b). Such effect, however, was not found with antidepressants such as the selective antagonist of dopamine D_2 and

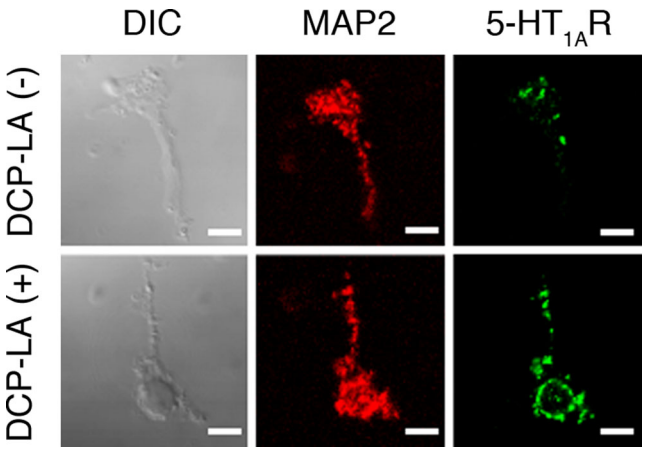

Fig. 4 DCP-LA increases presence of 5-HT_{1A} receptor on postsynaptic cells. Cells were mechanically dissociated from rat hypothalamic slices, and untreated (*negative sign*) and treated (*positive sign*) with DCP-LA (100 nM) for 20 min followed by immunostaining using antibodies against MAP2 and 5-HT_{1A}R. *DIC* differential interference contrast. *Bars*, 10 μm

D_3 receptors sulpiride, the SSRI sertraline, and the GSK-3β inhibitor Li_2CO_3 (Fig. 5c).

DCP-LA Ameliorates Depression-Like Behavior in Mice with Restraint Stress

In the forced-swim test to assess depression-like behavior, the immobility time for mice with restraint stress was significantly longer than that for nonstressed control mice (Fig. 6a–c). This is interpreted as restraint stress-induced depression-like behavioral change. DCP-LA shortened the prolonged immobility time in a dose-dependent (0.5–5 mg/kg) manner, reaching control levels at 1 mg/kg (Fig. 6a). By contrast, linoleic acid had no effect on the prolonged immobility time in mice with restraint stress (Fig. 6b). The antidepressants sulpride, sertraline, and Li_2CO_3 also shortened the prolonged immobility time in mice with restraint stress, with the efficacy similar to that for DCP-LA (Fig. 6c). Overall, these results indicate that DCP-LA has the potential to improve depression-like behavior.

Reduction in the cell surface localization of 5-HT_{1A} receptor in the hypothalamus highly correlated to prolongation of immobility time (R=0.728; Fig. 7b), while there was no significant relation between 5-HT_{1A} receptor in the hippocampus and immobility time (R=0.413; Fig. 7a), 5-HT_{2A} receptor in the hippocampus and immobility time (R=0.432; Fig. 7c), and 5-HT_{2A} receptor in the hypothalamus and immobility time (R=0.524; Fig. 7d). This suggests that reduction of cell surface localization of 5-HT_{1A} receptor in the hypothalamus is a critical factor responsible for depression-like behavior. Consequently, DCP-LA appears to ameliorate depression-like behavior by promoting translocation of 5-HT_{1A} receptor to the plasma membrane in the hypothalamus.

DCP-LA Stimulates Serotonin Release from Hypothalamic Slices

We subsequently examined the effect of DCP-LA on serotonin release from rat hypothalamic slices. No significant

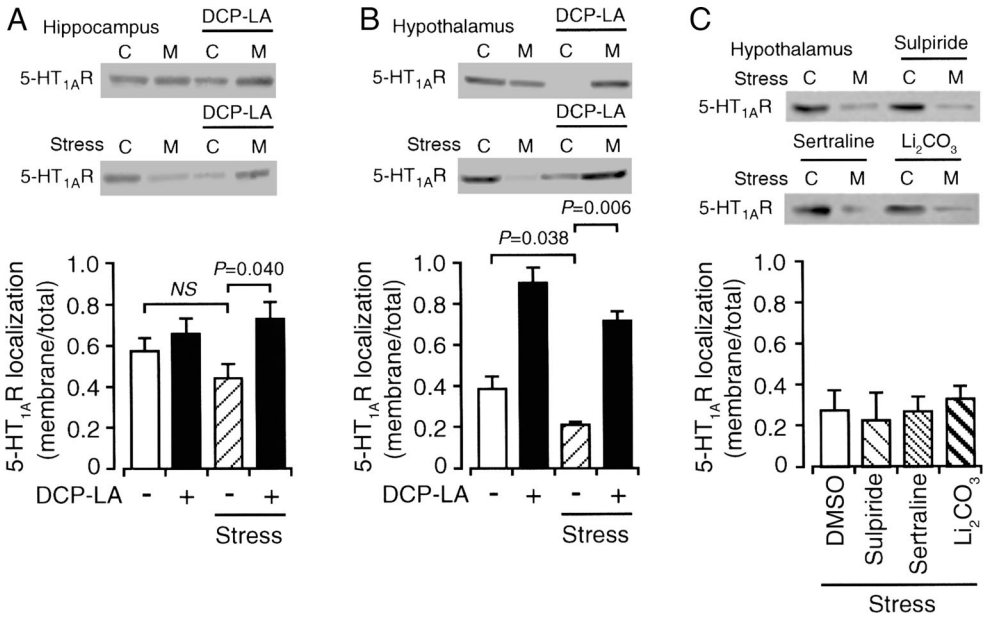

Fig. 5 DCP-LA restores restraint stress-induced reduction in the cell surface localization of 5-HT_{1A} receptor. DCP-LA (1 mg/kg) was orally administered to mice without and with restraint stress, and the hippocampus (**a**) and hypothalamus (**b**) were isolated after forced-swim test followed by Western blotting using a 5-HT_{1A}R antibody. (**c**) Sulpiride, sertraline, or Li_2CO_3 at a dose of 1 mg/kg was orally administered to mice without and with restraint stress, and the hypothalamus were isolated after forced-swim test followed by Western blotting using a 5-HT_{1A}R antibody. *C* cytosol, *M* membrane. In the graphs, each column represents the mean (±SEM) signal intensity for 5-HT_{1A}R on the plasma membrane relative to that for total cells (n=4 independent experiments). *P* values, Dunnett's test. *NS* not significant

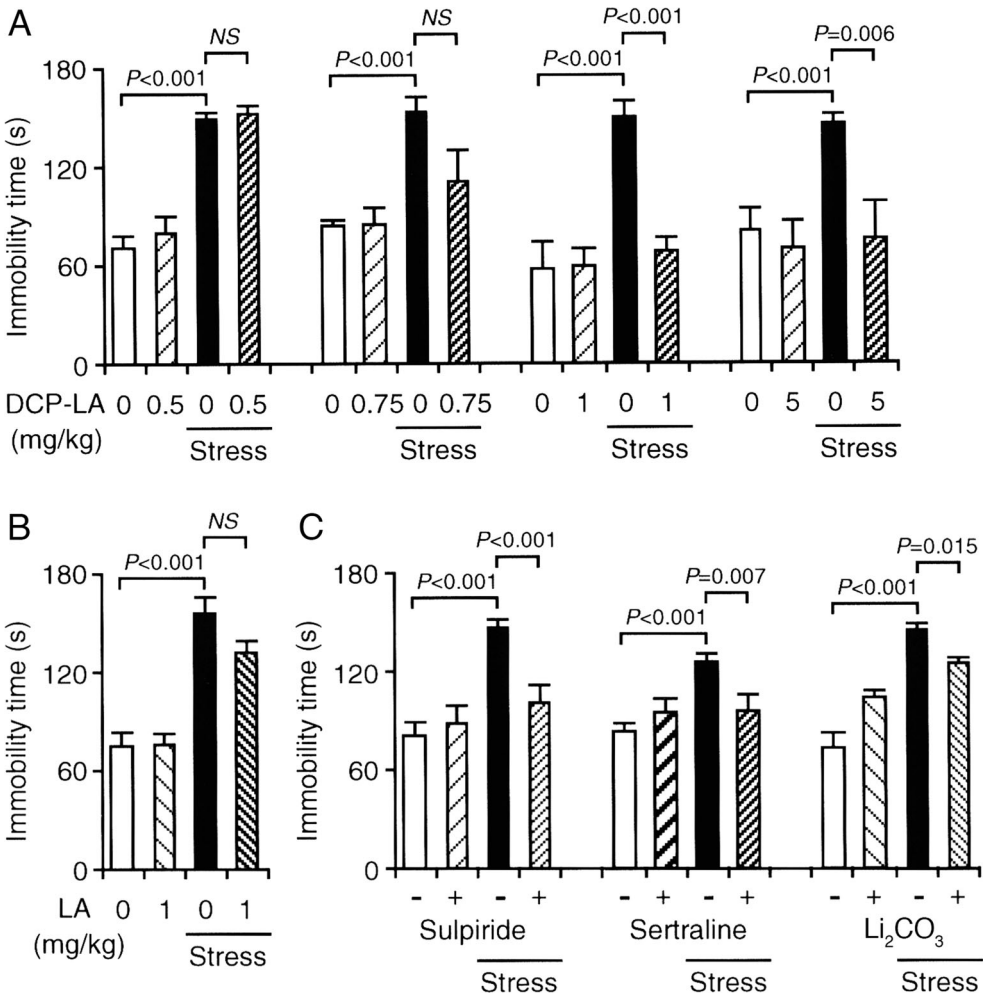

Fig. 6 DCP-LA ameliorates restraint stress-induced depressive disorder. Forced-swim test was carried out in mice without and with restraint stress, to whom DCP-LA at doses as indicated (**a**), linoleic acid (1 mg/kg; **b**), or sulpiride, sertraline, or Li_2CO_3 at a dose of 1 mg/kg (**c**) was orally administered, and immobility time was measured (n=5–15 independent mice). P values, Dunnett's test. *NS*, not significant

serotonin release was obtained with nicotine alone or DCP-LA alone (Fig. 8b). DCP-LA increased nicotine-triggered serotonin release in a concentration-dependent (1 nM–100 μM) manner (Fig. 8a). The effect of DCP-LA was inhibited by the PKC inhibitor GF109203X or the α7 ACh receptor antagonist α-BgTX (Fig. 8b). Taken together, these results indicate that DCP-LA stimulates serotonin release from hypothalamic slices in a PKC- and α7 ACh receptor-dependent manner. This, in the light of the fact that reduction/deficiency of serotonin causes depression, panic disorder, sleep disturbance, and mental irritation, suggests that DCP-LA could improve depressive disorder by stimulating serotonin release.

DCP-LA Inactivates GSK-3β

Emerging evidence has pointed to the role of GSK-3β in serotonin-sensitive anxiety and social behavior [9]. GSK-3β is activated by being phosphorylated at Tyr216 and inactivated by being phosphorylated at Ser9. 5-HT_{1A} receptor is shown to activate Akt followed by inactivation of GSK-3β through its phosphorylation at Ser9 [22]. In the present study, restraint stress did not affect phosphorylation of GSK-3β at Tyr216 both in the hippocampus and hypothalamus (Fig. 9a). By contrast, phosphorylation of GSK-3β at Ser9 in the hypothalamus for mice with restraint stress was significantly reduced (Fig. 9c), although Ser9 phosphorylation in the hippocampus was not affected (Fig. 9b). DCP-LA recovered reduced Ser9 phosphorylation in the hypothalamus for mice with restraint stress fully/further (Fig. 9c). DCP-LA also increased Ser9 phosphorylation in the hippocampus both for control and stressed mice (Fig. 9b) and in the hypothalamus for control mice (Fig. 9c). Collectively, these results indicate that DCP-LA inactivates GSK-3β or prevents stress-induced GSK-3β activation in the hypothalamus. This effect of DCP-LA could contribute to improvement of depression-related behavior.

Discussion

In the present study, DCP-LA increased cell surface localization of 5-HT_{1A} receptor in the hippocampus and

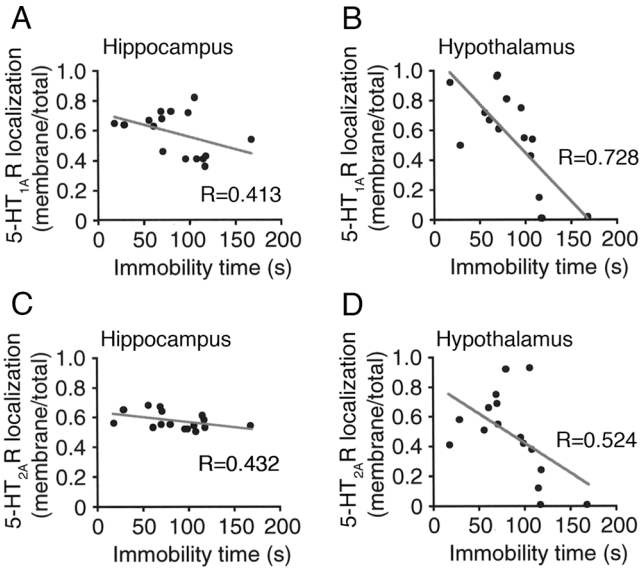

Fig. 7 Reduced cell surface localization of 5-HT_{1A} receptor in the hypothalamus correlates to prolonged immobility time. DCP-LA (1 mg/kg) was orally administered to mice without and with restraint stress, and forced-swim test followed by Western blotting using antibodies against 5-HT_{1A}R and 5-HT_{2A}R were carried out. The relation between 5-HT_{1A}R localization in the hippocampus and immobility time (**a**), between 5-HT_{1A}R localization in the hypothalamus and immobility time (**b**), between 5-HT_{2A}R localization in the hippocampus and immobility time (**c**), or between 5-HT_{2A}R localization in the hypothalamus and immobility time (**d**) was plotted. The linear regression analysis was carried out and the Pearson correlation coefficient (*R*) was calculated (n=15 independent mice)

hypothalamus for nonstressed normal rats, but such effect was not found in 5-HT_{2A} receptor. DCP-LA-induced increase in the cell surface localization of 5-HT_{1A} receptor was abolished by the vesicular exocytosis inhibitor BoTX-A, indicating that DCP-LA stimulates vesicular exocytosis of 5-HT_{1A} receptor, to increase cell surface localization of the receptor. DCP-LA serves as a selective and direct activator of PKCε [14, 15] and regulates α7 ACh receptor trafficking in a PKC-dependent manner [16, 17]. The effect of DCP-LA on 5-HT_{1A} receptor trafficking here, however, was not affected by the PKC inhibitor GF109203X, but it was clearly inhibited by a CaMKII inhibitor KN-93. This indicates that CaMKII plays a central role in DCP-LA-induced 5-HT_{1A} receptor translocation to the plasma membrane. In the cell-free assay, DCP-LA suppressed PP1 activity and directly bound to PP1. This raises the possibility that DCP-LA could indirectly activate CaMKII by preventing dephosphorylation of phosphorylated CaMKII due to PP1 inhibition. In our earlier study, DCP-LA stimulated exocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor in a CaMKII-dependent manner [21]. Likewise, DCP-LA might stimulate 5-HT_{1A} receptor exocytosis through indirect CaMKII activation due to PP-1 inhibition.

5-HT_{1A} receptor is localized both at presynaptic terminals and postsynaptic cells. DCP-LA did not increase presence of 5-HT_{1A} receptor in the synaptosomal fraction separated from the rat hypothalamus, suggesting that DCP-LA stimulates 5-HT_{1A} receptor exocytosis to the plasma membrane on postsynaptic cells. In the immunocytochemical analysis using acutely dissociated rat hypothalamic neurons without presynaptic components, DCP-LA accumulated immunoreactive signals for 5-HT_{1A} receptor along the cell surface. Overall, these results indicate that DCP-LA stimulates 5-HT_{1A} receptor exocytosis in a CaMKII-dependent manner, thereby increasing membrane localization of the receptor on postsynaptic cells. DCP-LA had no significant effect on membrane localization of 5-HT_{2A} both in the hippocampus and hypothalamus. It is presently unknown why DCP-LA preferentially translocates 5-HT_{1A} receptor, but not 5-HT_{2A} receptor. Plausible explanations for this are that of 5-HT receptors 5HT_{1A} receptor alone is phosphorylated by CaMKII, which triggers translocation of the receptor, or that CaMKII phosphorylates a

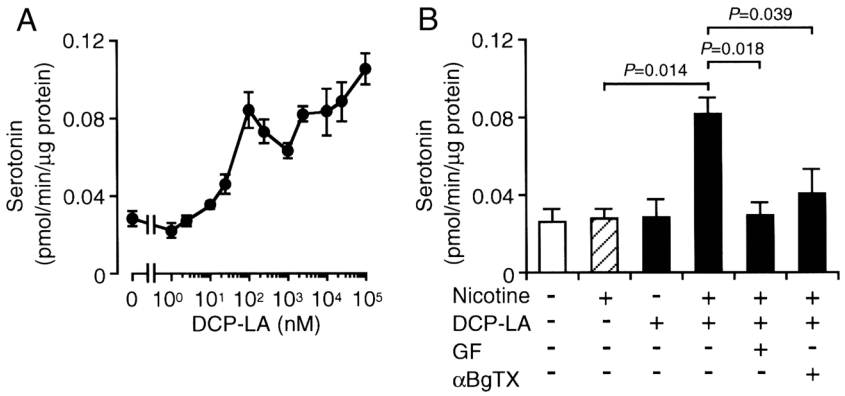

Fig. 8 DCP-LA stimulates serotonin release in a PKC- and α7 ACh receptor-dependent manner. **a** Rat hypothalamic slices were untreated and treated with DCP-LA at concentrations as indicated in the presence of nicotine (1 μM) for 20 min, and serotonin released was assayed. Each point represents the mean (±SEM) serotonin concentrations (n=6 independent experiments). **b** Slices were incubated in the absence (*negative sign*) and presence (*positive sign*) of nicotine (1 μM), DCP-LA (100 nM), GF109203X (GF; 100 nM), or α-BgTX (100 nM) for 20 min, and serotonin released was assayed. Each column represents the mean (±SEM) serotonin concentrations (n=6 independent experiments). *P* values, Dunnett's test

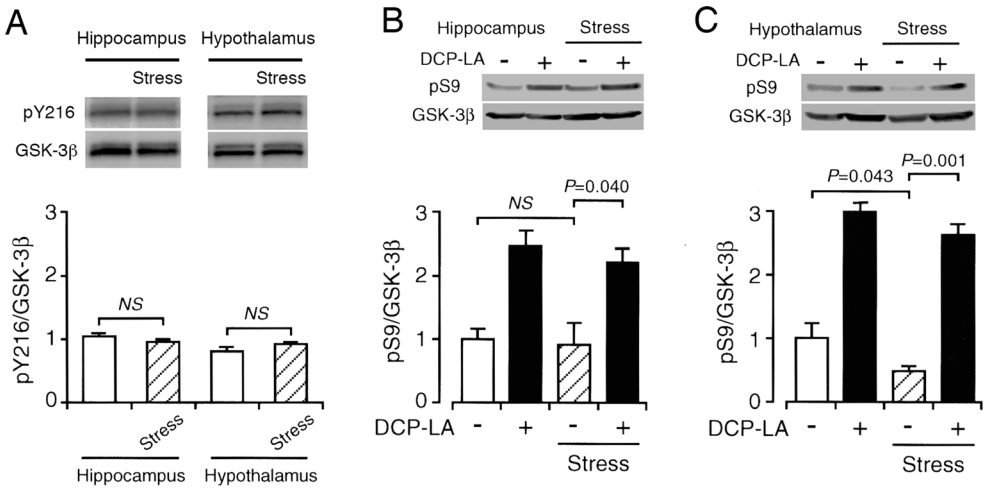

Fig. 9 DCP-LA inactivates GSK-3β. The hippocampal and hypothalamus were isolated from mice without and with restraint stress after forced-swim test, and then, Western blotting was carried out using antibodies against pY216 and GSK-3β (**a**). DCP-LA (1 mg/kg) was orally administered to mice without and with restraint stress, and the hippocampus (**b**) and hypothalamus (**c**) were prepared after forced-swim test followed by Western blotting using antibodies against pS9 and GSK-3β. In the graphs, each column represents the mean (±SEM) signal intensity for pY216 or pS9 relative to that for GSK-3β (n=4 independent experiments). *P* values, Dunnett's test. *NS* not significant

$5HT_{1A}$ receptor-specific adaptor protein intermediating between cargo containing $5HT_{1A}$ receptor and motor protein, to facilitate exocytosis of the receptor. To answer this question, we are currently carrying out further experiments.

Impairment of serotonergic neurotransmission is the major factor responsible for depression. Chronic stress has been shown to cause depressive-like behaviors, including passive behavioral coping and anhedonia in animal models, along with many other affective, cognitive, and behavioral symptoms. Intriguingly, chronic stress attenuates serotonergic neurotransmission, specifically mediated by 5-HT_{1A} receptor. In the present study, restraint stress significantly reduced cell surface localization of 5-HT_{1A} receptor in the hypothalamus, but not in the hippocampus. Then, the question addressing is why the effect of restraint stress on subcellular distribution of differs between the hippocampus and the hypothalamus. We have presently no plausible answer for this question. In the forced-swim test, restraint stress prolonged immobility time as compared with that for nonstressed control mice. This implies that restraint stress causes depression-like behavior. DCP-LA restored stress-induced reduction of cell surface 5-HT_{1A} receptor completely/furthermore. In addition, DCP-LA significantly shortened prolonged immobility time in mice with restraint stress, indicating that DCP-LA ameliorates depression-like behavior. In the linear regression analysis, reduction of cell surface localization of 5-HT_{1A} receptor in the hypothalamus highly correlated to prolongation of immobility time. Taken together, these results allow drawing a conclusion that DCP-LA ameliorates depression-like behavior by reversing stress-induced reduction of cell surface 5-HT_{1A} receptor in the hypothalamus.

Unlike DCP-LA, linoleic acid had no beneficial effect on restraint stress-induced depression-like behavior. This provides evidence that linoleic acid systemically applied is rapidly metabolized or adsorbed to adipocytes and skeletal muscle cells before arriving in the brain but that DCP-LA is actually capable of exhibiting stable bioactivities in the brain. All the investigated antidepressants sulpiride, sertraline, and Li_2CO_3 significantly shortened prolonged immobility time in mice with restraint stress, but they had no effect on 5-HT_{1A} receptor translocation. This suggests that DCP-LA ameliorates depression-like behavior by the mechanism distinct from that for antidepressants examined here.

DCP-LA stimulates presynaptic glutamate release, thereby facilitating hippocampal synaptic transmission [19]. Then, we wondered whether DCP-LA stimulates serotonin release. Expectedly, DCP-LA increased nicotine-triggered serotonin release from rat hypothalamic slices, and the effect was inhibited by the PKC inhibitor GF109203X or the α7 ACh receptor antagonist α-BgTX. α7 ACh receptor is preferentially localized at presynaptic terminals and regulates neurotransmitter release. DCP-LA enhances α7 ACh receptor activity in a PKC-dependent manner [13, 16]. A significant increase in the release of serotonin was obtained with just combined treatment with DCP-LA and nicotine, but solitary treatment with DCP-LA or nicotine had no effect. This, in the light of the fact that DCP-LA is not an agonist of α7 ACh receptor, suggests that α7 ACh receptor activation is required for DCP-LA-induced serotonin release. This also suggests that nicotine does not activate α7 ACh receptor sufficiently to stimulate serotonin release. Taken together, these results indicate that DCP-LA stimulates serotonin release by activating PKCε, to enhance presynaptic α7 ACh receptor activity. DCP-LA-induced increase in serotonin release should facilitate serotonergic neurotransmission, leading to improvement of depression-related behavior.

GSK-3β participates in serotonergic transmission-mediated signaling networks relevant to mental illnesses [9–11]. GSK-3β is inactivated by Ser9 phosphorylation and Tyr216 dephosphorylation but conversely activated by Ser9 dephosphorylation and Tyr216 phosphorylation. In the present study, restraint stress reduced phosphorylation of GSK-3β at Ser9 in the hypothalamus without affecting Tyr216 phosphorylation. This interprets that restraint stress induces GSK-3β activation in the hypothalamus. DCP-LA restored restraint stress-induced reduction of GSK-3β phosphorylation at Ser9 fully/furthermore. This indicates that DCP-LA-induced GSK-3β inactivation could be a factor for improvement of depression-related behavior. How DCP-LA increases GSK-3β phosphorylation at Ser9 is currently under investigation.

In conclusion, the results of the present study show that DCP-LA could ameliorate depression-like behavior by promoting translocation of 5-HT_{1A} receptor to the plasma membrane on postsynaptic cells in the hypothalamus, by stimulating serotonin release from hypothalamic slices, and by inactivating GSK-3β in the hypothalamus. DCP-LA, thus, might be developed as a new type of antidepressant and shed brilliant light upon treatment of depression.

Disclosure Statement The authors state that there are no actual or potential conflicts of interest.

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