RAPID-ACTING ANTIDEPRESSANT-LIKE EFFECTS OF ACETYL-L-CARNITINE MEDIATED BY PI3K/AKT/BDNF/VGF SIGNALLING PATHWAY IN MICE

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Abstract—The possible involvement of the PI3K/AKT/BDNF/VGF signaling in rapid-acting antidepressant-like effects of antidepressants has been explored progressively by more studies. However, whether this signaling participates in the antidepressant-like effects of acetyl-l-carnitine (ALC) has not been examined. Herein, we assessed the antidepressant-like effects of ALC using the forced swimming test (FST). Our results demonstrated the dose–effect relationship of acute administration of ALC (5, 25, 50 and 100 mg/kg, i.p.) and showed that it dose-dependently decreased the immobility time on FST of mice. In addition, ALC (100 mg/kg, i.p.) also reversed depressive-like behavior and the down-regulation of phosphorylated AKT (pAKT), brain-derived neurotrophic factor (BDNF) and neuropeptide VGF in the hippocampus and prefrontal cortex of mice induced by chronic unpredictable mild stress (CUMS) paradigm. Further, intra-cerebroventricular (i.c.v.) infusions of LY294002 (10 nmol/side), a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, significantly prevented the antidepressant-like effect of ALC (100 mg/kg, i.p.). In conclusion, our results demonstrated that ALC exerts rapid-acting antidepressant-like effects that might be mediated by the PI3K/AKT/BDNF/VGF signaling pathway.

Key words: acetyl-l-carnitine, brain-derived neurotrophic factor, phosphatidylinositol 3-kinase, depression, neuropeptide VGF.

INTRODUCTION

Depression is a common psychiatric disorder with high morbidity and mortality rates (Licinio and Wong, 2001). It is becoming one of the most prevalent public mental health problems due to treatment resistance and increased risk of suicide, producing a serious burden to patients and society (Kessler et al., 2003). In addition, although the monoamine hypothesis of depression has been the dominating pathophysiology of depression as well as targets of pharmacological treatments for the last decades, there are serious limitations to the current monoamine theory (Massart et al., 2012). However, studies suggest that these neurotransmitters are immediately affected by diverse antidepressants, but clinical improvements are not evident until few weeks later (Machado-Vieira et al., 2008). Therefore, additional novel rapid-acting antidepressants with different mechanism of actions are needed to enable clinicians to diversify treatment options for treatment of depression.

Acetyl-l-carnitine (ALC), the short-chain ester of carnitine, is endogenously produced within mitochondria and peroxisomes and it can readily cross the blood–brain barrier (Kido et al., 2001). In addition, ALC is found naturally in the central nervous system (CNS) and involved in several neural pathways, the brain energy and phospholipid metabolism, the activity of neurotransmitters and neurohormones, the synaptic morphology and multiple neurotransmitters (Vivoli et al., 2010; Schaevitz et al., 2012; Smeland et al., 2012). Moreover, in previous studies, ALC showed beneficial effects in major depression (Cuccurazzu et al., 2013; Nasca et al., 2013). Interestingly, although ALC’s exact mechanisms of action in treatment of depression is still not clear, animal and cellular models suggest that its neuroplasticity effect, neurotransmitter regulation and mGlu receptor could play an important role as its antidepressant action mechanism today (Tempesta et al., 1985; Nasca et al.,

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Abbreviations: ACSF, artificial cerebrospinal fluid; ALC, acetyl-l-carnitine; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CUMS, chronic unpredictable mild stress; DMI, desipramine; FST, forced swimming test; HDACs, histone deacetylases; ICR, imprinting control region; i.c.v., intra-cerebroventricular; i.p., intraperitoneally; mTOR, mammalian target of rapamycin; NIH, National Institutes of Health; OPT, open-field test; pAKT, phosphorylated AKT; PI3K, phosphatidylinositol 3-kinase; RIPA, radio-immunoprecipitation assay.

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delay in antidepressant efficacy (Adell et al., 2005) may be attributable to neural adaptive mechanisms to reverse the damage of stress in the hippocampus including changes in neurotrophic factors (Castren, 2005; Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). The neurotrophic hypothesis of depression is based on the stress-induced down-regulation and rapid-acting antidepressant-induced up-regulation of brain-derived neurotrophic factor (BDNF) and neuropeptide VGF (non-acronymic) expression in the brain (Russo-Neustadt et al., 1999; Adlard and Cotman, 2004; Thakker-Varia et al., 2007, 2010; Nasca et al., 2013; Ghasemi et al., 2014; Lin et al., 2014). Clinical findings have suggested that depression is associated with a reduction in BDNF levels in the hippocampus and serum (Laske et al., 2010). In addition to BDNF, other classes of genes induced by BDNF, revealed by previous studies that neuropeptides are important regulators of hippocampal function (Thakker-Varia and Alder, 2009). Thakker-Varia et al. (2007) discovered a role for the neuropeptide VGF in mediating synaptic activity of hippocampal cells and the neuropeptide VGF is up-regulated by both BDNF and 5-HT treatment and that VGF protein in the hippocampus is reduced in animals subjected to behavioral models of depression. A number of studies have suggested that the prefrontal cortex also implicated in mediating depressive-like behaviors (Muguruza et al., 2014). However, it is not known whether ALC increases BDNF and VGF expression in the hippocampus and prefrontal cortex of mouse. In addition, more and more evidence suggests that the phosphatidylinositol 3-kinase (PI3K)/AKT pathway is implicated in the pathophysiology of depression and in the antidepressant-like effect of different compounds (Kitagishi et al., 2012; Shi et al., 2012; Numakawa et al., 2013). Given the involvement of the PI3K/AKT signaling pathway in the regulation of depressive-like behaviors and antidepressant actions, it is necessary to demonstrate whether the rapid-acting antidepressant effects of ALC are dependent on PI3K/AKT-mediated signaling.

EXPERIMENTAL PROCEDURES

Animals

Experiments were conducted on young, healthy male imprinting control region (ICR) mice (22–25 g) born and reared in the animal facility of Ningbo University School of Medicine and Zhengzhou University of China. All animals were maintained at 22 ± 2 °C and 60% ± 5% relative humidity under a 12-h light/12-h dark cycle (lights on at 07:00 h) with ad libitum access to food and water when the stressors were not applied. All stressors were applied to animals outside of their housing area in a separate procedure room. All animal experiments were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Ningbo University School of Medicine.

Drugs

The drugs used included: acetyl-L-carnitine-(N-methyl-d3) hydrochloride (ALC, Fig. 1) and desipramine (DMI) were purchased from Sigma (St Louis, MO, USA). Both the ALC and DMI were dissolved in 0.9% saline (1% DMSO). These solutions, freshly prepared before administration, were given intraperitoneally (i.p.) in a volume of 10 ml/kg body weight. LY294002, a specific PI3K inhibitor, was purchased from Tocris Bioscience (Bristol, UK), dissolved in artificial cerebrospinal fluid (ACSF) to 10 nmol/μl and administrated by intra-cerebroventricular (i.c.v.) infusions. The i.c.v. injections were performed by employing a “free hand” method under light ether anesthesia according to the procedure described previously (Wang et al., 2014). In brief, animals were anesthetized with ketamine and xylazine (100 and 10 mg/kg i.p., respectively) and were placed in a stereotaxic frame with flat-skull position. The bregma coordinates used for injection were: ±1.0 mm bilateral, −0.3 mm posterior and −2.5 mm below. The cannula was anchored to the skull with dental cement and a stainless steel stylet was inserted into the guide cannulae to maintain the patency for microinjections. The mice were allowed to recover for 7 days and were handled every other day to reduce the stress associated with handling at the time of testing. The drugs were then microinjected into the bilateral ventricle of the mice brain. The injection cannula was left in place for another 60 s before being slowly withdrawn to avoid back flow (Lu et al., 2007; Chen et al., 2011; Xu et al., 2013; Wang et al., 2014). To verify the correct placement of the cannulae after the intra-ventricle delivery of the drug, the mice were killed after the behavioral tests, and cryostat sections were cut through the cortex to visualize the track of the cannula into the ventricle. Only animals with the correct cannula placement were used for further analysis.

Pharmacological treatment and experimental procedure

To test the rapid-acting antidepressant-like effects of acute administration of ALC, mice received i.p. injection of ALC (5, 25, 50 and 100 mg/kg, i.p.) or DMI (20 mg/kg) 30 min before the OFT and then 30 min later the FST was conducted. To investigate the effects of ALC on depressive-like behaviors induced by chronic unpredictable mild stress (CUMS) and the involvement of PI3K/AKT/BDNF/VGF signaling in the antidepressant-like
effect of ALC, after 21 days of CUMS treatment, the mouse were administrated with a high-effective dose of ALC (100 mg/kg, i.p.) or DMI (20 mg/kg, i.p.) 30 min before the open-field test (OFT) and 30 min later the forced swimming test (FST) was conducted. Immediately after the behavioral tests, the mice were killed by decapitation, and the hippocampus and prefrontal cortex were collected for the detection of pAKT, AKT, BDNF and VGF expressions by Western blotting. To further evaluate the participation of PI3K/AKT/BDNF/VGF signaling in the antidepressant-like effect of ALC, mice were pre-treated with LY294002 (10 nmol/side, i.c.v.) or ACSF 30 min before ALC administration (100 mg/kg, i.p.). The OFT or FST was carried out 30 min or 60 min respectively after ALC or vehicle administration.

CUMS

This animal model of stress consists of chronic exposure to variable unpredictable stressors, none of which is sufficient alone to induce long-lasting effects (Lin et al., 2014). Briefly, CUMS consisted of exposure to a variety of unpredictable stressors (randomly), including (1) 24-h food deprivation, (2) 24-h water deprivation, (3) 1-h exposure to an empty bottle, (4) 7-h cage tilt (45°C), (5) overnight illumination, (6) 24-h soiled cage (200-ml water in 100-g sawdust bedding), (7) 6-min forced swimming at 12°C, (8) 2-h physical restraint, and (9) 24-h exposure to a foreign object (e.g., a piece of plastic). All stressors were applied individually and continuously, day and night. The control animals were housed in a separate room and had no contact with the stressed groups. To prevent habituation and to ensure the unpredictability of the stressors, all stressors were randomly scheduled over a 1-week period and repeated throughout the 3-week experiments (as shown in Table 1 for detailed protocol).

Behavioral tests

OFT. To ensure that the alterations in the duration of immobility were not due to the changes in the motor activity, the locomotor activity of the mice was assessed in an OFT immediately before the FST. This was performed as previously described (Zhang et al., 2013) with minor modifications. The animals were individually placed in a white Plexiglas box (50 × 50 × 39 cm) with the floor divided into four identical squares in a dim room. Line crossings (with all four paws placed into a new square) and rears (with both front paws raised from the floor) were recorded in a 5-min period. After each test, the apparatus was cleaned with 5% ethanol solution to hide animal clues. The light inside the apparatus was maintained at minimum to avoid any anxiety behavior.

FST. The FST was performed as previously described (Sarkisyan et al., 2010). The experiments were carried out in a sound-attenuated room lighted by white light (40-W lamp). Briefly, mice were individually placed in a clear plastic cylinder (height: 25 cm; diameter: 10 cm; containing 10 cm of fresh water at 23 ± 2°C). During the test session, each individual mouse was placed in the cylinder for 6 min, and the duration of the immobility was scored during the last 4 min. The time during which the mouse made only small movements necessary to keep the head above water was considered as duration of immobility. To remove the influence of potential alarm substances on the tested behavior, fresh water was introduced prior to each test. All test sessions were recorded with a video camera positioned such that the entire apparatus was in full sight. Trained observers, who were blinded to the treatments, scored the recordings of the test sessions.

Immunoblot analyses. Western blot analysis was performed as previously described (Zhang et al. 2013). In brief, brain tissues were homogenized in a radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; Upstate, Temecula, CA, USA) containing protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA) and then centrifuged at 15,000 × g for 30 min. Samples (80 μg protein each) were separated using SDS–PAGE and subsequently transferred to PVDF membranes (0.22 μm; Millipore, Temecula, CA, USA). The samples were then incubated overnight with rabbit anti-pAKT (1:1000; Millipore, CA, USA), anti-AKT (1:1000; Millipore, Temecula, CA, USA), anti-BDNF (1:500; Millipore, Temecula, CA, USA), anti-VGF (1:500; Millipore, Temecula, CA, USA), and anti-β-actin antibodies (1:1000; Cell signaling, MA, USA) at 4°C. Afterward, the membranes were incubated with Alexa Fluor 700 conjugated goat anti-rabbit antibody (1:10,000; Invitrogen, Eugene, OR, USA) for 60 min. Detection and quantification of specific bands were performed using a fluorescence scanner (Odyssey Infrared

<table>
<thead>
<tr>
<th>Stressor</th>
<th>Duration</th>
<th>Day</th>
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<tr>
<td>Food deprivation</td>
<td>24-h</td>
<td>Monday</td>
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<tr>
<td>Exposure to a foreign object</td>
<td>24-h</td>
<td>Monday</td>
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<tr>
<td>Water deprivation</td>
<td>24-h</td>
<td>Monday</td>
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<tr>
<td>Forced swimming at 12°C</td>
<td>6-min</td>
<td>Tuesday</td>
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<tr>
<td>Soiled cage</td>
<td>24-h</td>
<td>Wednesday</td>
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<td>Overnight illumination</td>
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<tr>
<td>Food deprivation</td>
<td>24-h</td>
<td>Wednesday</td>
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<td>Cage tilt (45°C)</td>
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<td>Physical restraint</td>
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<td>Exposure to an empty bottle</td>
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<td>Cage tilt (45°C)</td>
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<td>Soiled cage</td>
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<td>Exposure to a foreign object</td>
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<td>Forced swimming at 12°C</td>
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Imaging System, LI-COR Biotechnology, Lincoln, NE, USA). For band stripping, the membranes were incubated with a stripping buffer (Chemicon, Temecula, CA, USA) for 15 min. All samples were analyzed at least in triplicate.

Quantitative RT-PCR. This was performed following the procedures described previously (Vahdati Hassani et al., 2014). Total RNAs were extracted from the hippocampus and prefrontal cortex of mice using High Pure RNA Tissue Kit according to the manufacturer’s instructions. The quantity and quality of the isolated RNAs were assessed using NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, USA). QRT-PCR was performed to analyze transcript levels of VGF using EXPRESS One-Step SYBR® GreenER® SuperMix Kit for one-step qRT-PCR according to the manufacturer’s instructions and a StepOne™ Real-Time PCR System (ABI, USA). The following real-time PCR protocol was used for all genes: activation of reverse transcriptase and cDNA synthesis (50 °C for 5 min), PCR activation (95 °C for 2 min), 40 cycles of denaturation (95 °C for 15 s) and annealing/extension (60 °C for 1 min). At the end of the PCR, a melting curve analysis was performed by gradually increasing the temperature from 60 to 95 °C with a heating rate of 0.3 °C/s. The primers used are as follows: VGF, forward 5'-GATGACGACGACGAAGAC-3' and reverse 5'-CGATGATGCTGACCACAT-3'; BDNF, forward 5'-AAAACCATAAGGACGCGGACTT-3' and reverse 5'-GAGGCTCCAAAGGCACTTGA-3'; β-actin, forward 5'-GGGAAATCGTGCGTGACATT-3' and reverse 5'-GCCGCAGTGGCCATCTC-3'. The data were analyzed by Software 2.2 using Ct value as the readout and normalized relative to levels of β-actin.

Statistical analyses. All measurements were performed by an independent investigator blinded to the experimental conditions. Data are expressed as the mean ± standard error of means (SEM). Data were analyzed by a one-way ANOVA or a two-way ANOVA followed by Newman–Keuls post hoc test using the GraphPad Prism software (Version 5.0, Prism software for PC, GraphPad). The criterion for significance was p < 0.05.

RESULTS

ALC administration dose-dependently decreases the immobility time in the FST of mice

In order to decide for the correct dose of our current work, we first assessed the dose–effect relationship of acute ALC administration using the FST in naïve mice. The mice received intraperitoneal injection of ALC (5, 25, 50 and 100 mg/kg, i.p.) or DMI (20 mg/kg, i.p.) 30 min before the OFT and then immediately followed by the FST 30 min later (Fig. 2A). A one-way ANOVA revealed that ALC significantly decreased immobility time \( F(5, 48) = 7.324, p < 0.001 \) in the FST, showing dose-dependent manner. To exclude the possibility that ALC induced locomotor activity alterations in these behavioral tests, we measured the effects of ALC on locomotor activity 30 min before the

Fig. 2. Dose–effect relationship of acute ALC administration in naïve ICR mice. (A) Protocol for experiments using the OFT and FST. The OFT was conducted 30 min after a single injection of vehicle, ALC or Desipramine (DMI) (i.p.). The FST was performed 30 min after the OFT. (B) Acute ALC treatment significantly decreased immobility time in the FST. (C and D) Acute ALC treatment had no effects on locomotor activity, reflected by the line crossing (C) and rearing (D) in mice. The data are expressed as mean ± SEM (n = 9 per group). *p < 0.05, **p < 0.01, compared with Vehicle-treated group.

FST. The mice treated with ALC (5, 25, 50 and 100 mg/kg, i.p.) or DMI (20 mg/kg, i.p.) did not differ from vehicle-treated mice in the number of line crossing \( F(5, 48) = 0.5959, p = 0.7031 \) or rearing \( F(5, 48) = 0.2593, p = 0.9330 \), indicating that reductions in immobility time in the FST was not attributable to alterations in locomotor activity.

ALC administration reverses depressive-like behaviors induced by CUMS in mice

This experiment investigated the effects of ALC on depressive-like behaviors induced by CUMS. Four groups of mice were used (n = 9 per group): (1) mice
not subjected to CUMS and treated with vehicle injection, (2) mice subjected to CUMS and treated with vehicle injection, (3) mice subjected to CUMS and treated with a single ALC (100 mg/kg, i.p.) injection, (4) mice subjected to CUMS and treated with a single DMI (20 mg/kg, i.p.) injection. Briefly, the animals were subjected to different stressors for 21 consecutive days. On day 22, the OFT was conducted 30 min after a single injection of vehicle, ALC or DMI. The FST was performed immediately 30 min after the OFT (Fig. 3A). The one-way ANOVA revealed significant differences in drug treatments in the FST \( F(3, 36) = 59.61, p < 0.0001; \) Fig. 3B]. Post hoc analyses indicated that exposure to CUMS in mice treated with vehicle significantly increased the immobility time in the FST \( p < 0.01 \) as compared to the non-stressed mice with the same treatment (vehicle). This was reversed by treatment with ALC \( p < 0.01 \) in the FST (Fig. 3B). In addition, an ANOVA indicated there were significant differences among all treatments in their effect on locomotor activity: line crossing \( F(3, 36) = 3.183, p = 0.0354; \) Fig. 3C and rearing \( F(3, 36) = 2.702, p = 0.0459; \) Fig. 3D]. Post hoc tests revealed vehicle-treated CUMS mouse exhibited a significant decrease in line crossing \( p < 0.05 \) and rearing \( p < 0.05 \) compared with non-stressed mouse with Vehicle treated. However, ALC treatment had no significant effect on line crossing \( p > 0.05 \) and rearing \( p > 0.05 \) compared with non-stressed mouse with Vehicle treated. Reversal of CUMS-induced behavioral despair by acute treatment confirmed the antidepressant potential of ALC.

**ALC administration reverses CUMS-induced down-regulation of pAKT, BDNF, and VGF in the hippocampus and prefrontal cortex of mice**

As shown in Fig. 4, CUMS significantly decreased the expression of pAKT \( F(3, 8) = 18.02, p = 0.0006 \) for the hippocampus; \( F(3, 8) = 7.945, p = 0.0088 \) for the prefrontal cortex; \( F(3, 8) = 17.15, p = 0.0008 \) for the prefrontal cortex; \( F(3, 12) = 19.22, p < 0.001 \) for the prefrontal cortex; \( F(3, 8) = 45.98, p < 0.001 \) for BDNF [Protein: \( F(3, 8) = 12.25, p = 0.0023 \) for the hippocampus; Protein: \( F(3, 8) = 45.98, p < 0.001 \) for the hippocampus; mRNA: \( F(3, 12) = 45.98, p < 0.001 \).

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**Fig. 3.** Effects of acute ALC administration on depressive-like behaviors induced by CUMS in mice. (A) Behavioral test procedure. Briefly, the mice were subjected to different stressors for 21 consecutive days. On day 22, the OFT was conducted 30 min after a single injection of vehicle, ALC or Desipramine (DMI) (i.p.). The FST was performed immediately 30 min after the OFT. (B) Acute ALC treatment significantly decreased immobility time in the FST. (C and D) Acute ALC treatment had no effects on locomotor activity, reflected by the line crossing (C) and rearing (D) in mice. The data are expressed as mean ± SEM (n = 9 per group). \(^*p < 0.05, \,**p < 0.01, \) compared with non-stress treated mice with Vehicle-administrated group; \( \dddot{p} < 0.01, \) compared with stress treated mice with ALC-administrated group.
for prefrontal cortex] and VGF [Protein: $F(3, 8) = 5.235$, $p < 0.01$, Fig. 4L; mRNA: $F(3, 12) = 19.58$, $p < 0.001$, Fig. 4N for prefrontal cortex] in the hippocampus and prefrontal cortex compared with the non-stressed mice treated with vehicle. In addition, ACL or DMI significantly reversed the CUMS-induced decreases in pAKT ($p < 0.01$ for both ACL and DMI in the hippocampus; Fig. 4B and $p < 0.01$ for both ACL and DMI in the prefrontal cortex; Fig. 4I), BDNF ($p < 0.01$ for both ACL and DMI in either the hippocampus or the prefrontal cortex; Fig. 4D, K, F, M), and VGF (Protein: $p < 0.05$ for both ACL and DMI; mRNA: $p < 0.01$ for both ACL and DMI in the hippocampus or the prefrontal cortex; Fig. 4E, L, G, N) in the hippocampus and prefrontal cortex. By contrast,

Fig. 4. ALC reverses the CUMS induced down-regulation of pAKT, BDNF and VGF in hippocampus or prefrontal cortex of mice. (A) and (F) representative immunoblots of pAKT, AKT, BDNF and VGF detected by Western blotting with tissues from the hippocampus (A) and prefrontal cortex (F); the rest panels are quantification of the immunoblotting bands of pAKT (B and G), AKT (C and H), BDNF (D and I), and VGF (E and J). The data are expressed as mean ± SEM ($n = 3$ per group). *$p < 0.05$, **$p < 0.01$, compared with non-stressed mice treated with Vehicle group; # $p < 0.05$, ## $p < 0.01$, compared with stressed mice treated with ALC group.

Fig. 5. PI3K/AKT/BDNF/VGF activity mediated the antidepressant-like effects of ALC in mice. (A) Experimental procedure for the assessment of the role of PI3K/AKT/BDNF/VGF signaling in the effects of ALC. Mouse were injected with LY294002 (10 nmol/side, i.c.v.) followed by ALC (100 mg/kg, i.p.) 30 min before OFT and the FST was conducted 30 min after the OFT. (B) Immobility time of mouse was measured. Pretreatment with LY294002 reversed the reduction of immobility time produced by ALC. (C and D) All the treatment had no effects on locomotor activity, reflected by the line crossing (C) and rearing (D) in mice. The data are expressed as mean ± SEM ($n = 9$ per group). *$p < 0.01$, compared with ACSF plus Vehicle group; **$p < 0.01$, compared with ACSF plus ALC group.
none of the treatments affected the AKT levels in the hippocampus and prefrontal cortex (Fig. 4C, J).

Inhibition of PI3K activity blocks the antidepressant-like effects of ALC in mice

To investigate the involvement of PI3K in the antidepressant-like effect of ALC, four groups of mice were used: (1) mice treated with ACSF (i.c.v.) plus vehicle (i.p.), (2) mice treated with LY294002 (i.c.v.) plus vehicle (i.p.), (3) mice treated with ACSF (i.c.v.) plus with ALC (i.p.), (4) mice treated with LY294002 (i.c.v.) plus with ALC (i.p.). 7 days after cannula implantation, mice were pre-treated with LY294002 (10 nmol/side, i.c.v.) or its vehicle (ACSF) 30 min before i.p. administration of ALC (100 mg/kg) or vehicle. The OFT was carried out 30 min after ALC treatment and the FST was conducted 30 min after the OFT (Fig. 5A). Fig. 5B shows the effect of inhibition of the upstream AKT activator PI3K by LY294002 (10 nmol/side, i.c.v.) in the antidepressant-like effect of ALC (100 mg/kg, i.p.) in the FST. The two-way ANOVA revealed significant differences for ALC treatment [F (1, 32) = 19.32; p = 0.0001], LY294002 treatment [F (1, 32) = 4.562; p = 0.0404] and ALC treatment × LY294002 interaction [F (1, 32) = 18.63; p = 0.0001]. Post-hoc analysis showed that the antidepressant-like effect of ALC was completely prevented by treatment of animals with the PI3K inhibitor LY294002. Fig. 5C shows that the administration of LY294002 alone or in combination with ALC was devoid of effect in the line crossing of OFT (ALC treatment [F (1, 32) = 0.6658; p = 0.4205], LY294002 treatment [F (1, 32) = 0.0623; p = 0.8045], ALC treatment × LY294002 interaction [F (1, 32) = 0.3318; p = 0.5687]. In addition, Fig. 5D also shows that the administration of LY294002 alone or in combination with ALC did not modify the rearing of mice in the OFT (ALC treatment [F (1, 32) = 0.6207; p = 0.4366], LY294002 treatment [F (1, 32) = 0.7334; p = 0.3982], ALC treatment × LY294002 interaction [F (1, 32) = 0.4693; p = 0.4982]).

Inhibition of PI3K activity blocks the up-regulation of pAKT, BDNF and VGF in the hippocampus and prefrontal cortex of mice produced by ALC

As shown in Fig. 6, a two-way ANOVA revealed significant effects of ALC treatment [pAKT: F (1, 8) = 12.87; p = 0.0071, Fig. 6B, BDNF: F (1, 8) = 37.07, p = 0.0003, Fig. 6D and VGF: F (1, 8) = 4.626, p = 0.0350, Fig. 6E in the hippocampus; pAKT: F (1, 8) = 17.23, p = 0.0032, Fig. 6J, BDNF: F (1, 8) = 19.13, p = 0.0024, Fig. 6L and VGF: F (1, 8) = 6.671, p = 0.0325, Fig. 6N in the prefrontal cortex]. LY294002 treatment [pAKT: F (1, 8) = 13.13, p = 0.0068, Fig. 6B, BDNF: F (1, 8) = 39.98, p = 0.0002, Fig. 6D and VGF: F (1, 8) = 7.819, p = 0.0233, Fig. 6E in hippocampus; pAKT: F (1, 8) = 24.13, p = 0.0012, Fig. 6J, BDNF: F (1, 8) = 24.77, p = 0.0011, Fig. 6L and VGF: F (1, 8) = 13.85, p = 0.0059, Fig. 6N in prefrontal cortex] and ALC treatment × LY294002 interaction [pAKT: F (1, 8) = 12.06, p = 0.0084, Fig. 6B, BDNF: F (1, 8) = 38.65, p = 0.0003, Fig. 6D and VGF: F (1, 8) = 5.998, p = 0.0400, Fig. 6E in the hippocampus; pAKT: F (1, 8) = 6.529, p = 0.0339, Fig. 6J, BDNF: F (1, 8) = 10.59, p = 0.0116, Fig. 6L and VGF: F (1, 8) = 13.60, p = 0.0061, Fig. 6N in the prefrontal cortex] on the expression of pAKT, BDNF and VGF in the hippocampus and prefrontal cortex respectively. Post-hoc analysis showed that the up-regulation effects of ALC on the expression of pAKT [p < 0.01 in the hippocampus and p < 0.01 in the prefrontal cortex; Fig. 6B, G], BDNF [p < 0.01 in the hippocampus and p < 0.01 in the prefrontal cortex; Fig. 6D, I] and VGF [p < 0.05 in the hippocampus and p < 0.01 in the prefrontal cortex; Fig. 6E, J] were completely prevented by treatment of animals with the PI3K inhibitor LY294002 in the hippocampus and prefrontal cortex respectively. By contrast, none of the treatments affected the AKT levels in the hippocampus (Fig. 6C) and prefrontal cortex (Fig. 6H).

DISCUSSION

ALC, an acylcarnitine form of essential dietary nutrient carnitine, may have a potential antidepressant effect with novel mechanism of action because of its diverse functions related to neuroplasticity (Jones et al., 2010). The beneficial effects of ALC in depression are supported in preclinical studies of animal and cellular models and in a series of randomized clinical trials (Nasca et al., 2013; Pulvirenti et al., 1990; Brennan et al., 2013; Bersani et al., 2013). The main findings of the present study were that acute treatment of ALC rapidly produces antidepressant-like effects as evidenced by reduced immobility time in the FST and we also provided the first evidence that the fast-acting antidepressant response of ALC in the FST of mice involves PI3K/AKT/BDNF/VGF signaling activation. In addition, our results strongly suggest that the antidepressant effects of ALC are similar to a rapid-acting antidepressant DMI, in the early stages of treatment.

Increasing evidence has shown that depression induced by stress may be associated with the decreased volume of cortical and limbic brain regions, and neurotrophic/growth factor support, most notably BDNF and VGF, suggest that the magnitude of the volume reduction and disruption of neurotrophic/growth factor are inversely correlated with the length of antidepressant treatment (Lin et al., 2014; Duman and Monteggia, 2006; Cattaneo et al., 2010). The beneficial effects of ALC in depression are supported in preclinical studies of animal and cellular models and in a series of randomized clinical trials (Nasca et al., 2013; Pulvirenti et al., 1990; Brennan et al., 2013; Bersani et al., 2013). The main findings of the present study were that acute treatment of ALC rapidly produces antidepressant-like effects as evidenced by reduced immobility time in the FST and we also provided the first evidence that the fast-acting antidepressant response of ALC in the FST of mice involves PI3K/AKT/BDNF/VGF signaling activation. In addition, our results strongly suggest that the antidepressant effects of ALC are similar to a rapid-acting antidepressant DMI, in the early stages of treatment.

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amygdala, basal forebrain, dorsal vagal complex, hindbrain, and ventral tegmental area of the midbrain (Salehi et al., 2006; Marosi and Mattson, 2014). VGF is expressed exclusively in neurons and in the adult VGF is detected in several areas in the brain including the olfactory system, cerebral cortex, hypothalamus and hippocampus as well as the adrenal medulla and motorneurons of the spinal cord (van den Pol et al., 1989, 1994). Further studies need to be conducted to find whether the other brain regions may be contributing to the antidepressant-like effects of ACL when systemic administration is used in mice.

Moreover, our results found that CUMS significantly decreased pAKT protein levels in the hippocampus and prefrontal cortex of mice respectively. We also found that acute ALC administration significantly increased the levels of pAKT, BDNF and VGF in the hippocampus and prefrontal cortex, which are signaling components associated with the therapeutic effects of antidepressants. As the downstream regulatory signaling pathway of ALC in our current work, PI3Ks are a large family of intracellular signal transducers. The role of the PI3K pathway has been implicated in the regulation of cell growth, survival, proliferation, and movement (Astle et al., 2011; Chan et al., 2011; Nedachi et al., 2011). Numerous studies have also implicated PI3K in depression and anxiety (Leibrock et al., 2013; Moretti et al., 2014). In addition, more and more studies have revealed that BDNF and its receptor, TrkB, have been extensively studied in association with depression and antidepressant treatments (Lin et al., 2014; Lindholm and Castreñ, 2014). BDNF-induced TrkB activation stimulates the PI3K/AKT signaling pathways, which play a principal role in promoting neuronal survival and synaptic plasticity and antidepressant-like actions (Pizzorusso et al., 2000; Rodgers and Theibert, 2002).

**Fig. 6.** Pretreatment with LY294002 blocks the effects of ALC on the expression of pAKT, AKT, BDNF and VGF in the brain of mice. (A) and (F) representative immunoblots of pAKT, AKT, BDNF and VGF detected by Western blotting with tissues from the hippocampus (A) and prefrontal cortex (F); the rest panels are quantification of the immunoblotting bands of pAKT (B and G), AKT (C and H), BDNF (D and I), and VGF (E and J). The data are expressed as mean ± SEM (n = 3 per group). ⁎p < 0.05, ⁎⁎p < 0.01, compared with ACSF plus Vehicle group; #p < 0.05, ##p < 0.01, compared with ACSF plus ALC group.
Particularly, major components of the PI3K/AKT pathway, including the mammalian target of rapamycin (mTOR) which play an important role in the production of BDNF, have been implicated in depression and the antidepressant response (Duman and Voleti, 2012). Furthermore, another interesting result of our present study was that i.c.v. infusions of LY294002, a specific PI3K inhibitor, blocked the antidepressant-like effects of ALC in the FST of mice, indicating that the downstream signaling pathways mediated by PI3K may play a principal role in depression therapy of ALC. Our results also suggest that BDNF and VGF mediated by PI3K/AKT are involved in the regulation of antidepressant activity of ALC. To our knowledge, the present study is the first to report that ALC activated the PI3K/AKT signaling pathway, leading to BDNF and VGF activation in depression animal model. Taken together, alterations in the expression of the proteins investigated in the present study may contribute to the molecular basis of stress-induced depressive-like behaviors and changes in the brain of mice. Consistent with our current work, previous studies (Cuccurazzu et al., 2013; Nasca et al., 2013) demonstrated that ALC treatment rapidly reversed the depressive-like behaviors in stressed animals and associated with BDNF (Nasca et al., 2013). In addition, it was demonstrated that the antidepressant-like effect of ALC was selectively associated with mGlu2 receptor (Cuccurazzu et al., 2013; Nasca et al., 2013). Growing evidences also demonstrated that mGlu receptor subtypes might be involved in the pathophysiology of depression, such as mGlu5 or mGlu7 (Chaki et al., 2013). Whether acetylating agents such as ALC regulate mGlu5 and mGlu7 receptors in the brain of depression animals is a question that warrants further investigation and these previous studies also have raised the question of whether mGlu2, mGlu5 and mGlu7 receptors down-regulate the PI3K/AKT/BDNF/VGF signaling in the rapid-acting antidepressant-like effect of ALC in the treatment of depression.

In conclusion, our present study is the first to report that ALC activates PI3K/AKT/BDNF/VGF signaling, which is impaired in the hippocampus and prefrontal cortex of depression animal model. Our results provide a clearer understanding of the molecular changes related to ALC-induced rapid-acting antidepressant effects. In addition, given that ALC is highly tolerated in the medical practice with histone deacetylases (HDACs) inhibitors, it is expected to become the rapid-acting antidepressant candidate.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CONTRIBUTORS

Chuang Wang, Qinwen Wang and Wenhua Zhou designed the study and wrote the protocol. Weiguo Wang, Yang Lu, Xin Zhao and Junfang Zhang administered the drugs and performed the behavioral tests. Xiaofei Wei and Xiaowei Chen performed the biochemical analysis, undertook the statistical analysis. Chuang Wang wrote the draft of the manuscript. All authors contributed to and have approved the final manuscript.

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