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Upregulation of mGlu2 Receptors via NF-κB p65 Acetylation Is Involved in the Proneurogenic and Antidepressant Effects of Acetyl-L-Carnitine

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Acetyl-L-carnitine (ALC) is a naturally occurring molecule with an important role in cellular bioenergetics and as donor of acetyl groups to proteins, including NF-κB p65. In humans, exogenously administered ALC has been shown to be effective in mood disturbances, with a good tolerability profile. No current information is available on the antidepressant effect of ALC in animal models of depression and on the putative mechanism involved in such effect. Here we report that ALC is a proneurogenic molecule, whose effect on neuronal differentiation of adult hippocampal neural progenitors is independent of its neuroprotective activity. The *in vitro* proneurogenic effects of ALC appear to be mediated by activation of the NF-κB pathway, and in particular by p65 acetylation, and subsequent NF-κB-mediated upregulation of metabotropic glutamate receptor 2 (mGlu2) expression. When tested *in vivo*, chronic ALC treatment could revert depressive-like behavior caused by unpredictable chronic mild stress, a rodent model of depression with high face validity and predictivity, and its behavioral effect correlated with upregulated expression of mGlu2 receptor in hippocampi of stressed mice. Moreover, chronic, but not acute or subchronic, drug treatment significantly increased adult born neurons in hippocampi of stressed and unstressed mice. We now propose that this mechanism could be potentially involved in the antidepressant effect of ALC in humans. These results are potentially relevant from a clinical perspective, as for its high tolerability profile ALC may be ideally employed in patient subpopulations who are sensitive to the side effects associated with classical antidepressants.
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INTRODUCTION

Although the role of adult hippocampal neurogenesis remains to be fully elucidated, several studies suggest its involvement in cognitive and emotional functions (Kempermann, 2008; Aimone *et al*, 2011; Couillard-Despres *et al*, 2011; Sahay *et al*, 2011) and its deregulation in neuropsychiatric disorders, including major depression disorder (MDD) (Hsieh and Eisch, 2010; Samuels and Hen, 2011). Based on the evidence that hippocampal neurogenesis can be downregulated by chronic stress, the hypothesis has emerged that this process may contribute to MDD pathophysiology (Pittenger and Duman, 2008; Hanson *et al*, 2011) and that it may be necessary for some of the effects of antidepressants in rodents (Santarelli *et al*, 2003; David *et al*, 2009) and humans (Boldrini *et al*, 2009). Endogenous acetyl-L-carnitine (ALC), aside from its role in cellular bioenergetics, modulates the activity of neurotrophic factors, hormones, and neurotransmitters in nervous tissues (Jones *et al*, 2010). Exogenously administered ALC can readily pass the blood–brain barrier (Inano *et al*, 2003) and it is neuroprotective at supraphysiological concentrations (Jones *et al*, 2010). In addition, the antinociceptive effects of ALC were demonstrated in rodent pain models (Chiechio *et al*, 2006, 2009). In humans, and particularly in the elderly, the beneficial effects of ALC were observed in mood disorders (Tempesta *et al*, 1987; Garzya *et al*, 1990; Bella *et al*, 1990; Zanardi and Smeraldi, 2006), with an unknown mechanism of action. Surprisingly, no published studies have evaluated, in animal models, the antidepressant activity of ALC. The drug can act as a donor of acetyl groups to proteins (Pettegrew *et al*, 2000), including the p65 member of NF-κB transcription factors (Chiechio *et al*, 2006), which are implicated in adult neurogenesis and neuroplasticity (Denis-Donini *et al*, 2008; Koo *et al*, 2010; Bonini *et al*, 2011; Grilli and Meneghini, 2012). Interestingly, ALC modulates gene expression of metabotropic glutamate receptor 2 (mGlu2) via NF-κB p65 acetylation and this mechanism has been proposed for ALC-mediated analgesic effects (Chiechio *et al*, 2006, 2009). Here we explored the possibility that ALC may promote adult

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neurogenesis and mGlu2 upregulation via NF-κB pathway activation and that this mechanism may contribute to its antidepressant efficacy.

MATERIALS AND METHODS

Animals

Male 5-month-old C57B/L6J mice from Charles River Laboratories (Calco, Italy) were utilized. Protocols were reviewed and approved by the local ethical committee on animal experimental studies.

Drugs

The following drugs were used: acetyl-L-carnitine and L-carnitine (Sigma-Tau, Pomezia, Italy); LY487379 (Tocris Bioscience, Bristol, UK); LY379268 and LY341495 (Ascent Scientific, Cambridge, UK); and JSH-23, SC-514, SN-50, and SN-50 M (Calbiochem-Merck KGaA, Darmstadt, Germany).

Cell Cultures and Immunocytochemical Analysis

For adult hippocampal neural progenitor cultures, the procedure for their maintenance, differentiation, and determination of apoptotic/necrotic rate was described elsewhere (Meneghini *et al*, 2010). To assess proliferation, neural progenitors were cultured for 2–24–72 h in the presence of ALC and vehicle. Bromodeoxyuridine (BrdU; 10 μM) was added to culture medium 2 h before cessation of treatment and then cells were fixed with 4% PFA. For immunofluorescence analysis, anti-BrdU (1 : 600, Novus Biologicals, Littleton, CO), anti-MAP-2 (1 : 600, Chemicon, Temecula, CA), anti-nestin (1 : 3000, Neuromics, Edina, MN), anti-GFAP (1 : 600, Millipore, Billerica, MA), or anti-NG2 (1 : 500, Abcam, Cambridge, UK) antibodies were used.

Western Blot Analysis

A total of 30 μg proteins were separated onto 8–10% SDS-PAGE gel. The primary antibodies used were: anti-mGlu2 (1 : 1000, Abcam), anti-mGlu3 (1 : 1000, Alomone Labs, Jerusalem, Israel), anti-PAR-1 (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Acetyl-NF-κB p65 (Lys310) (1 : 1000, Cell Signaling Technology, Danvers, MA), and anti-NF-κB p65 (1 : 1000, Santa Cruz Biotechnology). Densitometric analysis was performed by Quantity One software (Bio-Rad Laboratories), with samples normalized to α-tubulin (1 : 2000, Sigma Aldrich, Milan, Italy) or β-actin signals (1 : 2000, Sigma Aldrich).

UCMS Procedure and Behavioral Assessment

Upon arrival, C57B/L6J male mice ($n = 66$, 4–5 month old) were kept in the animal facility for 2 weeks before initiating the experimental procedure. The first 8 weeks of the UCMS regimen were drug free and treatment began from week 9 of UCMS and continued up to the end of behavioral testing. Vehicle (0.9% NaCl) or ALC (100 mg/kg) were administered s.c., once a day, for 3 weeks. In the first 5 days of treatment, a subgroup of mice ($n = 24$) was also given a daily dose of BrdU (150 mg/kg, Sigma Aldrich, i.p.). The stress regimen and behavioral analysis were applied as previously described (Koo *et al,* 2010; Valente *et al*, 2012).

In Vivo Neurogenesis Studies

For assessing the effect of drug treatment on cell proliferation, mice were treated for 21 days with ALC (100 mg/kg, s.c.) or vehicle. In the last day of treatment, mice received BrdU 150 mg/kg i.p. and were killed 2 h later. For acute (1 day) and subchronic (7 days) ALC treatment, mice were treated with BrdU on the first day of drug treatment and killed 21 days later. Mice were transcardially perfused and brain tissue prepared for immunofluorescence analysis with anti-BrdU (1 : 200; Novus Biologicals), anti-Neuronal Nuclei (NeuN; 1 : 150; Millipore), and anti-glial fibrillary acidic protein (GFAP; 1 : 100; Santa Cruz Biotechnology). Quantification of newborn hippocampal neurons and $BrdU^+$ cells was performed by modified unbiased stereology (Denis-Donini *et al*, 2008).

ALC Plasma Measurement

Quantification of ALC plasma levels was performed in samples from naive mice treated with vehicle and ALC 100 mg/kg s.c. for 21 days ($n = 6$/group), as previously described (Schaevitz *et al*, 2012).

Statistical Analysis

Data are reported as mean ± SD. In experiments with only two groups, Student's *t*-test was used. For experiments with more than two groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test, or by a two-way ANOVA (using stress and treatment as factors), followed by Bonferroni *post hoc* test. Statistical significance level was set for *P*-values < 0.05.

RESULTS

ALC Promotes Differentiation of Adult Hippocampal NPC Toward the Neuronal Lineage

$Nestin^+$ neural progenitor cells (NPCs) isolated from adult mouse hippocampi can be maintained in an undifferentiated proliferative state (Meneghini *et al*, 2013). Upon removal of growth factors from medium, they stop dividing and differentiate. By double immunolabeling for markers of neurons (MAP-2) and undifferentiated progenitors (nestin), the appearance of new neurons that are $MAP\text{-}2^+$ and $nestin^-$ cells can be evaluated. Under these experimental conditions, we tested the effects of ALC (0.01–1 mM). The drug significantly increased, in a concentration-dependent manner, the percentage of $MAP\text{-}2^+$ cells ($F(5, 48) = 139.2$, $P < 0.0001$; Figure 1a), with a maximal effect at 300 μM (% increase over vehicle-treated cells: $+148.9 \pm 22.8$). Exposure to 300 μM ALC also produced significant reduction of $GFAP^+$ astrocytes and $NG2^+$ oligodendrocyte precursors (% decrease over vehicle-treated cells: -47.4 ± 8.4 and -31.4 ± 4 for $GFAP^+$ cells ($P < 0.001$) and $NG2^+$ cells ($P < 0.001$), respectively; Figure 1b and c). In order to investigate whether ALC promoted cell survival, we

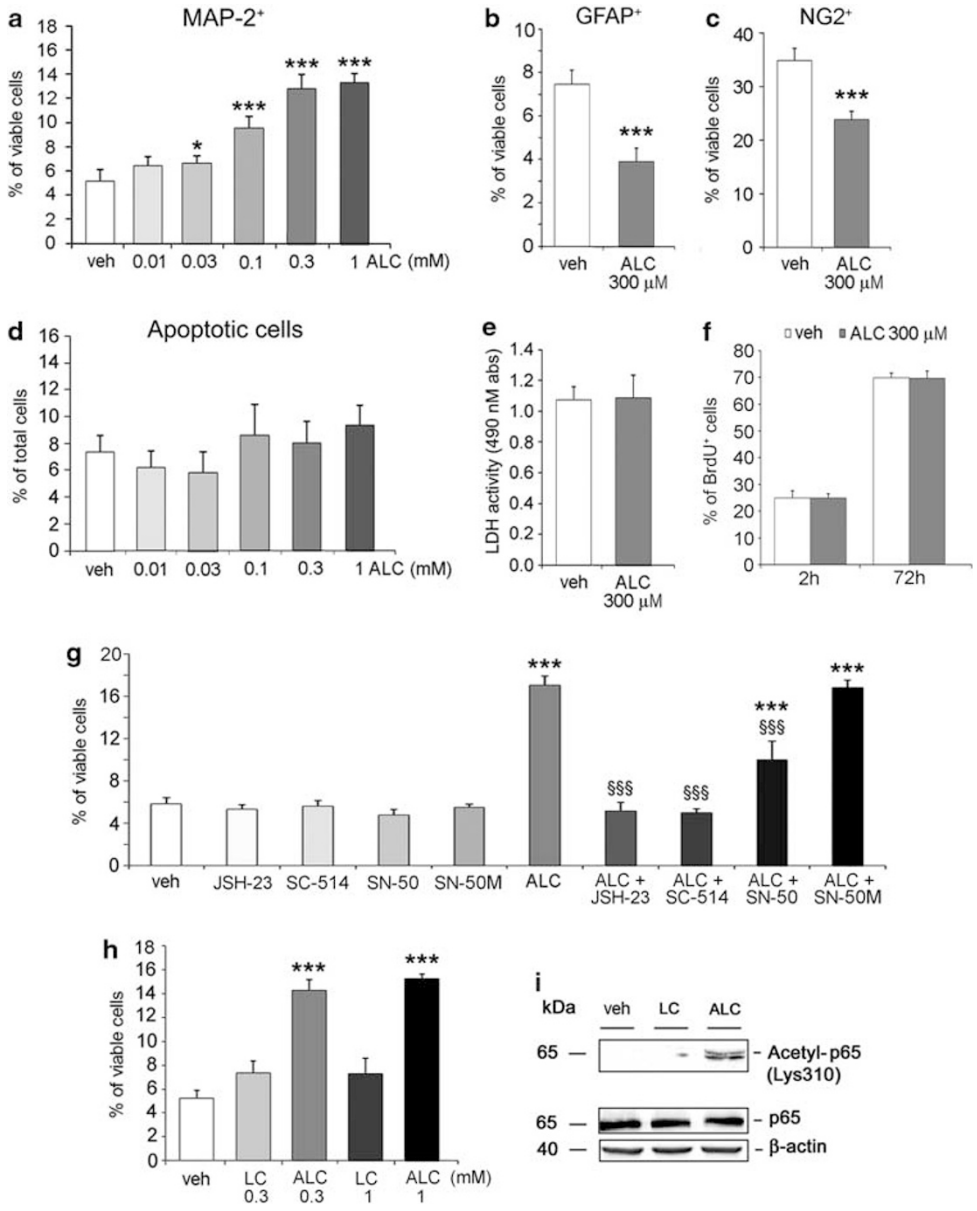

Figure 1 ALC proneurogenic effects on adult hippocampal neural progenitors involved activation of the NF-κB pathway and acetylation of p65. (a) The 24 h treatment with ALC (0.01–1 mM) promoted neuronal differentiation from adult hippocampal NPCs by significantly increasing the percentage of MAP-2^+ cells as compared with vehicle (veh). (b, c) ALC (300 μM) significantly reduced the percentage of $GFAP^+$ astrocytes (b) and $NG2^+$ oligodendrocyte precursors (c) derived from adult NPCs. (d, e) The percentage of apoptotic cells (d) and the amount of released LDH activity (e) were not significantly different in ALC- *vs* vehicle-treated cells. (f) The 2- and 72-h incubation of proliferating neural progenitors with ALC 300 μM did not affect BrdU incorporation as compared with vehicle-treated cells. BrdU (10 μM) was added to culture medium during the last 2 h of incubation. Data are expressed as %$BrdU^+$ cells over total viable cells. (g) NF-κB inhibitors JSH-23 (3 μM), SC-514 (3 μM), and SN-50 (10 μg/ml) counteracted the proneurogenic effects of 300 μM ALC. The inactive peptide SN-50M (10 μg/ml) had no effect. Data are mean ± SEM of $n = 2$ experiments in triplicate for (g) and $n = 3$ experiments in triplicate for all the others. (h) The 0.3 and 1 mM L-carnitine (LC) produced no effect on MAP-2^+ cells when compared with similar concentrations of ALC. (i) The 2-h treatment with ALC (300 μM), but not LC (300 μM) or vehicle, resulted in NF-κB p65 Lys310 acetylation in extracts of adult NPCs. Expression levels of total p65 and β-actin were also evaluated as internal controls. Data are mean ± SD of $n = 3$ experiments in triplicates. $^*P < 0.05$; $^{***}P < 0.001$ *vs* vehicle-treated cells, $^{§§§}P < 0.001$ *vs* ALC-treated cells. Data in (a, d, f, g, and h) were analyzed by one-way ANOVA followed by Tukey's *post hoc* test, whereas data in (b, c, and e) were analyzed by Student's *t*-test.

analyzed the number of apoptotic cells and the amount of LDH released by necrotic cells in culture medium in the presence of ALC or vehicle. Despite the fact that one-way ANOVA revealed a difference in the apoptotic rate ($F(5, 48) = 6.181$, $P = 0.0002$; Figure 1d), *post hoc* analysis confirmed no significant difference in the comparison between any ALC concentration and the vehicle group. No difference was also observed in LDH activity in vehicle- *vs* ALC-treated cultures (Figure 1e). In addition, exposure to 300 μM ALC for 2 and 72 h had no effect on the proliferation rate of adult hippocampal neural progenitors, compared with vehicle, as assessed by BrdU incorporation (Figure 1f). Similarly, no significant difference was reported between ALC and vehicle after 24 h of incubation (data not shown).

Overall, these data demonstrated that ALC promoted neuronal differentiation of adult hippocampal NPC at the expenses of nonneuronal lineages and that neither neuroprotective nor proliferative effects were involved in drug activity *in vitro*.

NF-κB Pathway Activation and p65 Acetylation Are Involved in ALC-Mediated Proneurogenic Effects

NF-κB transcription factors are involved in CNS functions, including neuronal commitment and differentiation of adult NPC (Grilli and Memo, 1997; Denis-Donini *et al*, 2005, 2008; Koo *et al*, 2010). We then tested drugs interfering with activation of the NF-κB pathway: JSH-23 and SN50, cell-permeable blockers of nuclear translocation of NF-κB p65 and p50, respectively; SC-514, a reversible inhibitor of IκB kinase 2 (IKK2). JSH-23 and SC-514 at 3 μM completely abolished, whereas 10 μg/ml SN-50 partially counteracted 300 μM ALC-induced increase of MAP-2$^+$ cells in NPC cultures ($F(9,50) = 223.9$, $P < 0.0001$; Figure 1g). *Post hoc* analysis confirmed that, when applied alone, JSH-23, SC-514, and SN-50 had no effect on NPC differentiation (5.35 ± 0.44, 5.66 ± 0.51, and 4.81 ± 0.51, respectively) as compared with vehicle (5.85 ± 0.6; Figure 1g). Furthermore, SN50M, the mutant counterpart of SN-50, did not affect ALC activity on MAP-2$^+$ cells (ALC + SN50M: 16.86 ± 0.7; 300 μM ALC: 17.1 ± 0.9; Figure 1g). Overall, these data suggested the involvement of NF-κB signaling in the proneurogenic effects of ALC on adult hippocampal NPC.

Under similar experimental conditions, 300 μM and 1 mM L-carnitine (LC) had no effect on the generation of new neurons ($F(2,18) = 3.340$, NS; Figure 1h), unlike similar concentrations of ALC ($F(2,18) = 322$, $P < 0.0001$; Figure 1h), suggesting that acetylation may be involved in ALC proneurogenic activity. By using an antibody against Acetyl-NF-κB p65 (Lys310), we demonstrated that a 2-h treatment of hippocampal NPC with 300 μM ALC promoted p65 acetylation (Figure 1i). No p65 acetylation could be observed when cells were treated with either vehicle or 300 μM LC (Figure 1i).

Group II mGlu Receptors, and in Particular the mGlu2 Subtype, Are Involved in ALC-Mediated Effects on Adult Hippocampal NPC

The analgesic properties of ALC have been suggested to involve drug-induced upregulation of mGlu2 receptor expression in dorsal root ganglia neurons via acetylation of NF-κB p65 (Chiechio *et al*, 2006). In our culture model, the nonselective mGlu2/3 agonist LY379268 promoted neurogenesis, as shown by a concentration-dependent increase of MAP-2$^+$ cells ($F(6,56) = 106.2$, $P < 0.0001$; Figure 2a), with maximal effects elicited at 10 μM (% increase over vehicle-treated cells: $+164 \pm 21.7$). As at μM concentrations the agonist loses its selectivity for group II and may activate group I and II mGlu receptors, we then assessed whether the preferential mGlu2/3 antagonist LY341495 (0.1–7.5 nM) was able to counteract ALC proneurogenic effects on hippocampal NPC. In the presence of 300 μM ALC, LY341495 caused a concentration-dependent inhibition of drug-induced increase of MAP-2$^+$ cells ($F(7,64) = 94.97$, $P < 0.0001$; Figure 2b), with maximal inhibition at 7.5 nM (Figure 2b). Alone, 7.5 nM LY341495 produced a statistically significant reduction in the percentage of MAP-2$^+$ cells when compared with vehicle-treated cells (7.5 nM LY341495: 3.01 ± 0.98; veh: 5.41 ± 0.67, $P < 0.05$; Figure 2b) suggesting the presence of an endogenous glutamatergic tone. In order to dissect the functional contribution of specific group II receptor subtypes, we then tested the selective mGlu2-positive allosteric modulator (PAM), LY487379. Like ALC, the mGlu2 PAM (0.1–10 μM) increased the percentage of MAP-2$^+$ cells ($F(5,48) = 85.68$, $P < 0.0001$; Figure 2c), with a maximal effect elicited at 1 μM (% increase over vehicle-treated cells: $+192.8 \pm 28.8$). Representative images of MAP-2 immunolabeling experiments further supported the effects of a 24-h treatment of NPC with vehicle (Figure 2d), 300 μM ALC (Figure 2e), 10 μM LY379268 (Figure 2f), 1 μM LY487379 (Figure 2g), 7.5 nM LY341495 in presence of 300 μM ALC (Figure 2h), or 7.5 nM LY341495 alone (Figure 2i). We then tested whether a 24-h treatment with ALC was able to upregulate expression of mGlu2 receptors in NPC cultures under differentiating conditions (Figure 2j). Densitometric analysis demonstrated that 300 μM ALC increased mGlu2 protein levels ($F(3,8) = 11.78$, $P = 0.0026$; Figure 2j) (% increase over vehicle-treated cells: 51 ± 19.7) and that this effect was counteracted by JSH-23, which had no effect alone. Under the same experimental conditions, ALC treatment, in the presence or absence of JSH-23, had no effect on mGlu3 protein levels ($F(3,8) = 0.398$, NS; Figure 2j). Altogether, these data confirmed that ALC elicited its *in vitro* proneurogenic effects via NF-κB p65-mediated upregulation of mGlu2 expression.

Chronic ALC Treatment Reverts UCMS-Induced Depressive-Like Symptoms in Adult Mice

In rodents, unpredictable chronic mild stress (UCMS) has good face validity and predictivity (Willner *et al*, 1987; Mineur *et al*, 2006; Ibarguen-Vargas *et al*, 2008). A group of 22 adult male C57B/L6J mice was initially submitted to a 8-week UCMS regimen or maintained under control, non-stressful conditions. The entire procedure is schematically described in Figure 3a. Before starting, mice were analyzed for 3% sucrose preference (SP), and, as expected, they unequivocally preferred drinking sucrose over water (SP > 80%). After 8 weeks of UCMS, one-way ANOVA revealed a significant effect on SP ($F(3,40) = 31.09$, $P < 0.0001$). In particular, a *post hoc* test showed that stressed mice had significantly decreased SP values compared with their basal preference ($45 \pm 4\%$ and $80 \pm 2\%$, $P < 0.001$; Figure 3b), whereas unstressed mice displayed no difference between basal and 8-week values (78 ± 3 and $76 \pm 2\%$, respectively, NS; Figure 3c). Furthermore, SP between stressed and unstressed mice was also significantly different (45 ± 4 and $76 \pm 2\%$, respectively, $P < 0.001$). In parallel, the 8-week UCMS period increased immobility time of stressed compared with unstressed mice both in the TST (77 ± 9 and 35 ± 5 s, respectively, $P < 0.001$; Figure 3d) and in the FST (80 ± 5 and 51 ± 6 s, respectively, $P < 0.05$; Figure 3e) paradigms. Altogether, these data confirmed that UCMS elicited depressive-like symptoms in mice. Then, both UCMS and control mice were divided into two groups that were administered once daily s.c. either vehicle or ALC (100 mg/kg) for 21 days. Stress procedure continued to be

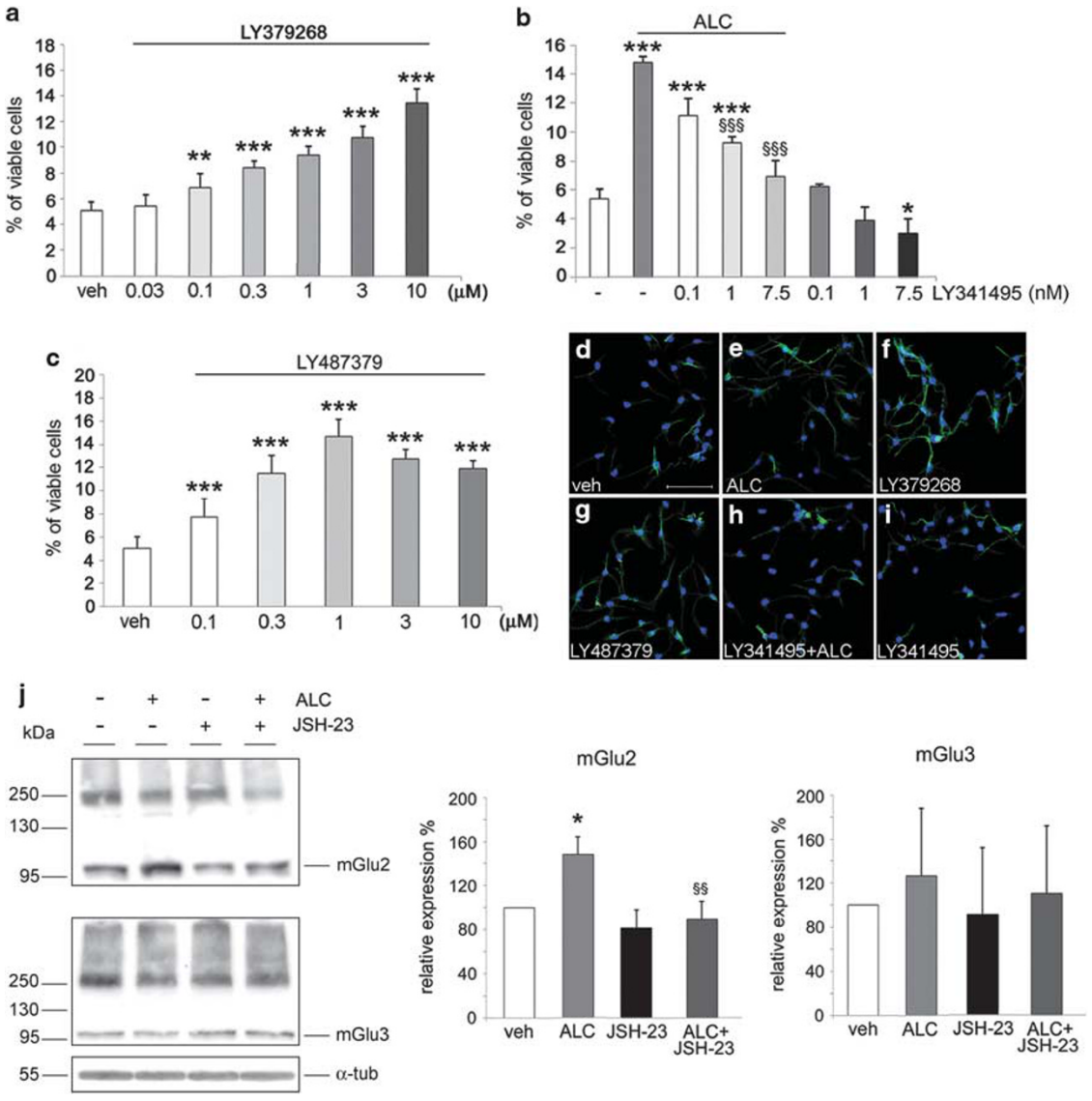

Figure 2 Group II mGlu receptors, and in particular the mGlu2 subtype, are involved in ALC proneurogenic effects. (a) The mGlu2/3 agonist LY379268 increased the percentage of MAP-2$^+$ cells in adult NPC cultures in a concentration-dependent manner. (b) The preferential mGlu2/3 antagonist LY341495 counteracted the effects of ALC (300 μM) on MAP-2$^+$ cells. (c) The selective mGlu2 PAM LY487379 increased the percentage of MAP-2$^+$ cells generated from adult NPCs. (a–c) Data are mean ± SD of $n = 3$ experiments in triplicates, analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* vehicle-treated cells and $^{§§§}P < 0.001$ *vs* ALC-treated cells. (d–i) Representative fluorescence microscopy images of MAP-2 immunolabeling (green) in cells treated for 24 h with vehicle (d), 300 μM ALC (e), 10 μM LY379268 (f), 1 μM LY487379 (g), 7.5 nM LY341495 + 300 μM ALC (h), and 7.5 nM LY341495 (i). Nuclei were counterstained with TOPRO (blue). Scale bar: 47.62 μm. (j) Immunoblot analysis and quantification of mGlu2 and mGlu3 protein levels in adult hippocampal NPCs treated for 24 h with vehicle, 3 μM JSH-23, 300 μM ALC in the presence of vehicle, or 3 μM JSH-23. Compared with vehicle, ALC treatment increased mGlu2 protein levels and JSH-23 counteracted these effects. No change in mGlu3 protein levels was observed in the presence of any treatment condition. Data represent the mean ± SD of $n = 3$ experiments from different cell preparations, normalized by α-tubulin expressions and analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. $^*P < 0.05$ *vs* vehicle-treated cells; $^{§§}P < 0.01$ *vs* ALC-treated cells.

applied during drug/vehicle treatment. The drug dose was selected so to correspond to a recommended dose (0.5 g/day) in humans. In addition, we confirmed that the dose regimen resulted in increased plasma concentration compared with vehicle-treated mice (297.5 ± 28.7 and 19.5 ± 1.8 μM in ALC- and vehicle-treated mice, $P < 0.001$). At the end of the drug administration period, mice were retested for SP and immobility time in the TST and FST. When SP was analyzed in experimental groups, a two-way ANOVA revealed a significant effect of ALC treatment ($F(1, 18) = 20.82$, $P = 0.0002$) and stress × ALC interaction ($F(1, 18) = 11.68$, $P = 0.0031$) in the absence of significant effect of stress ($F(1, 18) = 0.844$, NS). As shown in Figure 3b and c, chronic ALC treatment reversed UCMS-induced anhedonia (Figure 3b), with mice preferring sucrose (76 ± 2%) over water (Figure 3b). Conversely, ALC treatment had no effect on SP in unstressed mice (66 ± 2 and 61 ± 4% in ALC- and vehicle-treated control mice, respectively; Figure 3c). Importantly, chronic ALC administration had no effect on total fluid consumption in naive animals (data not shown). When we evaluated behavior in the TST, we found a significant difference of stress ($F(1, 18) = 9.309$,

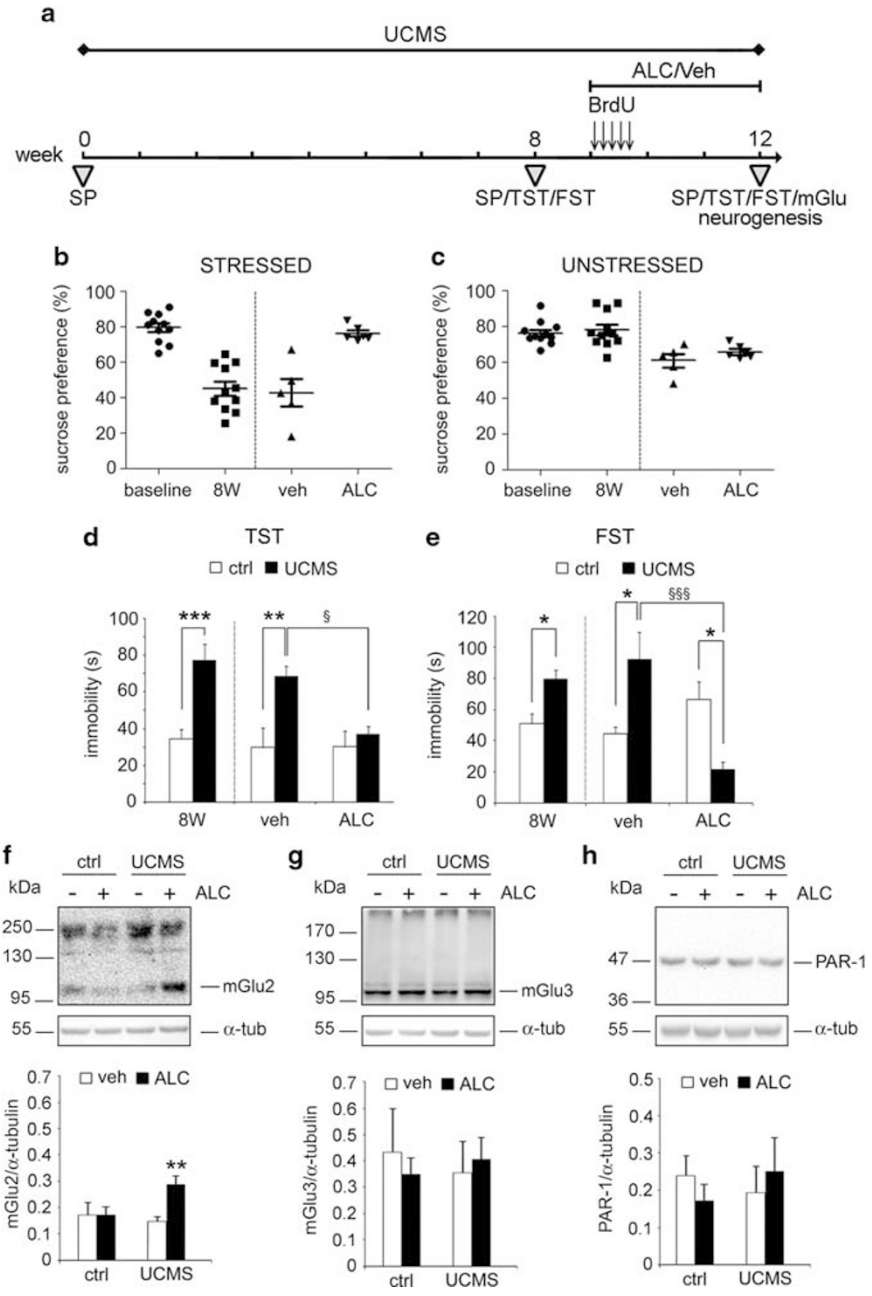

Figure 3 Chronic ALC treatment reverted UCMS-induced depressive-like behavior and upregulated mGlu2 protein levels in the hippocampi of stressed mice. (a) Schematic representation of the experimental procedure. (b, c) Sucrose preference (SP) in (b) stressed ($n=11$) and (c) unstressed ($n=11$) mice. Mice subjected to 8-week UCMS significantly reduced SP compared with their (b) baseline values ($P<0.001$) and with (c) unstressed mice ($P<0.001$). (b) Chronic ALC treatment (100 mg/kg, once a day, s.c.) reversed UCMS-induced anhedonia, with ALC-treated stressed mice ($n=6$) displaying SP values significantly higher than vehicle-treated stressed mice ($n=5$; $P<0.001$). (c) ALC ($n=6$) or vehicle treatment ($n=5$) had no effect on unstressed mice. (d, e) Behavioral analysis in the TST (d) and FST (e) following UCMS and after 21 days of saline/ALC treatment. After 8 weeks of stress, UCMS mice significantly increased time (in seconds) spent in immobility in both TST (d) and FST (e) as when compared with unstressed, control (ctrl) mice. ALC was able to revert stress-induced increase of immobility time in both TST (d) and FST (e), with ALC-treated UCMS mice immobility being significantly different from the vehicle-treated UCMS mice in both TST and FST. No significant difference was observed between vehicle- and ALC-treated unstressed mice in both TST (d) and FST (e). Data are mean ± SD (*$P<0.05$, **$P<0.01$, ***$P<0.001$, §$P<0.05$, §§§$P<0.001$). (f–h) Representative experiments of immunoblot analysis of mGlu2 (f), mGlu3 (g), and PAR-1 (h) levels in hippocampi of vehicle-treated unstressed, ALC-treated unstressed, vehicle-treated UCMS, and ALC-treated UCMS mice. Densitometric values are mean ± SD of mGlu2/α-tubulin, mGlu3/α-tubulin, and PAR-1/α-tubulin ratios from three independent experiments ($n=4$ mice/group). Data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. **P < 0.01 *vs* vehicle-treated stressed mice.

$P=0.0069$) and stress × ALC interaction ($F(1,18)=4.488$, $P=0.048$), whereas there was no overall effect of ALC treatment ($F(1,18)=4.192$, $P=0.055$). *Post hoc* analysis revealed that ALC, but not vehicle, reverted stress-induced increase in immobility (69 ± 6 and 37 ± 4 s for vehicle-stressed and ALC-stressed mice, respectively, $P<0.05$; Figure 3d). Similarly, in the FST, two-way ANOVA confirmed a significant effect of ALC treatment

($F(1,18)=5.387$, $P=0.032$) and stress × ALC interaction ($F(1,18)=19.37$, $P<0.0003$), but no effect of stress ($F(1,18)=0.025$, NS). The *post hoc* analysis showed that ALC-treated stressed mice reduced their immobility as compared with vehicle-treated stressed mice (22 ± 5 and 93 ± 17 s, respectively, $P<0.001$; Figure 3e). In addition, immobility in ALC-treated stressed mice was significantly reduced as compared with ALC-treated unstressed mice (22 ± 5 and 67 ± 12 s, respectively, $P<0.05$). On the other hand, no significant difference was observed between vehicle- and ALC-treated unstressed mice in both TST (Figure 3d) and FST (Figure 3e), whereas an increased immobility time in vehicle-stressed compared with vehicle-unstressed mice was present in both TST (69 ± 5.62 and 30 ± 10.57, respectively, $P<0.01$; Figure 3d) and FST (93 ± 17.2 and 45 ± 4.1, respectively, $P<0.05$; Figure 3e). We also observed no effect of chronic ALC administration on body weight in unstressed or stressed mice (data not shown). Altogether, these data demonstrated the efficacy of chronic ALC administration in reverting depressive-like symptoms induced by UCMS in adult mice.

Chronic ALC Treatment Increases mGlu2 Expression and Adult-Born Neurons in Hippocampi of Stressed Adult Mice

At the end of the entire experimental procedure, hippocampi were dissected for evaluation of mGlu2/3 protein levels. Western blot analysis demonstrated that ALC treatment significantly increased mGlu2 protein levels ($F(1,8)=13.39$, $P=0.0064$). ANOVA also showed a significant effect between stress × ALC interaction ($F(1,8)=12.68$, $P=0.0074$) and stress ($F(1,8)=5.581$, $P=0.04$). On the other hand, mGlu3 protein levels were not different in analyzing either stress × ALC interaction ($F(1,8)=1.05$, $P=0.335$) and stress or ALC treatment. Specifically, a *post hoc* test revealed that mGlu2 and mGlu3 expression levels in vehicle-treated control and UCMS mice were not significantly different (Figure 3f and g). Conversely, in stressed mice, ALC treatment resulted in increased mGlu2, but not mGlu3, protein levels compared with vehicle-treated mice (% increase: 86.6 ± 8.8; Figure 3f and g). Moreover, mGlu2 and mGlu3 expression levels were not significantly different in ALC- and vehicle-treated control mice (Figure 3f and g). As a negative control, we verified that ALC had no effect on the expression levels of an unrelated GPCR, PAR-1 (Figure 3h). An additional group of mice was submitted to a 8-week UCMS regimen or maintained under control conditions and stressed and unstressed mice were then divided into two groups ($n=6$ each) that were administered once daily either vehicle or ALC (100 mg/kg, s.c.) for 21 days. This time, the animals were also injected with BrdU (150 mg/kg, i.p.), for the first 5 days of drug treatment. At 21 days after the last drug/vehicle injection, mice were transcardially perfused and their brains processed for quantification of newly born $BrdU^+/NeuN^+/GFAP^-$ neurons in the dentate gyrus. Two-way ANOVA revealed no significant difference in stress × ALC interaction ($F(1,14)=1.490$, NS; Figure 4a–e) and of stress ($F(1,14)=2.036$, NS), whereas a significant effect of ALC treatment was observed ($F(1,14)=29.20$, $P<0.0001$). The *post hoc* analysis confirmed a significantly increased number of newborn neurons in ALC-treated stressed mice compared with vehicle-treated stressed mice (1403 ± 238 and 632 ± 215, respectively, $P<0.01$; Figure 4c–e). The number of new neurons was also increased in ALC-treated unstressed compared with vehicle-treated unstressed mice (1095 ± 299 and 608 ± 134, $P<0.05$; Figure 4a, b and e). Conversely, no significant difference was reported between vehicle-treated groups. Altogether, these data demonstrate that similar to classical antidepressants, chronic administration of ALC promotes hippocampal neurogenesis in both stressed and unstressed mice. When tested, chronic ALC treatment, compared with vehicle, had no significant effect on the proliferation rate of neural progenitors, as assessed by counting $BrdU^+$ cells in the SGZ (2291 ± 687 and 2379 ± 327 in ALC and vehicle-treated mice, respectively, $P=0.77$) and in the GCL (2221 ± 375 and 2241 ± 439 in ALC and vehicle-treated mice respectively, $P=0.93$) of naive animals killed 2 h after administration of the thymidine analog (Figure 4f). These results suggest that the proneurogenic effects of ALC are not mediated by increased proliferation of undifferentiated neural progenitors *in vivo*. As antidepressants usually exert their proneurogenic effects under a chronic, but not acute or subchronic, regimen (Malberg *et al*, 2000), we tested the effect of 1 or 7 days of ALC (and vehicle) administration on hippocampal neurogenesis in naive adult mice ($n=7$/group). A single ALC treatment had no significant effect on the number of new neurons, as compared with vehicle treatment (726 ± 187 and 866 ± 82 in ALC- and vehicle-treated mice, respectively; $P=0.21$). Although no significant difference was also observed in the number of $BrdU^+/NeuN^+/GFAP^-$ cells after 7 days of ALC/vehicle treatment (1308 ± 401 and 979 ± 324 in ALC- and vehicle-treated mice, respectively; $P=0.161$), a two-way ANOVA revealed a significant effect of duration of ALC treatment ($F(1,17)=6.697$, $P=0.0192$). In particular, a *post hoc* analysis confirmed an increased number of $BrdU^+/NeuN^+/GFAP^-$ cells after 7 days compared with 1 day of ALC treatment ($P<0.05$). No difference was observed between 1 and 7 days in vehicle-treated mice.

DISCUSSION

Several double-blind, placebo-controlled studies demonstrated that ALC treatment has beneficial effects in major depression and dystymia (Bella *et al*, 1990; Garzya *et al*, 1990; Gecele *et al*, 1991; Zanardi and Smeraldi, 2006). Here we show, for the first time, that ALC promotes neuronal differentiation of adult hippocampal progenitors *in vitro* without affecting proliferation or survival rate. Recent work demonstrated the involvement of upregulated mGlu2 gene expression in ALC-mediated analgesic activity via NF-κB p65 acetylation (Chiechio *et al*, 2009). Also in our experimental model, at least *in vitro*, the NF-κB pathway was responsible for mediating ALC proneurogenic effects, as interfering with its activation abolished drug activity. More specifically, ALC resulted in acetylation of p65 at Lys(310) in NPC cultures. Moreover, ALC treatment of hippocampal NPC resulted in a significant upregulation of mGlu2 protein levels and this effect was abolished by inhibiting p65 nuclear translocation. It is also interesting

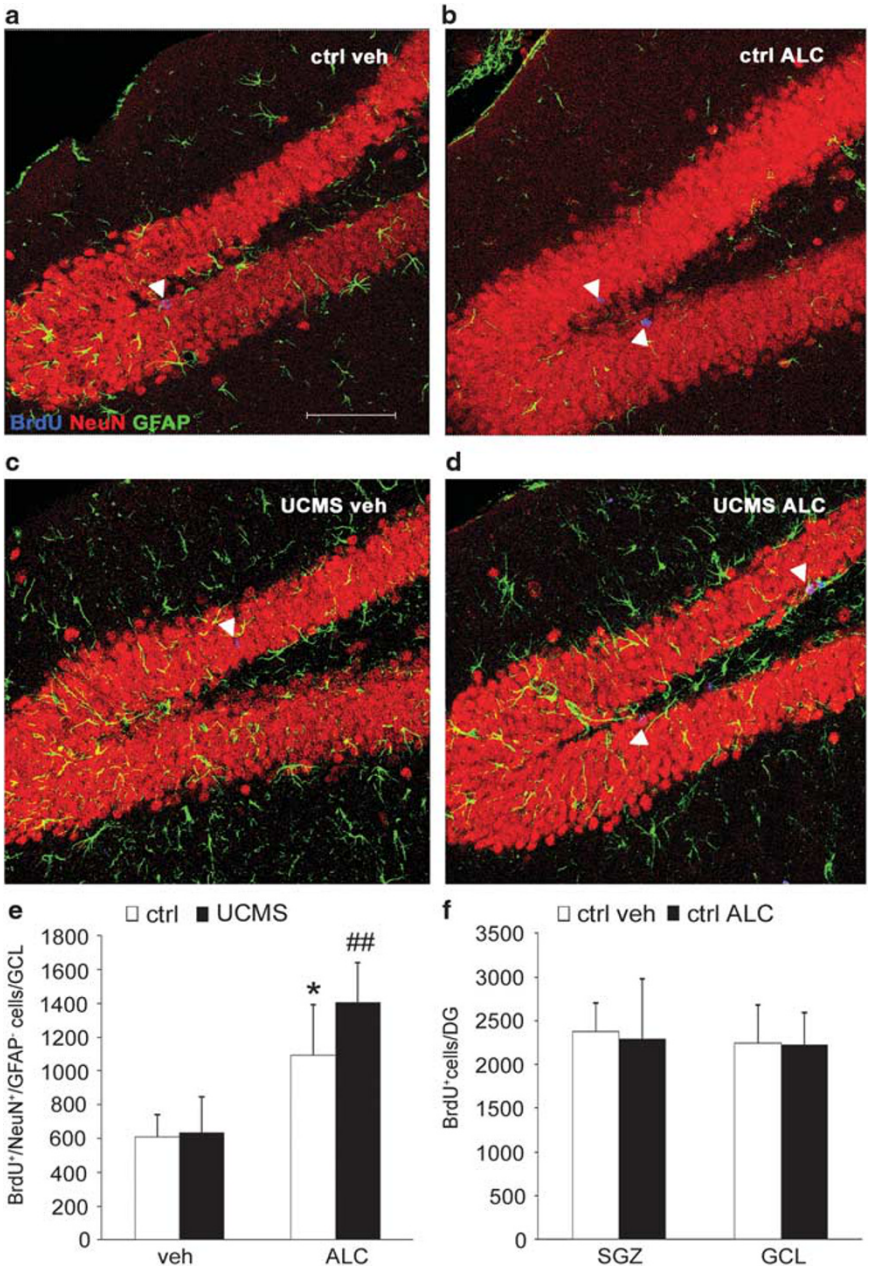

Figure 4 Chronic ALC treatment increased hippocampal neurogenesis *in vivo*. (a–d) Representative confocal microscopy images of immunofluorescent labeling for BrdU (blue)/NeuN (red)/GFAP (green) of hippocampal sections in control (Ctrl; a, b) and stressed (UCMS; c, d) mice subcutaneously injected with vehicle (a, c) or 100 mg/kg ALC (b, d) for 21 days. White arrowheads indicate $BrdU^+/NeuN^+/GFAP^-$ cells within the dentate gyrus. Scale bar: 75 μm. (e) Quantitative analysis of $BrdU^+/NeuN^+/GFAP^-$ cells in stressed and unstressed mice. The number of newly born hippocampal neurons was significantly increased in the granular cell layer (GCL) of ALC-treated stressed and unstressed mice compared with vehicle-treated stressed and unstressed mice, respectively. Data are expressed as mean ± SD of $n = 7$ mice/group, and were analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. $^*P < 0.05$ *vs* vehicle-treated unstressed mice; $^{\#\#}P < 0.01$ *vs* vehicle-treated stressed mice. (f) Proliferation rate in the dentate gyrus of mice treated with 100 mg/kg ALC or vehicle for 21 days. The number of $BrdU^+$ cells in the subgranular zone (SGZ) and GCL was not significantly different in vehicle- and ALC-treated mice killed 2 h after BrdU administration. Data are expressed as mean ± SD of $n = 7$ mice/group.

that in our culture model mGlu2 activation by a selective positive allosteric modulator promoted neurogenesis, whereas a preferential mGlu2/3 antagonist counteracted ALC-mediated proneurogenic effects. We are aware of the interpretative limitations of data generated by single mGlu ligands, as the distinct functions of mGlu2 and mGlu3 are not fully understood. For this reason, even if we did not observe significant effects of ALC on mGlu3 protein levels, at present we cannot exclude that this receptor subtype, which has been shown to be functionally expressed by adult NPC (Di Giorgi-Gerevini *et al*, 2005; Ciceroni *et al*, 2010), may also be involved. We also proved that chronic ALC reverted depressive-like behavior in a predictive animal model. Importantly, the utilized dose corresponds to the lowest recommended dose in humans (0.5 g/day) (Reagan-Shaw *et al*, 2008). Furthermore, this dose regimen effectively increased plasma levels of ALC in chronically treated mice, as previously shown (Schaevitz *et al*, 2012). We also

demonstrated that chronic ALC treatment correlated with increased mGlu2 protein levels in the hippocampi of stressed but not unstressed mice. The role of group II mGlu receptors in depression is currently debated, as both mGlu2/3 agonists and antagonists can produce antidepressive effects in rodent models (Chaki *et al*, 2013). This apparent paradox could in part rely on the fact that mGlu2/3 subtypes may play different functions and/or may be differentially (de)regulated in depressive disorders. Although at present we have no information as to where mGlu2 upregulation may affect in the hippocampal circuit, it is known that these receptors are in the preterminal regions of perforant pathway axons and mossy fibers, where they have little effect on glutamate release under normal conditions but they may potentiate a negative feedback control under conditions of excessive glutamate release, which is associated with depression (Bonanno *et al*, 2005; Sanacora *et al*, 2008). Interestingly, THIIC, a novel potent and selective allosteric modulator of mGlu2, recently demonstrated robust antidepressant properties *in vivo* (Fell *et al*, 2011), suggesting a contribution of that subunit to the pathophysiology of depression. We also demonstrated that chronic ALC administration increased formation of adult-born neurons in the hippocampus of adult mice *in vivo*, a property shared by classical antidepressants. Unlike mGlu2 effects, chronic ALC treatment correlated with increased hippocampal neurogenesis in both stressed and unstressed mice. In this respect, ALC is similar to classical antidepressants that also promote neurogenesis in naive mice when chronically administered (Malberg *et al*, 2000). Interestingly, unlike other classical antidepressants (Santarelli *et al*, 2003), ALC did not affect proliferation of neural hippocampal progenitors *in vitro* and *in vivo*. Finally, like classical antidepressants, ALC did not significantly increase neurogenesis when administered acutely (1 day) or subchronically (7 days). Although no significant effects were reported between vehicle and ALC, we observed a significant increase in the number of newly generated neurons in hippocampi of 7-day ALC-treated compared with 1-day ALC-treated mice. Future experiments should investigate whether these observations may underlie a more rapid onset of proneurogenic effects of ALC compared with classical antidepressants.

The involvement of NF-κB p65 acetylation in the proneurogenic and potentially antidepressive effects of ALC is in apparent contrast with the observation that p65 activation has been proposed to mediate decreased hippocampal neurogenesis in response to chronic stress and interleukin-1 (Koo *et al,* 2010). The fact that both induction and inhibition of adult neurogenesis may rely on NF-κB p65 is likely to reflect the complexity within the NF-κB signaling pathway. Indeed, NF-κB proteins represent a family of several transcription factors whose members, including p65, can combine to form hetero- and homodimers of different composition, which can be differentially activated in a given cell type and exert different, even opposite, functions through activation of different sets of gene targets (Grilli and Memo, 1997). In addition, little information is currently available on the contribution of p65 acetylation to the NF-κB-mediated transcriptional program. In this respect, in the future it will be important to identify the full set of gene targets activated by ALC in the hippocampus.

Although at present we cannot prove a causal relationship between ALC behavioral effects and drug-mediated effects on mGluR2 expression and neurogenesis *in vivo*, it is remarkable that ALC-mediated proneurogenic and antidepressant activity may be elicited through the mechanism mediating its analgesic effects. Interestingly, upregulation of mGlu2, via acetylation of NF-κB p65, is also triggered by other analgesic drugs such as MS-275 and SAHA, which are histone deacetylase (HDAC) inhibitors (Chiechio *et al*, 2009). In addition, HDAC inhibitors are also proneurogenic (Kim *et al*, 2009) and elicit antidepressant-like effects (Gundersen and Blendy, 2009; Covington *et al*, 2009). Altogether, these and our observations would potentially imply that mGlu2 upregulation, via p65 acetylation, may also be involved in these pharmacological activities of HDAC inhibitors.

Of course, at present, we cannot exclude additional mechanisms participating in the antidepressant effects of ALC, including modulation of the glia-derived growth factor artemin (Di Cesare Mannelli *et al*, 2011) and increased hippocampal levels of noradrenaline (Smeland *et al*, 2012).

In conclusion, here we propose novel mechanisms that could be potentially involved in the antidepressant effects of ALC in humans, namely upregulation of mGlu2 levels and promotion of neurogenesis in the hippocampus, via NF-κB p65 acetylation. The demonstration that ALC is an antidepressant in a predictive model of disease may have clinical relevance. ALC is indeed very well tolerated in humans (Thal *et al*, 1996, 2000) and it could be employed in patient subpopulations who are sensitive to toxicity associated with currently available antidepressants.

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