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# Resveratrol Up-regulates AMPA Receptor Expression via AMP-activated protein kinase – mediated Protein Translation

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## Abstract

Resveratrol is a phytoalexin that confers overall health benefits including positive regulation in brain function such as learning and cognition. However, whether and how resveratrol affects synaptic activity remains largely unknown. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are glutamatergic receptors that mediate the majority of fast excitatory transmission and synaptic plasticity, and thus play a critical role in higher brain functions, including learning and memory. We find that in rat primary neurons, resveratrol can rapidly increase AMPAR protein level, AMPAR synaptic accumulation and the strength of excitatory synaptic transmission. The resveratrol effect on AMPAR protein expression is independent of sirtuin 1 (SIRT1), the conventional downstream target of resveratrol, but rather is mediated by AMP-activated protein kinase (AMPK) and subsequent downstream phosphoinositide 3-kinase (PI3K)/Akt signaling. Application of the AMPK specific activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) mimics the effects of resveratrol on both signaling and AMPAR expression. The resveratrol-induced increase in AMPAR expression results from elevated protein synthesis via regulation of the eukaryotic initiation factor (eIF) 4E/4G complex. Disruption of the translation initiation complex completely blocks resveratrol-dependent AMPAR up-regulation. These findings indicate that resveratrol may regulate brain function through facilitation of AMPAR biogenesis and synaptic transmission.

### Keywords

resveratrol; αamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptor, AMPAR); AMP-activated protein kinase (AMPK); sirtuin 1 (SIRT1); phosphoinositide 3-kinase (PI3K); protein translation

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## 1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural polyphenolic compound that is present at high levels in grape skin, red wine and nuts (Rocha-Gonzalez et al., 2008). Resveratrol has been broadly used as a dietary supplement and shows beneficial effects on general health and cognitive function (Chung et al., 2012; Witte et al., 2014; Kodali et al., 2015). Resveratrol has also been shown to protect neurons from cell death under several pathological conditions including glutamate-induced excitotoxicity (Chung et al., 2011), oxidative stress (Fukui et al., 2010), ischemia and epilepsy (Wu et al., 2009). Importantly, resveratrol can ameliorate the symptoms associated with several neurodegenerative diseases including Alzheimer's disease (AD) (Vingtdeux et al., 2008; Wang et al., 2008), Parkinson's diseases (PD) (Jin et al., 2008) and Huntington's diseases (Parker et al., 2005; Sinclair, 2005).

Resveratrol is known as an activator of sirtuins (SIRTs), a class of $NAD^+$-dependent protein deacetylases (Howitz et al., 2003; Smith et al., 2009). Among the seven members in SIRT family (SIRT1–7), SIRT1 is shown to be the primary target of resveratrol (Baur et al., 2006). SIRT1 activation is thought to underlie most of the beneficial effects associated with resveratrol (Baur and Sinclair, 2006). In addition, resveratrol has been shown to activate the metabolic regulator AMP-activated protein kinase (AMPK) (Dasgupta and Milbrandt, 2007), the primary bioenergy sensor responsible for the maintenance of cellular energy homeostasis (Hardie, 2007).

AMPA receptors (AMPAR) are ligand-gated glutamatergic ion channels that mediate the majority of excitatory neurotransmission in the brain (Shepherd and Huganir, 2007). Alterations in AMPAR synaptic accumulation serve as the molecular mechanisms underlying the expression of synaptic plasticity, including long-term potentiation (LTP) , long-term depression (LTD) and homeostatic synaptic plasticity (Malenka, 2003; Malinow, 2003; Turrigiano, 2008; Pozo and Goda, 2010). AMPAR abundance in the cell is determined by the balanced processes of protein translation and degradation (Zhang et al., 2009; Lin et al., 2011). Changes in AMPAR function and its dynamic trafficking have direct impacts on synaptic transmission and cognitive functions (Lee et al., 2003; Kessels and Malinow, 2009). Aberrant AMPAR function is also associated with the cognitive impairments related to multiple neurological diseases (Chang et al., 2006; Hsieh et al., 2006). However, whether resveratrol has a role in glutamate receptor expression and synaptic localization remains unknown.

Here we report that resveratrol treatment induces an increase in AMPAR protein level and its synaptic localization, leading to an enhancement in the strength of synaptic transmission. The resveratrol effect on AMPAR requires the activity of AMPK and the downstream PI3K/Akt pathway, but is independent of SIRT1. Furthermore, we show that the resveratrol-induced increase in AMPAR level is not a consequence of suppressed protein degradation, rather, it results from stimulated protein synthesis mediated by the eIF4E/4G translation initiation complex. Consistent with the role of AMPK, the effect of resveratrol on AMPAR expression and signaling cascades can be reproduced by application of an AMPK-specific activator. These findings demonstrate a novel effect for resveratrol on glutamate receptor

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regulation, a process that may underlie the cognitive benefits of resveratrol on brain function.

## 2. Methods and Materials

### 2. 1. Antibodies, Plasmids and Reagents

Antibodies, plasmids and other reagents were obtained from the following resources. Antibodies: anti-GluA1Ct (rabbit) was made by OriGene; anti-GluA2/3 (rabbit), anti-NR1Ct (mouse), anti-PSD95 (mouse), anti-eIF4e (rabbit) were from Millipore; anti-αTubulin (mouse) was from Sigma-Aldrich; anti-phosphoAMPKαThr172 (rabbit), anti-Phospho-AktSer473 (rabbit), anti-eIF4g (rabbit) and anti-Akt (rabbit) were from Cell Signaling; anti-SIRT1 (rabbit) was from Abcam. Chemicals: Resveratrol, MG132, Leupeptin, Cycloheximide, Anisomycin, Actinomycin D, Compound C, LY 294002, STO609, Dimethyl sulfoxide (DMSO) were from Sigma-Aldrich; EX527 and 4EGI-1 were from Millipore; 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR) was from Enzo Life Sciences; Plasmids and siRNAs: pcDNA was purchased from Invitrogen; EGFP was purchased from Clontech Laboratories. αAMPK kinase dead (AMPK K.D.) and PI3 kinase dominant negative form (PI3K D.N.) were generously provided by Prof. Lewis C. Cantley (Harvard Medical School, Boston, MA); G-CaMP3 was generously provided by Prof. Loren L. Looger (Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA) *via* his deposit at Addgene (#22692); siRNAs (Scramble and SIRT1) were purchased from Santa Cruz Biotechnology.

### 2.2. Primary Culture of Neurons

Cortical and hippocampal brain tissues were dissected out from E18 rat fetus brains of either sex and prepared for primary culture. Tissues were first digested with papain (0.5mg/ml in HBSS) at 37°C for 20 minutes, then gently triturated with a Pasteur pipette in trituration buffer (0.1% DNase, 1% ovomucoid/1% bovine serum albumin in HBSS) until neurons were fully dissociated. Dissociated cortical neurons were then counted and plated into either 6-well plates or 60mm Petri dishes (Greiner Cellstar). Hippocampal neurons were plated on 18mm circular coverslips (Carolina, 0.1mm thick) in 60mm Petri dishes (5 coverslips/dish). Both dishes and coverslips were coated with poly-L-lysine (Sigma-Aldrich, 100 μg/ml in Borate buffer) overnight at 37°C then washed three times with sterile DI water and left in plating medium [MEM (500 mL) containing 10% fetal bovine serum (FBS), 5% horse serum (HS), 31 mg L-cysteine, and 1% penicillin/streptomycin and L-glutamine mixture (1% P/S/G); Invitrogen] before cell plating. Plating medium was replaced by feeding medium (Neurobasal medium supplemented with 1% HS, 2% B-27 and 1% P/S/G) the day after cell plating. Neurons were maintained in feeding medium with FDU (10 μM) supplemented at DIV5 to suppress glial growth until experimental use.

### 2.3. Western Blot Analysis of Protein Abundance

Two week old cortical neurons cultured in either 60 mm or 6-well plates were treated by resveratrol, AICAR or other chemicals as stated. For blockade/occlusion experiments, unless specifically stated, the cells were pre-treated for one hour prior to resveratrol treatment then remained in the bath until cell lysis. Cells in control groups were treated with the appropriate

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vehicle solvents (saline or DMSO). After treatment, neurons were lysed in Laemmli 2X sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and boiled for 10-min at 95°C for SDS page electrophoresis. After separation in SDS page, proteins were transferred to PVDF immunoblotting membrane (Bio-rad) and probed for different targets with the stated antibodies. Immunoblots were visualized using a chemiluminescence detection system (GE Healthcare) and exposed to Fuji medical X-ray films (Fisher Scientific), scanned and analyzed using the NIH ImageJ program.

### 2. 4. Immunocytochemistry

Low density (54,000/coverslip) hippocampal neurons were washed once in ice-cold ACSF and fixed for 10-min in 4% paraformaldehyde / 4% sucrose solution on ice. To stain total protein, cell membranes were permeabilized for 8-min in 0.3% Triton-X-100 (FisherBiotech) in phosphate buffered saline (PBS), rinsed three times in 1X PBS then subjected to a blocking procedure (1-hr in 10% goat serum PBS). To stain surface protein, cells were blocked in goat serum without permeabilization. After blocking, cells were incubated with primary antibodies (in 5% goat serum PBS) for 2-hr, washed and incubated with Alexa Fluor-conjugated fluorescent secondary antibodies (1:700, Life Technologies) for an additional hour. Cells were then mounted to microscopy glass slides with ProlongGold anti-fade mounting reagent (Life Technologies) for subsequent visualization.

### 2. 5. Transfection of Neurons or Human Embryonic Kidney (HEK) cells

DIV11 hippocampal neurons cultured on 18 mm coverslips were transferred to a 12-well plate and transfected by Lipofectamine 2000 (Life Technologies) with target plasm id DNA or siRNA per the manufacturer's suggestion. For one coverslip, 0.9 μL Lipofectamine 2000 and 1 μg plasmid DNA or siRNA (0.7μg DNA/siRNA plus 0.3μg EGFP) were first separately diluted in 50μL 1X MEM medium then mixed and incubated at room temperature for 20-min to form the transfection complex. The transfection complex was added to the coverslips in 500 μL feeding medium per well and incubated at 37°C for 4-hr before the medium was removed and replaced by new feeding medium. New feeding medium was half-to-half mixed with old feeding medium in the dish to avoid neurotoxicity. Neurons were then cultured 3 more days for the target proteins to express. Neurons were fixed and subjected to a standard immunocytochemistry protocol. HEK cells were cultured and split into 6-well plates (1 million / well) to grow overnight prior to transfection. The transfection process for HEK cells is identical to that described for neurons except that 4 μL Lipofectamine 2000 was mixed with 4 μg target plasmid to transfect each well of cells. Medium was changed 4 hr post-transfection and HEK cells were further cultured an additional 24-hr to ensure target protein expression before cells were harvested for Western blot analysis. HEK cells were cultured in the following medium: 1X DMEM with 10% FBS, 1% P/S and 1% L-Glutamine.

### 2. 6. Neuronal Cell Image Collection and Analysis

Mounted coverslips were kept in the dark at 4°C before imaging. Using a Carl Zeiss inverted fluorescent microscope, neuronal cell images were collected with a 63× oil-immersion objective (numerical aperture, 1.4) and collected with AxioVision Release 4.5 software. The

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exposure time of the fluorescence signal was adjusted manually to ensure that the signal intensity was within the full dynamic range by using a glow scale look-up table. Once an exposure time was established, it was used throughout the duration of the image collection process for all samples. Neuron images were quantified using NIH ImageJ. For quantification, GluA1 puncta ranging in size from 5 −20 pixels were first selected using a thresholding function before both mean puncta size and intensity were automatically measured. GluA1 total intensity was calculated by multiplying the value obtained for mean puncta size by the value obtained for fluorescence intensity.

### 2. 7. Live Neuron Imaging

For G-CaMP3 imaging in live neurons, a plasmid containing the cDNA of G-CaMP3 was transfected into DIV 11 hippocampal neurons followed by 3 days of incubation to ensure G-CaMP3 protein expression prior to imaging. One coverslip with cultured neurons was placed into a sealed live imaging chamber and maintained at 37°C on a temperature controller (Tempcontrol 37-2 digital) for the duration of the imaging session. Imaging duration of one coverslip was restricted to no longer than 30 minutes to ensure the health of neurons.

### 2. 8. Electrophysiology

For miniature excitatory postsynaptic current (mEPSC) recordings, 2-w old cultured hippocampal neurons on coverslips were first treated with resveratrol (40 μM) for 4 hours, then transferred to a recording chamber with extracellular solution containing 140mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 2.5mM $CaCl_2$, 11mM glucose, and 10mM Hepes (305 mOsm, pH 7.4), which was supplemented with TTX (1 μM) to block action potentials, APV (50 μM) to block NMDARs and bicuculline (20 μM) to block $GABA_A$ receptor-mediated mIPSCs. Whole-cell voltage clamp recordings were made with patch pipettes filled with intracellular solution containing 110 mM Cs-methanesulfonate, 10 mM CsCl, 10 mM Hepes, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM $Na_2$-GTP, and 10mM sodium phosphocreatine (295 mOsm, pH 7.4), with the membrane potential clamped at −70mV. Recordings started 5 minutes after establishing whole-cell configuration to ensure equilibration between the pipette solution and the cytosol. mEPSCs were recorded with an Axopatch 200B amplifier and displayed and recorded digitally on a computer for subsequent off-line analysis with Clampfit.

### 2. 9. Reverse Transcription PCR (RT-PCR)

After treatment, total RNA from cultured cortical neurons was isolated using the QIAamp RNA Blood mini kit (Qiagen). The same amount of RNA (500 ng) from each sample was reverse-transcribed to cDNA with 0.5 μg oligo$(dT)_{12–18}$ primer, 200 μM dNTP, 100 mM DTT and 200 units of SuperScript™ II Reverse Transcriptase (Life Technologies) following the manufacturer's instructions. RT-PCR was then performed with the following primers: GluA1: GCTTCATGGACATTGACTTA and ATCTCAAGTCGGTAGGAGTA (a 673 bp fragment); GAPDH: TGAAGGTCGGTGTGAACGGATTTGGCCG and CCATGTAGGCCATGAGGTCCACCACCC (a 983 bp fragment). GAPDH was used as the internal control for GluA1. The RT-PCR products were then subject to 1% Agarose gel electrophoresis, imaged and quantified with NIH ImageJ program.

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### 2. 10. Data Analysis and Statistics

All values are presented as mean ± S.E. and analyzed using Student's *t* test to compare the statistical difference between control and treatment groups. $p < 0.05$ is considered as statistically significant. *p* values are presented as $p > 0.05$, $p < 0.05$ or 0.01.

### 2. 11. Animal Use

All the procedures involving animal use in this study were in compliance with the policies of the Institutional Animal Care and Use Committee (IACUC) at Boston University, following the National Institutes of Health (NIH) guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Approved experimental protocol number: 11–039. The number of animals used in this study has been minimized.

## 3. Results

### 3. 1. Resveratrol treatment rapidly increases AMPAR levels in neurons

To investigate the potential effects of resveratrol on AMPARs, we incubated 2 wk-old cultured cortical neurons with varying concentrations of resveratrol (10 – 40 pM) for 4 hr and evaluated AMPAR protein levels *via* western blot. We found that 4 hr of either 20 μM or 40 μM resveratrol treatment significantly increased the total amount of GluA1 subunits (20 μM: 157.2 ± 7.0%, $n = 5$, $p < 0.01$; 40 μM: 168.1 ± 8.2%, $n = 5$, $p < 0.01$) (Fig. 1*A*). Based on this result, we used 40 μM as the working concentration of resveratrol for all subsequent experiments. We next investigated the temporal profile of resveratrol treatment on AMPAR expression. The time course of resveratrol treatment showed that application of 40 μM resveratrol enhanced protein abundance of both GluA1 and GluA2/3 from 1 hr to 8 hr. Changes of GluA1 and GluA2/3 peaked at 4 hr with an increase of 152.2 ± 4.7% ($n = 4$, $p < 0.01$) and 132.4 ± 5.1% ($n = 4$, $p < 0.01$), respectively (Fig. 1*B*). Resveratrol also led to down-regulation of postsynaptic scaffolding protein, PSD95 ($n = 3$, $p < 0.05$) (Fig. 1*B*). In contrast, resveratrol did not significantly alter the expression of the N-Methyl-D-aspartic acid (NMDA) receptor subunit GluN1, indicating AMPAR-specificity of the resveratrol effect. In addition to total AMPAR protein levels, the amount of synaptic AMPARs were also increased following 4 hr resveratrol treatment, as evidenced by immunostaining of GluA1 together with PSD95 as a synaptic marker (130.2 ± 2.9%, $n = 50$ cells, $p < 0.01$) (Fig. 1*C*). Furthermore, surface biotinylation experiments utilizing cultured cortical neurons also showed that resveratrol treatment significantly increased the level of surface AMPARs (150.8 ± 5.8%, $n = 4$, $p < 0.01$) (Fig. 1*D*). To investigate the resveratrol effect *in vivo*, we did intraperitoneal (IP) injection of resveratrol (30 mg/kg body weight) in mice (Wang et al., 2002). Consistent with the effect *in vitro*, a marked increase in AMPAR expression was observed 5 hrs after resveratrol injection in all three brain regions including prefrontal cortex (PFC, 201.7 ± 4.0%, $p < 0.01$), hippocampus (Hippo, 138.7 ±11.4%, $p < 0.01$) and cerebellum (Cere, 224.5 ± 28.7%, $p < 0.05$) ($n = 4$ mice), comparing with the injection of vehicle control (Fig. 1*E*).

As glutamatergic receptors mediate most of the excitatory synaptic transmission, changes in AMPAR expression are expected to alter synaptic function. To examine the functional impacts of resveratrol treatment, we analyzed AMPAR-mediated miniature excitatory

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postsynaptic currents (mEPSCs) by whole-cell patch-clamp recordings in cultured hippocampal neurons. Compared with the vehicle control, 4 hr resveratrol treatment significantly increased the amplitude of mEPSCs (Ctrl: 10.9 ± 0.5 pA; Resv: 13.7 ± 0.7 pA; 126.1 ± 4.8%, $n = 8$ cells, $p < 0.01$) (Fig. 1*F*).

### 3. 2. Resveratrol increases AMPAR expression by facilitating receptor protein translation

Intracellular AMPAR levels are controlled by a dynamic balance between protein synthesis and degradation. Therefore, the observed resveratrol-induced increase in AMPAR expression levels may result from the suppression of receptor degradation and/or enhanced protein synthesis. To determine the involvement of protein degradation, we respectively inhibited two major degradation organelles: Leupeptin (Lep, 20 μM) to inhibit the lysosome and MG132 (MG, 10 μM) to block proteasomal activity. While 4 hr treatment with each inhibitor individually resulted in a significant increase in AMPAR expression, none of the inhibitors occluded the resveratrol-induced AMPAR increase (Fig. 2*A*, *B*). Moreover, resveratrol treatment in conjunction with the protein degradation inhibitors (Lep or MG) showed additive effects as compared to treatment with resveratrol or the inhibitors alone (Fig. 2*A*, *B*). These results indicate that the effect of reseveratrol on AMPAR protein expression is independent of receptor degradation.

To examine the role of protein synthesis, we utilized the protein synthesis inhibitors cycloheximide (CHX, 100 μM) or anisomycin (Ani, 30 μM). Consistent with an effect on protein synthesis, the resveratrol-mediated increase in AMPAR expression was abolished in either cycloheximide or anisomycin-treated neurons (Fig. 2*C*, *D*). Next, we wanted to determine whether resveratrol altered AMPAR gene transcription. To this end, we pretreated neurons with actinomycin D (ActD, 25 μM) for 1 hr to block mRNA transcription. Unlike cycloheximide and anisomycin, ActD did not affect the resveratrol-induced increase in AMPARs (147.0 ± 10.9%, $n = 4$, $p < 0.05$) (Fig. 2*E*). To directly examine AMPAR gene transcription, we performed reverse transcription PCR (RT-PCR) to quantify GluA1 mRNA levels in cultured cortical neurons. As shown in Fig. 2*F*, GluA1 mRNA was not changed following resveratrol treatment (101.6 ± 6.1%, $p > 0.05$, $n = 3$). Together, these data indicate that resveratrol enhances AMPAR expression *via* up-regulation of receptor mRNA translation, without changing mRNA transcription or receptor degradation.

### 3. 3. Resveratrol regulates AMPAR expression in a SIRT1-independent manner

As a well-known activator of the sirtuin family (Howitz et al., 2003; Smith et al., 2009; Dal-Pan et al., 2011), resveratrol's effects have often been shown to be mediated by the sirtuins (Baur and Sinclair, 2006; Pallas et al., 2009). Therefore, we hypothesized that the observed effects of resveratrol on AMPAR expression were also mediated by the sirtuins. Among the seven members of mammalian sirtuins (SIRT1–7), resveratrol primarily targets SIRT1 (Baur and Sinclair, 2006; Witte et al., 2009). In cultured cortical neurons, the SIRT1-specific inhibitor EX527 (1.2 μM)(Napper et al., 2005) was applied 1 hr prior to and during resveratrol treatment. Surprisingly, in the presence of EX527, resveratrol remained to be able to increase GluA1 protein expression to a level similar to that of resveratrol alone (Resv: 141.4 ± 14.4%, $n = 4$, $p < 0.05$; Resv+EX527: 141.9 ± 16.7%, $n = 4$, $p < 0.05$) (Fig.

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3*A*). Also, EX527 treatment alone did not have significant effect on GluA1 expression ($n$ = 4, $p > 0.05$) (Fig. 3*A*).

To exclude any potential side effects of EX527, we utilized a siRNA directed against SIRT1 (siSIRT1) to specifically knockdown endogenous SIRT1. Compared to the scrambled siRNA control, expression of siSIRT1 dramatically decreased endogenous SIRT1 levels in hippocampal neurons and HEK 293T cells (22.3 ± 7.3%, $n$ = 4, $p < 0.01$) (Fig. 3*B*). In hippocampal neurons co-transfected with siSIRT1 and EGFP, resveratrol significantly increased synaptic GluA1 accumulation (132.3 ± 6.5%, $n$ = 18 cells, $p < 0.05$) while siSIRT1 itself had no effect on synaptic GluA1 ($n$ = 18 cells, $p > 0.05$) (Fig. 3*C*). These data demonstrate that resveratrol up-regulates AMPAR protein expression *via* a SIRT1-independent mechanism.

### 3. 4. Resveratrol up-regulates AMPARs through activating AMPK and PI3K/Akt pathways

In addition to SIRT1 activation, resveratrol has also been shown to target AMPK (Dasgupta and Milbrandt, 2007). Resveratrol exerts certain physiological effects *via* stimulating AMPK activity, such as improving mitochondrial function and glucose tolerance (Chung et al., 2012). However, whether the resveratrol-AMPK pathway exists in neurons remains unclear. To examine this cascade, we probed phosphorylated AMPK at Thr172, an indicator of AMPK activation. We found that resveratrol (1–8 hr) incubation significantly increased the level of AMPK Thr172 phosphorylation (224.1 ± 30.0% of the control at 4 hr, $n$ = 4, $p < 0.01$) without affecting total AMPK protein levels (Fig. 4*A*), indicating that resveratrol causes AMPK activation in neurons. The temporal profile of AMPK phosphorylation also matched the changes in AMPAR expression (Fig. 1*B* and Fig. 4*A*).

AMPK is coupled to a large number of downstream effectors (Hardie, 2007), among which PI3K/Akt is one of the major signaling cascades. PI3K/Akt signaling plays a critical role in protein synthesis and growth (Schratt et al., 2004), and is involved in AMPAR trafficking and synaptic plasticity (Man et al., 2003). Our recent work has demonstrated that in neurons PI3K/Akt serves as the signaling cascade downstream of AMPK (Amato et al., 2011). To examine the possibility that resveratrol regulates the PI3K/Akt pathway *via* AMPK activation, we probed resveratrol-treated neuronal lysates with an antibody specific for phosphorylated Akt at Ser473, a hallmark of Akt activation and thus an indicator for PI3K activity (Sarbassov et al., 2005). Indeed, we observed a direct correlation between AMPK phosphorylation and the time-dependent increase in Akt phosphorylation in neurons with resveratrol treatment (186.0 ± 14.4% at 4 hr, $n$ = 3, $p < 0.01$) (Fig. 4*A*).

To further confirm the involvement of AMPK in resveratrol effects on AMPAR expression, we first used the specific AMPK inhibitor, Compound C (CC, 20 μM), to block AMPK activity in neurons. Treatment with CC abolished both resveratrol-induced AMPAR increases (CC: 78.2 ± 14.1%; CC+Resv: 79.2 ± 11.6%, $n$ = 4) and AMPK phosphorylation (Fig. 4*B*). In support of PI3K as the effector downstream of AMPK, treatment of cortical neurons with the specific PI3K inhibitor, LY294002, blocked resveratrol-induced Akt phosphorylation and GluA1 increases (Fig. 4*C*).

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To further confirm the involvement of the AMPK-PI3K–Akt system, in cultured hippocampal neurons we overexpressed a kinase dead form of AMPKα1 subunit (AMPK K.D., D157A point mutation) and a dominant negative PI3K mutant (PI3K D.N.) to suppress endogenous AMPK or PI3K activity, respectively. Overexpression of AMPK K.D. significantly decreased the synaptic GluA1 accumulation (68.3 ± 6.7%, $n = 22$ cells, $p < 0.05$) and completely abolished resveratrol-induced increases in GluA1 synaptic accumulation (69.2 ± 9.6%, $n = 22$ cells, $p < 0.05$) (Fig. 4*D*). Similar to AMPK K.D., overexpression of PI3K D.N. in hippocampal neurons also markedly decreased synaptic GluA1 accumulation (71.1 ± 9.6%, $n = 22$ cells, $p < 0.05$) and blocked resveratrol-induced increase in GluA1 synaptic accumulation (87.1 ± 7.4%, $n = 22$ cells, $p < 0.05$) (Fig. 4*D*). In contrast, control cells expressing only EGFP showed a significant increase in GluA1 expression in response to resveratrol treatment (133.9 ± 7.3%, $n = 22$ cells, $p < 0.05$) (Fig. 4*D*). In line with the dominant negative effect of the mutant AMPK or PI3K, overexpression of AMPK K.D. or PI3K D.N. in HEK cells dramatically reduced AMPK or Akt phosphorylation levels, respectively (data not shown).

Previous studies have implicated calcium/calmodulin-dependent kinase kinase β (CaMKKβ) as the upstream activator of AMPK (Hawley et al., 2005). By using the fluorescent calcium indicator protein G-CaMP3 (Nakai et al., 2001; Hou et al., 2011), we monitored changes of intracellular calcium in transfected hippocampal neurons. 4 hr incubation with resveratrol caused a marked increase in G-CaMP3 intensity, indicating an elevation of intracellular calcium (Fig. 4*E*). Furthermore, to determine the involvement of CaMKK in the resveratrol-mediated effect, we used the specific CaMKK inhibitor, STO609. When STO609 (STO, 40 μM) was applied for 1 hr prior to and during resveratrol treatment, resveratrol-induced AMPK phosphorylation and the subsequent increase in AMPAR abundance were completely abolished (GluA1 abundance by STO: 77.9 ± 9.2%, $n = 5$, $p < 0.05$; STO+Resv: 79.9 ± 10.0%, $n = 5$, $p < 0.05$) (Fig. 4*F*). Moreover, STO609 alone caused a decrease in AMPAR abundance, indicating that the constitutive activity of CaMKK is important in the maintenance of AMPAR proteostasis.

### 3. 5. Involvement of the eIF4F translation initiation complex in resveratrol-induced increases in AMPARs

Our data indicated an involvement of protein synthesis in resveratrol-induced increase in AMPAR expression, however the molecular details surrounding the translational regulation of AMPARs remain unclear. In eukaryotes, an important initiation step in protein translation is the recruitment of the ribosome to the mRNA, an action which requires proper assembly of the eukaryotic initiation factor 4F (eIF4F) complex (Sonenberg and Hinnebusch, 2009). eIF4F is assembled by the cap-binding protein eIF4E, eIF4A and the scaffolding protein eIF4G. Also involved is the endogenous translation repressor, 4E–BP, that binds to eIF4E and prevents the formation of the eIF4F complex. Interestingly, 4E–BP has been implicated in protein synthesis required for long-term potentiation (L-LTP) (Banko et al., 2005) and in the PI3K/Akt signaling pathway (Sonenberg and Hinnebusch, 2009), suggesting a potential role for eIF4F complex in resveratrol-induced AMPAR translation. To test this possibility, we first investigated whether resveratrol regulates the expression of the initiation complex components. As shown in Fig. 5*A*, resveratrol treatment significantly increased both eIF4E

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(154.8 ± 16.9% $n = 3$, $p < 0.05$) and eIF4G levels (159.7 ± 8.1% $n = 3$, $p < 0.01$). Moreover, overnight pretreatment with the eIF4E-4G interaction inhibitor, 4EGI-1 (22 μM), completely abolished the resveratrol-induced AMPAR increase (4EGI-1: 71.2 ± 10.8%, $n = 4$, $p < 0.05$; 4EGI-1+Resv: 59.5 ± 6.7%, $n = 4$, $p < 0.01$) (Fig. 5*B*). LY pretreatment completely blocked resveratrol-induced eIF4E and 4G up-regulation ($n = 3$ of each, Fig. 5*C*), indicating that the resveratrol effect on the initiation complex resulted from resveratrol-dependent signaling. These data strongly indicated the requirement of the eIF4F complex activity in resveratrol-stimulated AMPAR synthesis.

### 3. 6. Pharmacological activation of AMPK mimics the effects of resveratrol

Our data indicate that AMPK is the primary upstream effector in the resveratrol-induced molecular events. If so, activation of AMPK is expected to mimic the resveratrol effects. To test this notion, we utilized a conventional AMPK activator, AICAR, to specifically activate the AMPK pathway in neurons (Amato et al., 2011). Similar to resveratrol, AICAR treatment (2 mM, 2–4 hr) significantly increased total amount of GluA1 (2 hr: 144.5 ± 9.9%, $n = 3$, $p < 0.05$; 4 hr: 185.4 ± 8.6%, $n = 3$, $p < 0.01$) and the level of AMPK phosphorylation (2 hr: 139.3 ± 10.6%, $n = 3$, $p < 0.05$; 4 hr: 219.3 ± 16.1%, $n = 3$, $p < 0.01$) (Fig. 6*A*). Consistently, AICAR treatment significantly increased Akt phosphorylation and the protein amounts of eIF4E/G (Fig. 6*A*). Similar to resveratrol, the AICAR-induced GluA1 increase was also blocked by the protein synthesis inhibitors cycloheximide ($n = 3$) and anisomycin ($n = 3$) (Fig. 6*B*) and was occluded by resveratrol co-application ($n = 4$) (Fig. 6*C*). Furthermore, both the AMPK inhibitor CC (20 μM) and PI3 kinase inhibitor LY (20 μM) blocked an AICAR-induced increase in GluA1 (Fig. 6*D, E*). All together these data show that AICAR utilizes a similar molecular mechanism as resveratrol to increase AMPAR expression in neurons. Interestingly, while LY dramatically inhibited Akt phosphorylation on Ser 473, it did not affect AICAR-caused AMPK activation, indicating that the PI3K–Akt signaling cascade lies downstream of AMPK (Fig. 6*E*).

## 4. Discussion

In this study, we investigated the effect of resveratrol on AMPAR expression and the underlying molecular mechanisms. We show that resveratrol increases AMPAR expression in neurons. Specifically, resveratrol stimulates receptor protein translation without affecting receptor mRNA transcription or the rate of protein degradation. Upstream of the signaling cascade, resveratrol causes an elevation of intracellular $Ca^{2+}$ and activation of CaMKKβ, leading to the activation of AMPK. Consistent with our previous findings (Amato et al., 2011), AMPK activation results in up-regulation of the downstream PI3K/Akt pathway, leading to eIF4E/4G–mediated AMPAR protein translation. A schematic cartoon illustrating this signaling pathway has been shown in Fig. 7. Given the crucial role for AMPARs in synaptic activity and synaptic plasticity, elevated AMPAR expression and synaptic transmission may play an important role in mediating resveratrol's beneficial effects in brain functions including learning, memory and cognition.

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### 4. 1. Regulation of AMPAR abundance by the AMPK pathway

Resveratrol has conventionally been thought to activate SIRT1 to achieve its promiscuous biological effects (Baur and Sinclair, 2006). However, recent studies have demonstrated that AMPK, another downstream effector of resveratrol, also plays an important role in mediating the effects of resveratrol (Chung et al., 2012; Cho et al., 2014). In line with this, we find that the AMPK signaling pathway, but not SIRT1, is required for resveratrol-mediated AMPAR up-regulation. As one of the most important excitatory receptors in the brain, the abundance and turnover of AMPARs are highly regulated by many different molecules and signaling pathways. For the first time, we show a link between the bioenergy sensor AMPK and AMPAR regulation. These findings may further explain many beneficial effects that resveratrol imparts to the brain. Specifically, energy-lacking conditions such as calorie restriction (CR) or physical exercise that often activate AMPK are known to be implicated in cognitive improvements and mental health (Gillette-Guyonnet and Vellas, 2008; Witte et al., 2009). The involvement of AMPK in CR is especially interesting. AMPK contributes to CR by stimulating mitochondrial biogenesis and positive regulation on metabolism (Canto and Auwerx, 2011). CR is also the most consistent intervention that can increase life span and improve ageing-related cognitive deteriorations in animals (Fontana et al., 2010; Dal-Pan et al., 2011). In line with this notion, resveratrol is an established CR mimetic that possesses all these effects as well (Chung et al., 2012). It will be interesting to investigate whether CR itself can also enhance AMPAR synthesis similar to the resveratrol effect.

### 4. 2. PI3K/Akt pathway and neuroprotection with resveratrol

Resveratrol is well known for protecting neurons from cell death under many pathological conditions in the brain (Gupta et al., 2002; Sinha et al., 2002; Wang et al., 2002; Tsai et al., 2007; Fukui et al., 2010; Chung et al., 2011). However, the underlying mechanisms mediating the neuroprotective effect remain less clear. In our study, we find that resveratrol activates the PI3K/Akt signaling pathway *via* activation of AMPK. Given the crucial role of the PI3K/Akt pathway in cell protection (Jo et al., 2012), our findings provide insight into a novel mechanism of resveratrol-mediated neuroprotection. Intriguingly, unlike what is observed in neurons, resveratrol inhibits the PI3K/Akt pathway in many peripheral tissues (Poolman et al., 2005; Venkatachalam et al., 2008). The tissue type specificity may explain why resveratrol’s protective role occurs exclusively in neurons. .

### 4. 3. eIF4E/4G–mediated regulation of AMPAR translation

We provide the first evidence indicating a direct role for the translation initiation complex in AMPAR translation. In agreement with our findings, a recent study has revealed that knock-out of 4E–BP, the endogenous inhibitor of the eIF4F complex, resulted in an increase in AMPARs (Ran et al., 2013). Interestingly, the expression of NMDARs, another type of glutamate receptor usually co-localizing with AMPARs, is not affected by resveratrol. It is not clear how neurons achieve the specificity on AMPARs. Possibly, certain regulatory components other than the core eIF4F subunits participate in the initiation process, which may determine the specificity in the selection of target mRNAs.

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We find that resveratrol up-regulates total and synaptic AMPARs due to facilitated protein translation, however, the subcellular sites of resveratrol's effects remain unclear. Changes in local AMPAR levels could result from intracellular receptor trafficking or local protein synthesis, or both. Thus, in addition to the cell body, resveratrol may regulate AMPAR local synthesis in the dendrites. Indeed, mRNAs of AMPAR subunits reside in the dendrites and spines and are subjected to activity-dependent local synthesis (Grooms et al., 2006). Furthermore, dendritic localization has been shown for the signaling molecules involved in the effects of resveratrol, including AMPK, PI3K/Akt (Yoshii and Constantine-Paton, 2007; Potter et al., 2010) and the protein translation machinery including the eIF4E/4G elongation complex (Takei et al., 2004). Given the requirement of mRNA translation, probably of AMPARs, in late-LTP (Bramham, 2008; Gobert et al., 2008), it is intriguing whether resveratrol can facilitate or enhance the expression of LTP, which may also be implicated in resveratrol's beneficial role in brain function.

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1. Resveratrol increases AMPAR levels in neurons *via* facilitated receptor protein translation
2. Elevated AMPAR expression by resveratrol is mediated by AMPK, but not SIRT1
3. PI3K and the initiation factors eIF4E/G are required for resveratrol's effect on AMPARs.
4. AMPK activator AICAR mimics the effect of resveratrol on AMPAR regulation
5. Intraperitoneal injection of resveratrol leads to an increased expression of AMPARs *in vivo*.

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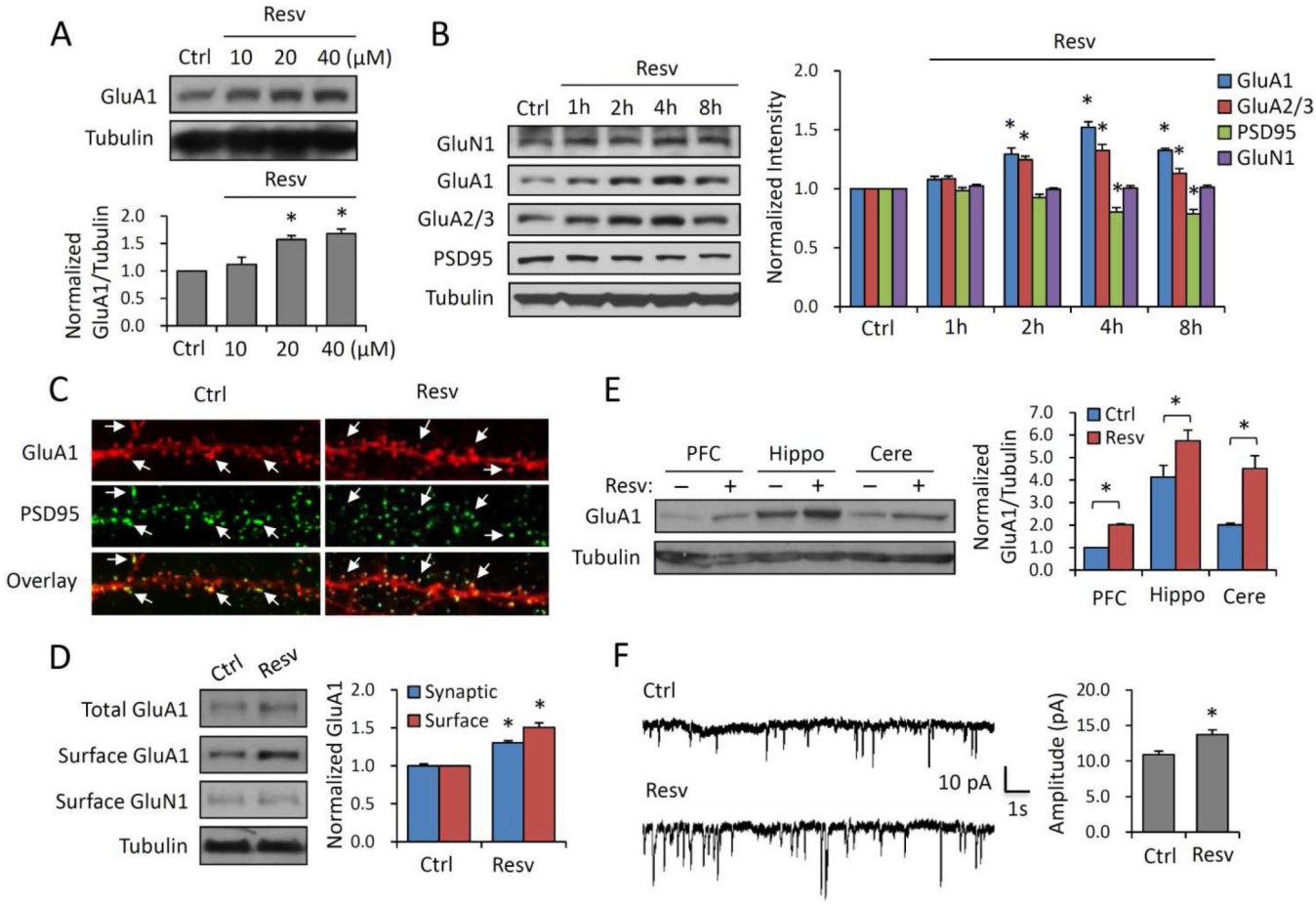


**Figure 1.**

Resveratrol enhances AMPAR expression in neurons. A. Western blots of cortical neurons treated with resveratrol at different dosage (10 – 40 μM) showing increased level of AMPAR GluA1 subunits. Resveratrol at 40 μM induced the most dramatic increase of GluA1. B. Time course of resveratrol (40 μM, 1 – 8 hr) treatment showed increased levels of AMPAR GluA1 and Glu2/3 subunits in cultured cortical neurons. No change was observed in the expression of NMDAR GluN1 subunits and tubulin. C, D. Synaptic staining or surface biotinylation of AMPARs showed resveratrol treatment (40 μM, 4 hr) significantly increased synapses (C) and cell-surface (D) localized GluA1. Surface GluN1 showed no change. PSD95 (C, green) was stained as the marker of excitatory synapses. Synaptic GluA1 puncta (C, red) overlapping with PSD95 were measured. Cell-surface AMPAR amount is quantified as the ratio of surface: total GluA1 (D). E. I.P. injection of resveratrol (30 mg/kg body weight, 5 hr) into C57BL/6 wild-type mice dramatically increased the total expression levels of AMPARs in PFC, hippocampus and cerebellum ($n = 4$), comparing with mice injected with vehicle control. F. Whole-cell voltage patch-clamp recording showed 4 hr resveratrol (40 μM) treatment significantly increased the average amplitude of the mEPSCs (126.1 ± 4.8%, $n = 8$ cells, $p < 0.01$). Bar graphs represent mean ± S.E., *$p < 0.05$.

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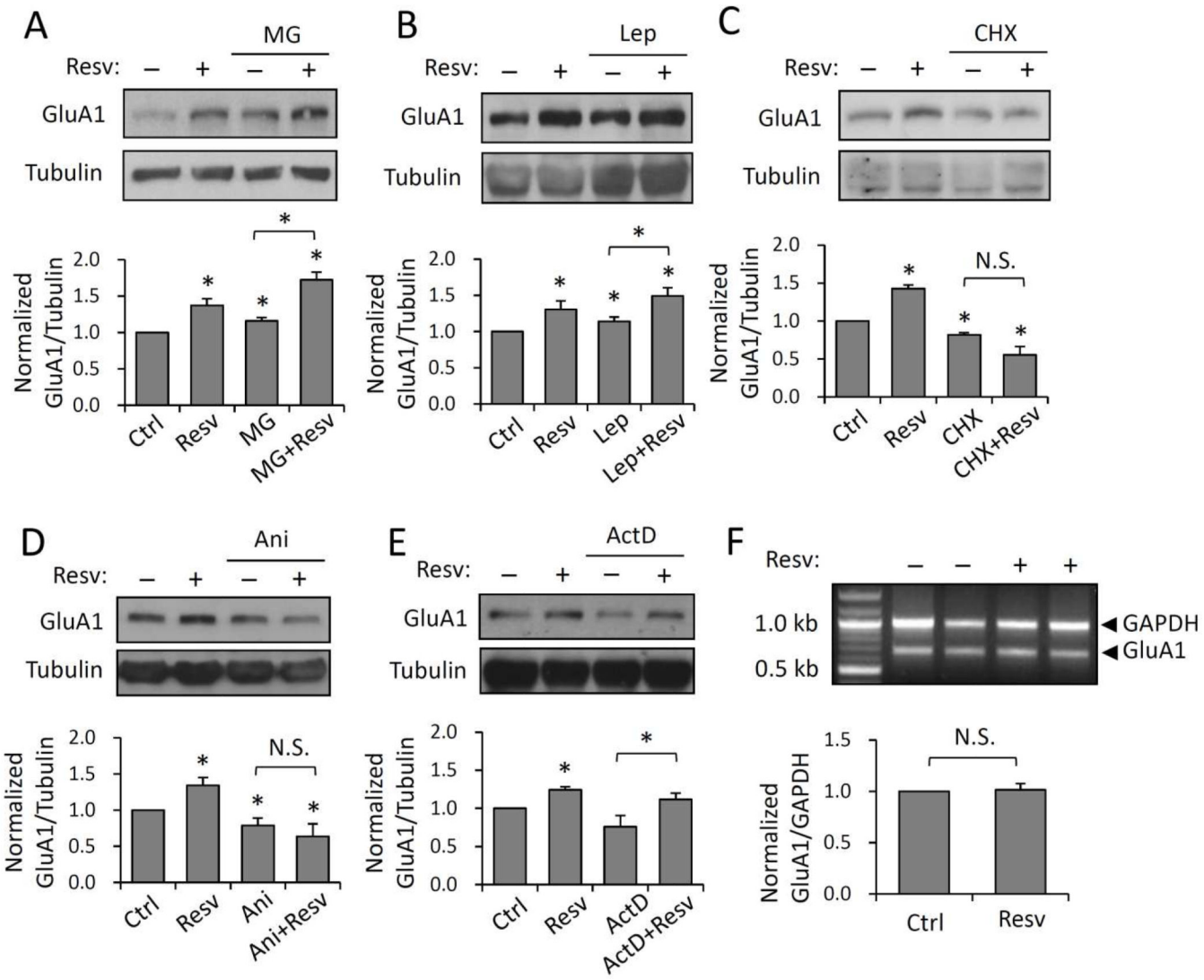


**Figure 2.**

Resveratrol increases AMPAR expression *via* regulation on protein translation. A, B. Cortical neurons were incubated with resveratrol together with proteasome inhibitor MG132 (MG, 10 μM, $n = 3$) or lysosome inhibitor leupeptin (Lep, 20 μM$n = 4$) to block protein degradation. Resveratrol effect on AMPAR expression persisted in the presence of the inhibitors (MG + Resv: 172.3 ± 10.5%, $n = 3$, $p < 0.05$; Lep + Resv: 149.4 ± 10.8%, $n = 4$, $p < 0.01$). C, D. Suppression of protein translation by cycloheximide (CHX, 100μM) or anisomycin (Ani, 30 μM) completely abolished the resveratrol effect (CHX+Resv: 55.3 ± 11.0%, $n = 4$, $p < 0.05$; Ani+Resv: 63.6 ± 17.4%, $n = 4$, $p < 0.05$). E. Application of transcription inhibitor actinomycin D (ActD, 25 μM, $n = 4$) did not block the resveratrol effect on AMPARs. F. RT-PCR showed that 4 hr resveratrol treatment (40 μM) did not change the expression level of GluA1 mRNA. Bar graphs represent mean ± S.E., $^*p < 0.05$.

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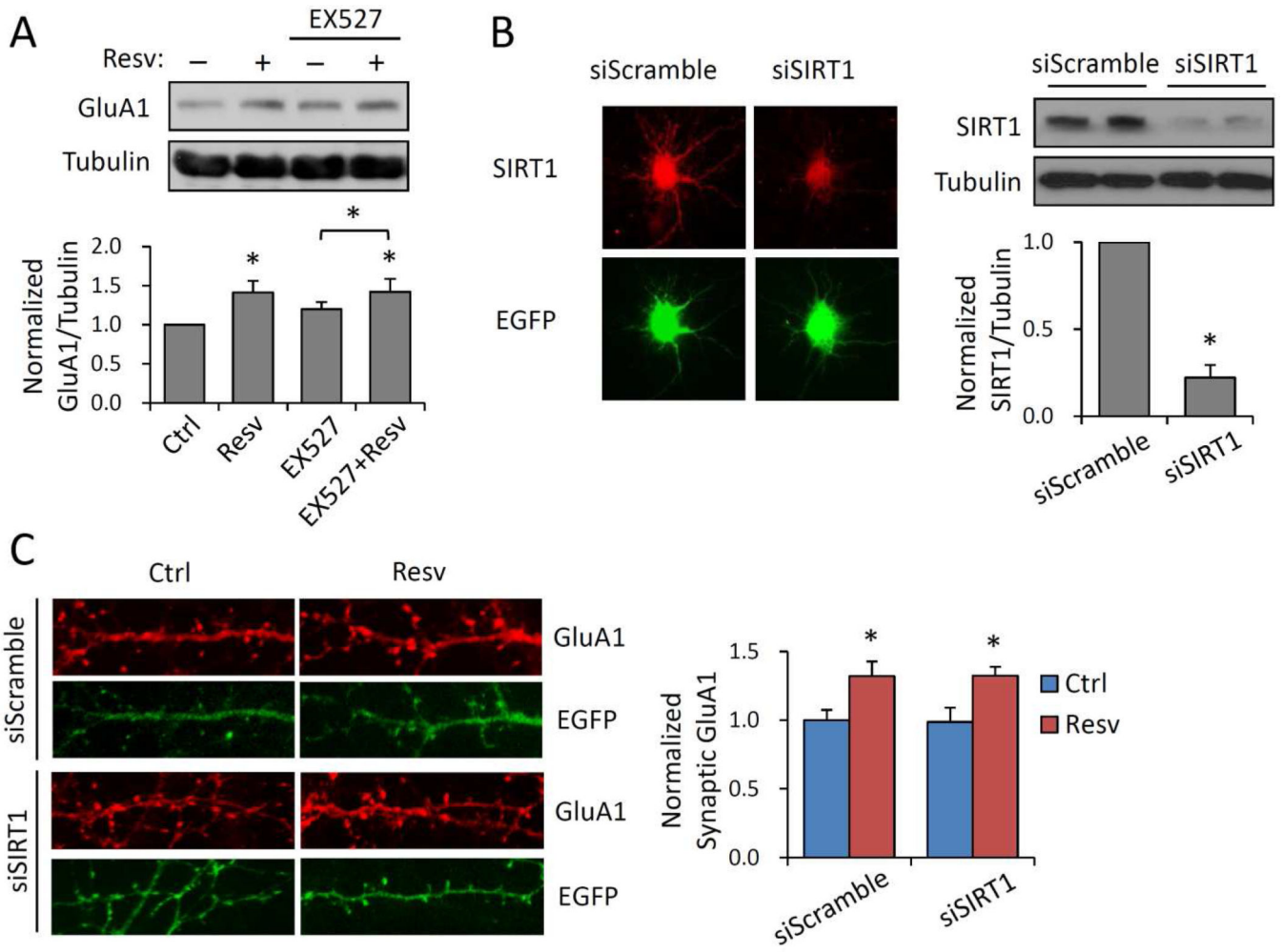


**Figure 3.**

Resveratrol-induced AMPAR up-regulation is independent of SIRT1 activity. A. SIRT1-specific inhibitor EX527 (1.2 μM) treatment did not affect resveratrol-induced increase in AMPAR amount. B. Hippocampal neurons or HEK 293T cells were transfected with EGFP together with SIRT1-specific siRNA (siSIRT1) or a scrambled control (siScramble). Immunostaining (left panel) or blotting (right panel) of endogenous SIRT1 showed significantly reduced expression in cells transfected by siSIRT1 (22.3 ± 7.3%, $n = 4$, $p < 0.01$). C. GluA1 (red) was immunostained in hippocampal neurons transfected with EGFP together with siSIRT1 or siScramble. Knockdown of SIRT1 by siRNA did not affect the resveratrol effect on AMPAR expression. Bar graphs represent mean ± S.E., *$p < 0.05$.

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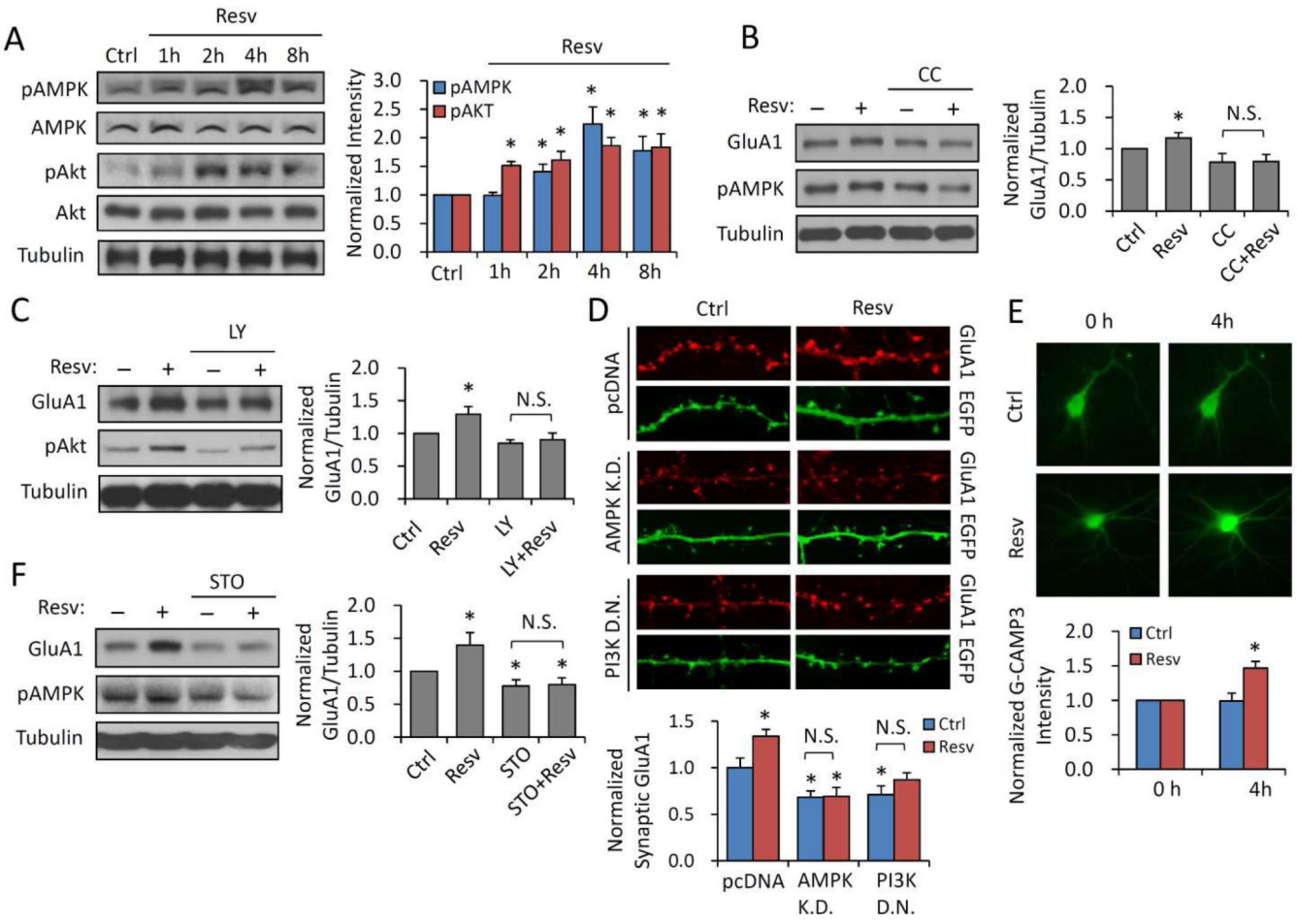


**Figure 4.**

Resveratrol increases AMPAR expression through AMPK and PI3K/Akt signaling cascades. A. Western blot analysis of resveratrol-treated cortical neurons. Resveratrol caused time-dependent phosphorylation (and activation) of AMPK and Akt. Total AMPK, Akt and tubulin showed no change. B, C. Cortical neurons were treated with resveratrol in the presence of AMPK inhibitor Compound C (CC, 20 μM) or PI3K inhibitor LY 294002 (LY, 20 μM), respectively. Resveratrol effect on AMPAR expression was abolished by each individual inhibitor. $n = 4$ each, $*p < 0.05$. D. GluA1 was immunostained (red) in neurons transfected with EGFP plus kinase dead AMPK (AMPK KD.) or dominant negative PI3K (PI3K D.N.). No increases in GluA1 puncta intensity by resveratrol was detected in cells expressing AMPK KD. or PI3K D.N. while normal response was observed in pcDNA transfected cells. Bar graphs represent mean ± S.E., $*p < 0.05$. E. G-CaMP3 imaging in live hippocampal neurons showed 47.8 ± 10.0% ($n = 13$ cells, $p < 0.05$) an increase in average fluorescent intensity in resveratrol-treated (40 μM, 4h) hippocampal neurons as compared to DMSO-treated (1:1000, 4h) control neurons. F. CaMKK inhibitor STO609 (STO, 40 μM) pre-treatment abolished resveratrol-induced GluA1 increase and AMPK phosphorylation.

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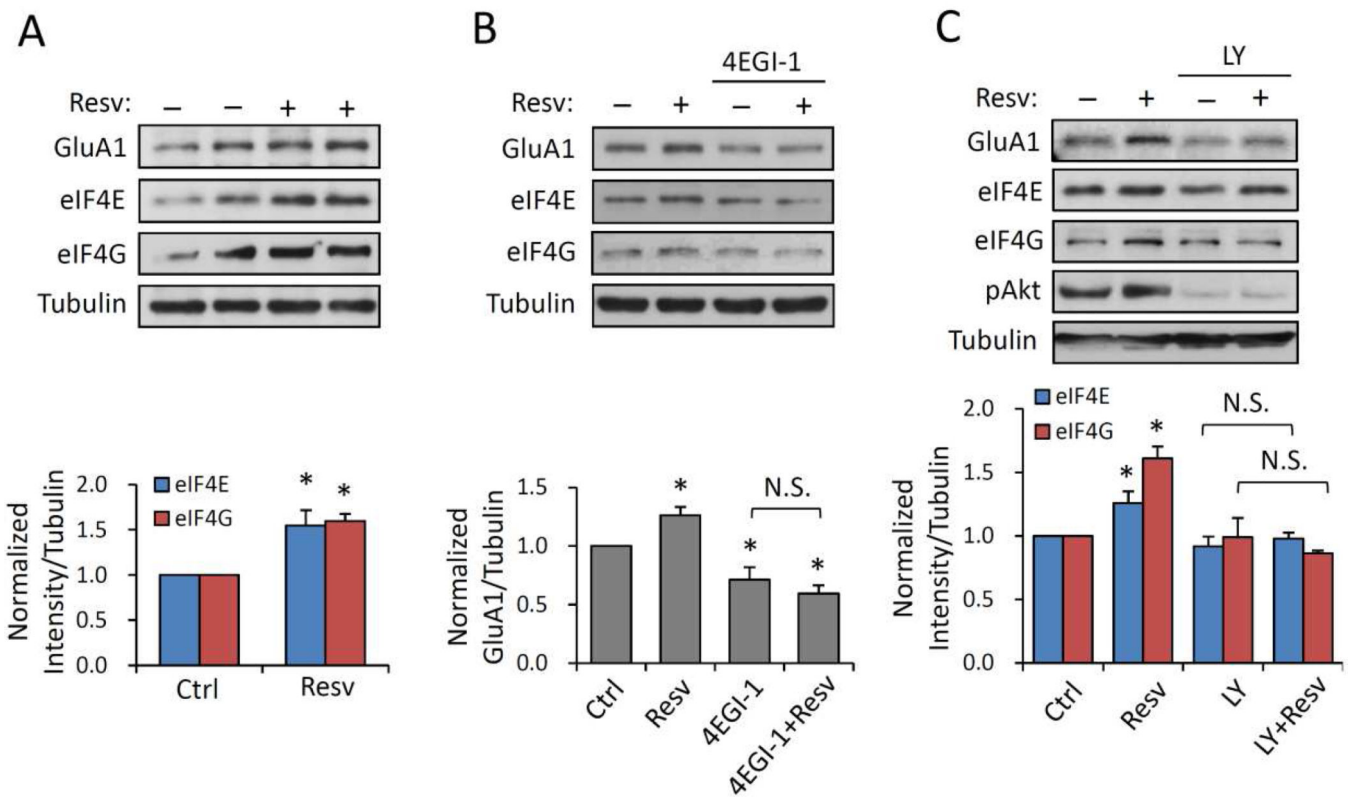


**Figure 5.**

Involvement of the translation initiation complex eIF4F in resveratrol-induced AMPAR up-regulation. A. Resveratrol treatment significantly increased protein levels of both eIF4E and eIF4G, as well as GluA1. Tubulin as a control showed no change. B. Application of a specific eIF4E and 4G interaction blocker 4EGI-1 (22 μM) abolished resveratrol-induced AMPAR increase ($n = 4$, $p < 0.05$). C. Application of PI3K inhibitor LY blocked resveratrol-induced eIF4E and 4G up-regulation ($n = 3$ of each, $p < 0.05$). Bar graphs represent mean ± S.E., *$p < 0.05$.

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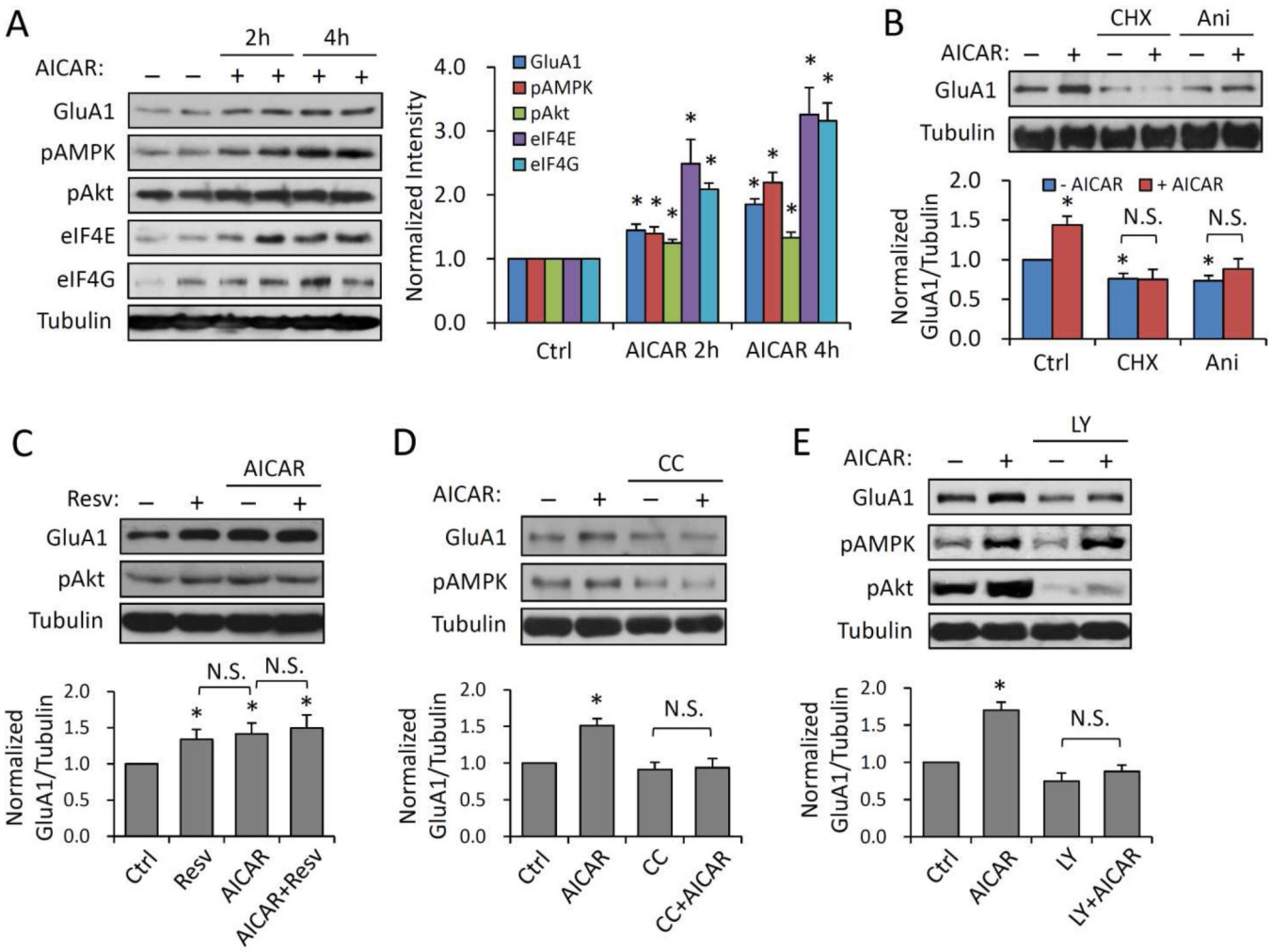


**Figure 6.**

AMPK activation by AICAR treatment mimics resveratrol-dependent signaling and alterations in AMPAR expression. A. In cultured cortical neurons, activation of AMPK by AICAR (2 mM) led to an increase in GluA1, eIF4E, eIF4G, and the activation of AMPK (pAMPK) and Akt (pAkt) pathways. B. AICAR-induced AMPAR increase was blocked by protein synthesis inhibitor cycloheximide (CHX, 100 μM) ($n$ = 3) or anisomycin (Ani, 30 μM) ($n$ = 3). C. Cortical neurons were treated with resveratrol or together with AICAR. In the presence of AICAR, no further increase in GluA1 amount was induced by resveratrol ($n$ = 4). D, E. AICAR effect on AMPAR increase was blocked by either AMPK inhibitor CC (20 μM) ($n$ = 3) or PI3K inhibitor LY 294002 (LY, 20 μM) ($n$ = 3). Bar graphs represent mean ± S.E., *$p$ < 0.05.

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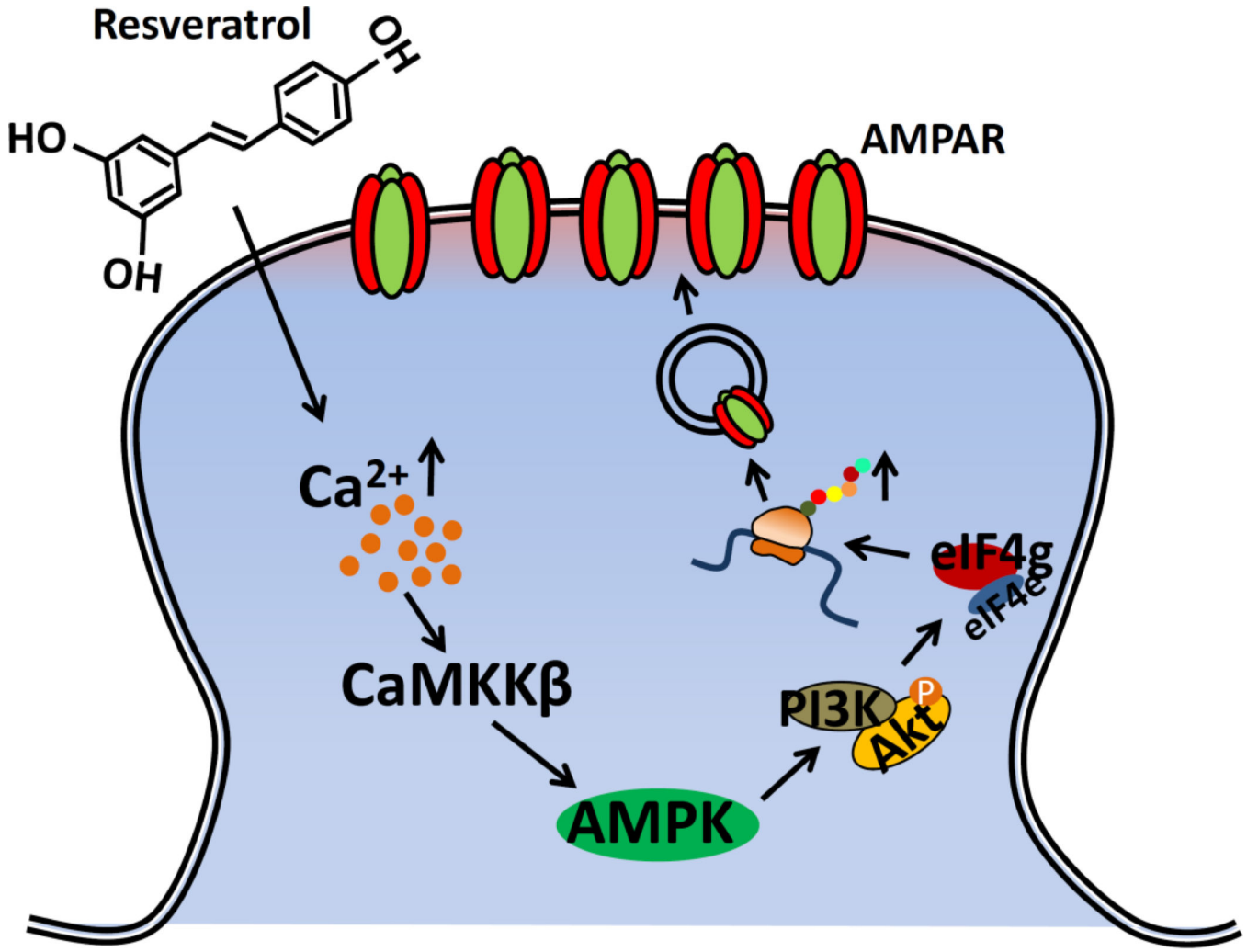


**Figure 7.**

A schematic illustration of the signaling cascades underlying resveratrol-induced AMPAR up-regulation. Resveratrol increases intracellular calcium level that leads to an activation of CaMMKβ and consequently the AMPK pathway. AMPK activates its downstream PI3K/Akt signaling cascades and eIF4E/G complex, leading to an enhanced translation of AMPAR subunits.