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Latrepirdine is a potent activator of AMP-activated protein kinase and reduces neuronal excitability.

Petronela Weisová  
*Royal College of Surgeons in Ireland*

S P. Alvarez  
*Royal College of Surgeons in Ireland*

Seán M. Kilbride  
*Royal College of Surgeons in Ireland*

Ujval Anilkumar  
*Royal College of Surgeons in Ireland*

B Baumann  
*Royal College of Surgeons in Ireland*

*See next page for additional authors*

Citation

Authors
Petronela Weisová, S P. Alvarez, Seán M. Kilbride, Ujval Anilkumar, B Baumann, Joaquin Jordán, Tytus Bernas, Heinrich J. Huber, Heiko Düssmann, and Jochen HM Prehn
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Latrepirdine/Dimebon is a small-molecule compound with attributed neurocognitive-enhancing activities, which has recently been tested in clinical trials for the treatment of Alzheimer’s and Huntington’s disease. Latrepirdine has been suggested to be a neuroprotective agent that increases mitochondrial function, however the molecular mechanisms underlying these activities have remained elusive. We here demonstrate that latrepirdine, at (sub)nanomolar concentrations (0.1 nM), activates the energy sensor AMP-activated protein kinase (AMPK). Treatment of primary neurons with latrepirdine increased intracellular ATP levels and glucose transporter 3 translocation to the plasma membrane. Latrepirdine also increased mitochondrial uptake of the voltage-sensitive probe TMRM. Gene silencing of AMPKα or its upstream kinases, LKB1 and CaMKKβ, inhibited this effect. However, studies using the plasma membrane potential indicator DisBaC3(3) demonstrated that the effects of latrepirdine on TMRM uptake were largely mediated by plasma membrane hyperpolarization, precluding a purely ‘mitochondrial’ mechanism of action. In line with a stabilizing effect of latrepirdine on plasma membrane potential, pretreatment with latrepirdine reduced spontaneous Ca2+ oscillations as well as glutamate-induced Ca2+ increases in primary neurons, and protected neurons against glutamate toxicity. In conclusion, our experiments demonstrate that latrepirdine is a potent activator of AMPK, and suggest that one of the main pharmacological activities of latrepirdine is a reduction in neuronal excitability.

**Keywords:** AMP-activated protein kinase; bioenergetics; Ca2+ homeostasis; glutamate excitotoxicity; mitochondria; plasma membrane potential

**INTRODUCTION**

Latrepirdine/Dimebon has been safely used as an anti-histaminergic agent for the treatment of allergies and travel diseases in Russia for more than 25 years. Latrepirdine has been shown to improve cognition in rodent models,1,2 and to enhance memory in rhesus monkeys.3 Latrepirdine was also successfully tested in a Phase 2 study of patients with mild-to-moderate Alzheimer’s disease (AD).4 The potential mechanisms of latrepirdine’s neurocognition-enhancing activities are unrelated to its anti-histaminergic properties, and have been attributed to mitochondrial-enhancing or -stabilizing activities.5,6 However, these apparent ‘mitochondrial’ activities have been poorly characterized at a molecular level. Despite this shortfall, latrepirdine was subsequently tested in two Phase 3 trials in patients with AD, and in a Phase 2/3 trial in patients with Huntington’s disease. All three studies failed to observe any beneficial activity of latrepirdine when studied at a relatively advanced disease stage.7,8

Latrepirdine has been suggested to enhance or stabilize mitochondrial membrane potential (ΔΨm), an important indicator of mitochondrial function, in primary cortical neurons and human SH-SY5Y neuroblastoma cells.9 Latrepirdine has also been shown to increase cellular ATP levels, to protect SH-SY5Y cells against serum starvation-induced cell death, and to reduce Ca2+ -induced swelling of rat brain mitochondria.9,5 In the present study, we set out to explore the potential mechanisms underlying the reported mitochondrial activities of latrepirdine. We here describe that latrepirdine is a very potent, small-molecule activator of the intracellular energy sensor, AMP-activated protein kinase (AMPK), acting at low, (sub-)nanomolar concentration ranges. We furthermore demonstrate that the molecular actions of latrepirdine include profound changes on plasma membrane potential and neuronal excitability, and investigate the conditions in which latrepirdine may confer protection against excitotoxic neuronal injury.

**MATERIALS AND METHODS**

Supplementary Information includes materials and a detailed description of techniques not described in the main text.

**Immunofluorescence**

As previously described,10 cerebellar granule neurons (CGNs) were harvested from a 24-well plate using trypsin and fixed in 1% formalin for 20–25 min at 4°C in the absence of a permeabilization step. Cells were incubated with a rabbit polyclonal GLUT 3 antibody (Millipore Bioscience Research Reagents, Billerica, MA, USA), diluted 1:250 in PBS and 0.1% BSA for 1 h; cells were washed and incubated with an Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Invitrogen, Biosciences, Dublin, Ireland)
diluted 1:250 for 1 h. After washing the cells three times with PBS/0.1% BSA, samples were analyzed immediately by flow cytometry on a Partec CyFlow ML (Münster, Germany) followed by analysis using FloMax software. In all cases, a minimum of 10^5 events were acquired.

Preparation of primary CGNs
Murine or rat cerebellum was extracted from postnatal day 7–8 pups and CGNs were prepared as described previously. Briefly, cells were cultured on poly-D-lysine-coated glass Willco dishes (Amsterdam, The Netherlands), 6-well plates and 24-well plates at a density of 1 x 10^5 cells per ml, or on 96-well plates (Corning) at a density of 50,000 cells per well in 100 µl, and maintained at 37 °C in a humidified atmosphere of 5 % CO2/95% air. Experiments were carried out after 7 days in culture when cells became sensitive to glutamate excitotoxicity. All animal work was carried out with ethics approval from the RCSI Research Ethics Committee and under the license obtained from Irish government granted to the authors under the Cruelty to Animal Act, 1976. A record of killed pups was taken down and annual report was submitted to the Irish Department of Health and Children.

Preparation of mouse neocortical neurons
Primary cultures of cortical neurons were prepared from E16 to E18 as described previously. To isolate the cortical neurons, hysterecomies of the uterus of pregnant female mice were performed using an abdominal injection of 40 mg kg^-1 pentobarbital (Dolethal) as lethal anesthesia. The cerebral cortices were pooled in a dissection medium on ice (PBS with 0.25% glucose and 0.3% bovine serum albumin). The tissue was incubated with 0.25% trypsin–EDTA at 37 °C for 15 min. After the incubation, the trypsinization was stopped by the addition of medium containing sera. The cells were immediately stored at -80 °C until further use.

Glutamate excitotoxicity
Preparation of primary CGNs
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Glutamate toxicity
After 7–8 days in culture, primary neurons were treated with glutamate/glycine at concentrations of 100 µM/10 µM for 10 min in experimental buffer composed of 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH2PO4, 5 mM NaHCO3, 20 mM HEPES, 1.2 mM Na2SO4 supplemented with glucose (15 mM) and CaCl2 (1.2 mM) at pH 7.4. Cultures were rinsed with 1.2 mM CaCl2 at pH 7.4. Cultures were maintained at 37 °C. Treatment with glutamate/glycine was performed on days 6–8 in culture on poly-D-lysine-coated glass Willco dishes (Amsterdam, The Netherlands). Cells were maintained at 37 °C. 30 min prior to recording. Treatment was applied to the experimental buffer and returned to preconditioned media.

Measurement of ATP
Cells were treated in 24-well plates, the medium was aspirated, 200 µl of hypotonic lysis buffer (Tris acetate buffer, pH 7.75) was added. Samples were immediately stored at −80 °C. ATP measurements were performed using the ENLUTEN ATP assay system (Promega, Southampton, UK) as per the manufacturer’s instructions. Luminescence was recorded using a Tecan Genios well plate reader (Männedorf, Switzerland) in luminescence mode. ATP content values were calculated by a concentration standard curve with the DNA/CaPi coprecipitate. The cells were then washed with pre-warmed (36.5 °C) Hank’s balanced saline solution (HBSS) washing buffer to ensure that the precipitates have dissolved completely. Finally, cells were incubated in conditioned neuronal tissue culture medium. shRNA targeting ampk a1/a2 (pVF-AMPK-shRNA) and scrambled sequence (pVF-Control-shRNA) were prepared and used as described previously. For inhibition of LKB1 and CaMKKβ, neurons were transfected with a vector (pGFP-V-RS) expressing either a commercial rat shRNA targeting lkb1 5’-TGGTGCTGATTCTCAGCTGAATGGCTGTG-3’ (Gene ID 25048, Origene) or camkkβ 5’-CCTGGAATTCCTCCAGAACAGCAACACAA-3’ (Gene ID 24245, Origene, Rockville, MD, USA). Cells were used for experiments 48 h after transfection. Efficiency of knockdown of AMPK, LKB1 and CaMKKβ was examined by western blotting.

Statistics
Data are given as means ± s.e.m. For statistical comparison, one-way analysis of variance between groups and Student-Newman-Keuls post hoc test were carried out on SPSS software (SPSS GmbH Software, Munich, Germany). Where the P-value was <0.05, groups were considered to be significantly different.
RESULTS

Pretreatment with (sub)nanomolar concentrations of latrepirdine provides neuroprotection against glutamate excitotoxicity

Excitotoxicity caused by glutamate receptor overactivation has been shown to contribute to neuronal injury and neurodegeneration in both acute and chronic neurodegenerative disorders, including stroke, AD and Huntington’s disease.13–16 To characterize potentially neuroprotective concentrations of latrepirdine against glutamate excitotoxicity in high throughput and at the single-cell level, we employed a Cellomics ArrayScan high-content screening platform. This technique uses automated epifluorescence microscopy and allowed quantification of effects of latrepirdine on neuronal survival over a wide concentration range over time within the same well plate. Because a previous study has reported protective properties of latrepirdine against serum starvation-induced cell death in SHSY-5Y neuroblastoma cells in a pretreatment paradigm,5 we pretreated CGNs with 0.01–100 nM latrepirdine for 24 h. Latrepirdine was then washed out and neurons exposed to excitotoxicity using a model of glutamate-induced injury that has been extensively characterized in our laboratory.10–12,17–20 We identified a narrow, (sub)-nanomolar concentration window of around 0.1 nM, in which latrepirdine conferred a protection against glutamate excitotoxicity (Figure 1a).

We next verified the data obtained from the automated high-content screening assay by manually scoring the percentage of pyknotic nuclei. Pretreatment with latrepirdine (0.01, 0.1 and 1 nM) for 24 h resulted in a significant neuroprotection against glutamate excitotoxicity, with the higher concentration of latrepirdine (100 nM) showing no protective activity (Figure 1b), confirming the Cellomics data set. The concentration of latrepirdine that showed most potent attenuation of cell death was 0.1 nM, thus we used

Figure 1. Latrepirdine pretreatment mediates neuroprotection against excitotoxic injury at (sub)nanomolar concentrations. (a) High-content time-lapse screening of cell death following glutamate excitation. Murine cerebellar granular neurons plated in a 96-well plate were pretreated with a range of concentrations of latrepirdine (0.01–100 nM) for 24 h as indicated. Cells were stained with Hoechst 1 h before treatment with glutamate/glycine (for 10 min at indicated concentrations) after which cells were washed twice with high Mg$$^{2+}$$ buffer and preconditioned medium (now containing PI) was replaced. The plate was then immediately placed within the Cellomics imaging chamber (Time 0) and imaged at 1-h intervals over 24 h. Cells were categorized and analysis was carried out using Cell Profiler as described in the Materials and Methods. Data presented are representative traces from thousand of cells, and experiments were carried out on three independent neuronal cultures. (b) Murine cerebellar granular neurons were plated in 24-well plates and following pretreatment with latrepirdine (0.01–100 nM as indicated) for 24 h, cells were exposed to glutamate/glycine 100 μM/10 μM for 10 min. After treatment, cells were washed twice with high Mg$$^{2+}$$ buffer and incubated in preconditioned medium for a further 24 h. Pyknotic nuclei were counted as apoptotic, as determined by Hoechst 33358 staining (1 μg ml$$^{-1}$$) and expressed as a percentage of total (n = 4 independent experiments in triplicate). Data are presented as mean ± s.e.m. *P ≤0.001 indicates difference between glutamate-only treated and latrepirdine (0.01–1 nM)-pretreated glutamate-treated neurons.
this concentration of the compound for all further experiments. Treatment with latrepirdine alone did not affect cell viability at any concentration (Figure 1b). We then examined whether acute treatment with Dimebon exerted any protective activity in the context of glutamate toxicity. Interestingly, acute (10 min) pretreatment with latrepirdine (0.1 nM) failed to provide any neuroprotection against glutamate excitotoxicity (Supplementary Figure 1). Collectively, these data suggest that Dimebon can act as neuroprotectant against glutamate excitotoxicity when applied at nanomolar concentrations and in a pretreatment paradigm, however fails to provide any neuroprotection when added concomitantly to excitotoxic stress.

Latrepirdine increases mitochondrial TMRM uptake
In light of previous findings that linked neuroprotective and neurocognitive effects of latrepirdine to improved mitochondrial bioenergetics,5,9,21,22 we next explored whether the protective effects of latrepirdine pretreatment against glutamate toxicity were related to changes in mitochondrial membrane potential (ΔVm), an indicator of mitochondrial bioenergetics. Neurons were imaged by time-lapse confocal microscopy using the lipophilic, cationic probe TMRM (10 nM) that is taken up into negatively charged mitochondria, following Nernstian behavior, and is thus also sensitive to changes in plasma membrane potential.17,23 TMRM uptake in single cells can be followed by time-lapse microscopy and under non-quench conditions an increase in fluorescence intensity is indicative of increased uptake.17 Latrepirdine increased mitochondrial TMRM fluorescence intensity significantly after 60 min (Figures 2a and b), in accordance with data previously obtained in cortical neurons and SH-SYSY cells.3 However, there was no difference in TMRM fluorescence intensity during exposure to glutamate when compared to untreated neurons (Supplementary Figure 2), suggesting that glutamate-induced membrane depolarization is not affected by latrepirdine.

We next conducted a series of experiments to explore whether the increase in TMRM uptake in response to latrepirdine was solely due to changes in membrane potentials, or partially due to an increase in mitochondrial mass or biogenesis. Neither the increase in TMRM uptake in response to latrepirdine was solely due to changes in membrane potentials, or partially due to an increase in mitochondrial mass or biogenesis. Neither the increase in TMRM uptake in response to latrepirdine was solely due to changes in membrane potentials, or partially due to an increase in mitochondrial mass or biogenesis.

Latrepirdine activates AMPK and reduces neuronal excitability
We have previously detected a direct link between increased TMRM uptake, pre-conditioning, and activation of an evolutionarily conserved metabolic sensor, AMPK.10,27,28 Moreover, a recent study has demonstrated that AMPK activation leads to plasma membrane hyperpolarization through phosphorylation of a voltage-sensitive potassium channel.29 We therefore set out to

Figure 2. Latrepirdine increases TMRM fluorescence intensity and hyperpolarizes ΔVp. (a) Representative single-cell traces of changes in TMRM fluorescence intensity following latrepirdine treatment. Latrepirdine (0.1 nM) was added to cells on the confocal microscope stage and TMRM fluorescence intensity was imaged at 5-min intervals over 240 min. Analysis was carried out using Metamorph software and average pixel intensity per cell at each time point is shown. (b) Quantification of average TMRM fluorescence intensity per cell at selected time points, represented as mean ± s.e.m. *P < 0.05, indicates the difference between 0 min and 60, 120, 240 min after latrepirdine addition (n = 65 cells). This experiment was carried out on three independent cultures with similar results obtained. (c) Representative images of CGNs loaded with DisBAC2(3) (1 μM) and treated with latrepirdine (0.1 nM) on a confocal microscope stage showing decreased fluorescence intensity after 210 min. DisBAC2(3) is a bis-barbituric acid oxonol compound that is incorporated into the plasma membrane as a function of ΔVp. Plasma membrane hyperpolarization is characterized as an extrusion of the probe with subsequent decrease in fluorescence, whereas depolarization results in increased fluorescence. Scale bar, 10 μm. (d) The DisBAC2(3) traces from 105 cells treated with latrepirdine (0.1 nM) were averaged. Neurons were treated with latrepirdine (0.1 nM) at 0 min and fluorescence intensity imaged at 2-min intervals over 240 min. Image analysis was carried out as described in Supplementary Information. (e) Quantification of DisBAC2(3) (1 μM) fluorescence intensity (FL.int.) in vehicle-treated versus latrepirdine (0.1 nM) treated CGNs from selected time points. Average DisBAC2(3) fluorescence intensity is represented as mean ± s.e.m. *P < 0.0001, difference between vehicle-treated and latrepirdine-treated (0.1 nM) neurons stained with DisBAC2(3) (n = 105 cells). This experiment was carried out on three independent cultures with similar results obtained. (f) The corresponding ΔVp values (in mV) for each time point in the mean DisBAC2(3) traces were calculated as described in the Materials and Methods. (g) TMRM fluorescence intensity i) reaches a stable baseline when the dye equilibrates across plasma and mitochondrial membranes and increases. fluorescence intensity corresponds to increased uptake driven by either ii) plasma membrane potential (ΔVp) hyperpolarization or iii) mitochondrial membrane potential (ΔVm) hyperpolarization. (h) The kinetics of the mean TMRM intensity caused by changes in ΔVp were calculated according to the Nernstian equation of the TMRM concentrations in the buffer, cytosol and mitochondria (see Materials and Methods for equations). Thereby, it was calculated that the measured TMRM kinetics (blue line) indicated that there was a slight latrepirdine-induced depolarization of the ΔVp (−1.6% at 240 min), as the TMRM signal is lower than the expected increase due to the change in cytosolic TMRM after the ΔVp hyperpolarization.
examine whether latrepirdine had a direct effect on the activity of AMPK. Indeed, treatment of CGN cultures with latrepirdine increased the levels of phospho AMPK (Thr 172), indicative of elevated AMPK activity (Figures 3a and b).

Previous studies have identified enhanced glucose transporter 3 (GLUT 3) plasma membrane localization and elevated ATP levels in response to AMPK activation in neurons. Therefore, we tested whether latrepirdine treatment altered GLUT 3 translocation in CGNs by examining GLUT 3 cell surface expression. Treatment of neurons with latrepirdine (0.1 nM) led to a significant increase GLUT 3 translocation as evidenced by immunofluorescence and flow cytometry analysis (Figures 3c and d). We also detected a significant increase in neuronal ATP levels after 24-h latrepirdine treatment (Figure 3e), suggesting that Dimebon-induced AMPK activation may enhance neuronal bioenergetic function or decrease ATP utilization.

Latrepirdine-induced hyperpolarization of ΔΨᵢ requires AMPK and its upstream kinases LKB1 and CaMKKβ. To assess whether increased AMPK signaling directly mediated latrepirdine-induced changes in cellular physiology, we employed small hairpin RNA (shRNA) technology to suppress AMPKα expression 24 h before latrepirdine treatment as reported previously (Figure 4a). Experiments were conducted using TMRM uptake as read-out, and were evaluated on the single-cell level as the shRNA constructs co-expressed GFP. Live-cell confocal imaging microscopy of neurons with suppressed AMPKα (AMPKα shRNA) revealed a complete suppression of latrepirdine-induced alterations in TMRM uptake when compared with control shRNA-transfected neurons (Figures 4b and c). Changes in DisBAC2(3) fluorescence intensity were also reduced in AMPKα shRNA-transfected neurons (Figure 4d). We then addressed the question as to whether the upstream AMPK kinases (AMPKK), LKB1 and
CaMKKβ30–32 could modulate the latrepirdine-induced changes in TMRM uptake. Transfection of neurons with shRNA plasmids targeting LKB1 (LKB1 shRNA) or CaMKKβ (CaMKKβ shRNA) also led to a significant depletion of neuronal LKB1 and CaMKKβ levels (Figure 4a). Gene silencing of either LKB1 or CaMKKβ prevented neuronal TMRM uptake in latrepirdine-treated neurons, suggesting that the activity of both kinases was required for the latrepirdine-induced increase in plasma membrane potential (Figures 4b and c). We also pharmacologically inhibited AMPK using the small-molecule inhibitor Compound C. 10,12,33,34 Treatment with Compound C (10 μM) prevented the latrepirdine-induced changes in DisBAC 2(3) and TMRM (Figures 4e and f).

Collectively, these results suggested that the latrepirdine-induced hyperpolarization of the plasma membrane potential required AMPK.

Pretreatment with latrepirdine attenuates cytosolic Ca2+ influx during glutamate excitation and decreases spontaneous Ca2+ elevations in neurons

Glutamate excitotoxicity is characterized by excessive Ca2+ influx through NMDA receptors, leading to intracellular Ca2+ overload.35 Indeed, glutamate-induced Ca2+ elevations critically depend both on the magnitude of plasma membrane potential depolarization36 as well as ATP-dependent Ca2+ extrusion.37 Our observations of plasma membrane hyperpolarization and the changes in cellular bioenergetics in response to latrepirdine posed the question whether protection by pretreatment with latrepirdine may be mediated by reduced neuronal Ca2+ overloading during glutamate excitation. CGN neurons were pretreated with latrepirdine (0.1 nM), and changes in cytosolic Ca2+ levels were monitored by confocal microscopy using Fluo-4 AM. CGNs pretreated for 24 h with latrepirdine, and then exposed to glutamate and glycine (100 μM/10 μM for 10 min) significantly attenuated cytosolic Ca2+ influx (Figures 5a and b). Quantification of peak fluo-4 fluorescence (Figure 5b) during the glutamate exposure showed a robust attenuation of Ca2+ influx in CGN neurons pretreated with latrepirdine (0.1 nM) for 24 h compared with vehicle-pretreated neurons. This finding was furthermore confirmed by the observation that pharmacological activation of AMPK with AICAR (0.1 mM, 24 h before glutamate excitation) also led to a significant attenuation of cytosolic Ca2+ levels during NMDA receptor overactivation in cortical neurons (NMDA alone: 5516.72 ± 1126.52 fluorescence intensity units, n = 70 cells vs. AICAR pretreated 3174.34 ± 1152.78 fluorescence intensity units, n = 67 cells, P < 0.001). Collectively, these data suggested that pharmacological activation of AMPK may be a key mechanism by which latrepirdine protects against glutamate excitotoxicity.

Figure 3. Latrepirdine induces AMPK activation and GLUT 3 translocation and increases ATP levels. (a) CGNs were treated with latrepirdine (0.1 nM) and were lysed for protein quantification at indicated time points. The level of phosphorylated AMPK (Thr 172) relative to the total level of AMPK, which corresponds to activation status of the enzyme, was examined by western blotting and (b) quantified using Image J (at 24-h timepoint post latrepirdine administration). This experiment was repeated three times with similar results. (c) Representative immunofluorescence images of plasma membrane localization of GLUT 3 in vehicle and latrepirdine (0.1 nM)-treated neurons. Scale bar, 10 μm. (d) Quantification of GLUT 3 plasma membrane localization in vehicle and latrepirdine (0.1 nM)-treated CGNs by immunofluorescence by flow cytometry. The mean fluorescence intensity of each population was obtained, and data are presented as mean ± s.e.m. of n = 3 populations. *P < 0.001 indicates difference between vehicle versus latrepirdine-treated neurons. This experiment was done in triplicate from 3 independent cultures. (e) CGNs were treated with latrepirdine (0.1 nM for 24 h), lysed and intracellular ATP content was determined as described in Materials and Methods. Data are shown as mean ± s.e.m. and the experiment was repeated on three independent preparations. *P < 0.01 indicates difference between vehicle versus latrepirdine-treated neurons.
AMPK activation with latrepirdine pretreatment affects Ca\(^{2+}\) handling in primary neurons in response to glutamate excitotoxicity. Interestingly, acute pretreatment with latrepirdine (0.1 nM, 10 min before glutamate) did not attenuate Ca\(^{2+}\) influx (Supplementary Figures 4A and B), suggesting that latrepirdine did not act directly on glutamate receptors.
Having observed that latrepirdine activates AMPK and also hyperpolarizes neuronal plasma membrane potential, we next turned our attention to the effects of latrepirdine on neuronal excitability, which was recently shown to be directly regulated by AMPK activity.29 To address this, we measured spontaneous Ca\(^{2+}\) oscillations in single-cortical neurons using high-frequency time-lapse confocal microscopy. The addition of 0.1 nM latrepirdine caused a significant attenuation of spontaneous Ca\(^{2+}\) spiking in the absence of Mg\(^{2+}\) compared to vehicle (Figure 5c). Quantification of the effect of latrepirdine showed an average reduction of spiking frequency from 14.0 ± 0.3 to 8.9 ± 0.5 min\(^{-1}\) (Figure 5d). The frequency of spiking was significantly lower in the presence of Mg\(^{2+}\) (2 mM), and addition of Tetrodotoxin (TTX, 1 µM) completely abolished spontaneous Ca\(^{2+}\) spiking (Figure 5d). In accordance with previous evidence indicating that activation of AMPK reduces neuronal excitability,29 acute exposure of the cells to the AMPK activator AICAR (0.1 mM) reduced the Ca\(^{2+}\)-spiking frequency from 13.8 ± 0.1 to 6.1 ± 0.7 min\(^{-1}\) (Figure 5e). Direct comparison of changes in frequency of oscillations induced by each compound revealed that 0.1 nM latrepirdine reduced neuronal excitability as potently as 0.1 mM AICAR, as no significant difference was found between the two groups (Figure 5f).

DISCUSSION

Most of the current therapeutic strategies for the treatment of AD are designed to target NMDA receptor overactivation, or to target β amyloid itself by interfering with its synthesis, aggregation or

Figure 5. Latrepirdine pretreatment attenuates the increase in cytosolic Ca\(^{2+}\) during glutamate excitation and reduces spontaneous Ca\(^{2+}\) oscillations. (a) Average single-cell traces of changes in fluorescence intensity of the cytosolic Ca\(^{2+}\) indicator Fluo-4 AM in response to glutamate excitation. CGNs pretreated with latrepirdine (0.1 nM for 24 h were loaded with Fluo-4 AM (3 µM) and mounted on the confocal microscope stage. Glutamate excitation (glutamate/glycine [100 µM/10 µM] for 10 min immediately followed by addition of MK 801 [2.5 µM]) was induced as indicated. Analysis was carried out using Metamorph software and average pixel intensity per population at each timepoint is shown. (b) Quantification of area under the Fluo-4 AM curve during glutamate excitation in prolonged latrepirdine-pretreated neurons. Vehicle: n = 30 cells; latrepirdine: (n = 45 cells). Data are shown as mean ± s.e.m. *P < 0.001 compared with vehicle-pretreated neurons that were glutamate treated. (c) Murine cortical neurons were cultivated on glass bottom dishes, incubated with 5 µM Fluo-4-AM for 45 min at 37 °C, washed, perfused with experimental buffer supplemented with 2 mM MgCl\(_2\) and placed on the heated stage of a LSM 510 microscope. Images were taken at 5 Hz, optical slice thickness set to 3.5 µM. The buffer was replaced with MgCl\(_2\)-free buffer at time 0 and either vehicle or latrepirdine (0.1 nM) was added after 120 s of imaging as indicated. Typical Fluo-4 kinetics are shown as change in fluorescence intensity divided by the mean overall fluorescence intensity (ΔF/F). (d) Quantification of Ca\(^{2+}\) spike frequency after MgCl\(_2\) washout, treatment with latrepirdine (0.1 mM, n = 176 cells) or (e) AICAR (0.1 mM) (n = 88 cells) addition followed by complete block using tetrodotoxin (TTX, 1 µM) (significant difference P < 0.05, paired t-test). (f) Quantification of changes of the Ca\(^{2+}\)-spiking activity due to addition of latrepirdine, AICAR, or vehicle (Control, n = 134 cells, latrepirdine, n = 176 cells, AICAR n = 88 cells). Kruskal–Wallis and subsequent Mann–Whitney tests showed a significant difference in latrepirdine and AICAR compared with control (*) but no significant difference in latrepirdine compared with AICAR (ns).
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degradation. Latrepirdine has gained significant interest as a novel class of therapeutic agents that target ‘mitochondria’, but has subsequently failed in clinical trials. We here demonstrate that latrepirdine is an activator of the energy sensor, AMPK, acting at surprisingly low, (sub-) nanomolar concentrations. We furthermore describe that the pharmacological activities of latrepirdine in primary neuron cultures include a pronounced effect on \(\Delta \psi_p\) and a strong inhibitory effect on neuronal excitability and glutamate-induced \(\text{Ca}^{2+}\) increases.

During conditions of increased energy demand, AMPK is activated as a protective response in an attempt to restore cellular homeostasis.\(^6\) Our findings that latrepirdine activates AMPK is in agreement with previous studies that demonstrated that latrepirdine improves neuronal energy metabolism\(^3\) and mitochondrial function.\(^2,22\) Supporting these findings, we demonstrate that latrepirdine triggers an increase in GLUT 3 translocation that was coupled with an increase in neuronal ATP levels. Latrepirdine has previously been shown to enhance cerebral glucose utilization in aged mice in vivo.\(^39\) We also observed that latrepirdine treatment led to a pronounced increase in mitochondrial uptake of the cationic dye, TMRR, suggestive of \(\Delta \psi_m\) hyperpolarization.\(^17\) This effect was abrogated in neurons in which AMPK\(\alpha\) expression was silenced, demonstrating the requirement of AMPK for the effect of latrepirdine on mitochondrial TMRR uptake. However, TMRR uptake into cells is determined by the Nerst potential across both plasma and mitochondrial membranes.\(^1,12,25\) Subsequent experiments indicated that latrepirdine also induced a strong \(\Delta \psi_p\) hyperpolarization. It is important to mention in this context that AMPK has previously been shown to hyperpolarize \(\Delta \psi_p\) through the phosphorylation of a voltage-sensitive potassium channel.\(^29\) Indeed, further quantitative analysis demonstrated that \(\Delta \psi_p\) hyperpolarization largely contributed to the latrepirdine-induced increase in TMRR uptake, precluding a pure ‘mitochondrial’ action of latrepirdine. As the plasma membrane Na\(^+\)/K\(^+\) ATPase uses at least 50% of neuronal ATP to maintain resting \(\Delta \psi_p\) a \(\Delta \psi_p\) hyperpolarization may also indirectly preserve cytosolic ATP expenditure, and thus may contribute to the increase in cellular ATP levels in response to latrepirdine treatment as observed in this and previous studies.

Our study also demonstrated that treatment with latrepirdine reduced glutamate-induced and ‘spontaneous’ cytosolic \(\text{Ca}^{2+}\) elevations, indicating that the latrepirdine-induced hyperpolarization of \(\Delta \psi_p\) correlated with decreased neuronal excitability. We noted a strong inhibition of glutamate-induced \(\text{Ca}^{2+}\) elevations following latrepirdine pretreatment, but not during acute treatment, suggesting that latrepirdine does not directly inhibit NMDA or other glutamate receptors. The inhibitory effect of latrepirdine pretreatment on glutamate-induced \(\text{Ca}^{2+}\) elevations could be due to plasma membrane potential hyperpolarization limiting NMDA receptor activation, or due to an improvement of neuronal ATP levels, enabling a faster removal of cytosolic \(\text{Ca}^{2+}\) via plasma membrane ATPases or the Na\(^+\)/K\(^+\) ATPase-driven Na\(^+\)/Ca\(^{2+}\) exchange. It is also possible that latrepirdine pretreatment alters NMDA receptor function or expression, however this requires further investigation. We also observed a potentially reduced spontaneous \(\text{Ca}^{2+}\) spiking in cultured cortical neurons, findings that are consistent with the pronounced hyperpolarizing effect of latrepirdine on \(\Delta \psi_p\). Latrepirdine’s inhibition of glutamate-induced \(\text{Ca}^{2+}\) elevations and neuronal excitability is also of interest in the context of the failed clinical trials of latrepirdine in AD patients. While NMDA receptor hyperactivity has been suggested to be associated with AD, it is likewise accepted that sufficient NMDA receptor activity requires to be maintained to exert a beneficial effect in AD patients.\(^31\)

While we observed a significant protective effect of prolonged latrepirdine pretreatment against glutamate excitotoxicity, acute pretreatment with latrepirdine failed to provide protection. Nor did acute pretreatment attenuate the glutamate-induced increase in cytosolic calcium, indicating that calcium influx may be the key signaling event that precipitates excitotoxic cell death. Prolonged pretreatment with latrepirdine activated AMPK, a kinase with both pro-survival,\(^10,42\) but also cell death-inducing activities.\(^28\) Our data demonstrate that there is a narrow range of latrepirdine concentrations that can exert a protective effect against excitotoxicity. This may reflect the moderate activation of AMPK within a pro-survival range, above which pro-death signaling occurs. We have recently shown that excessive or prolonged AMPK activation can lead to cell death through upregulation of pro-apoptotic BH3-only protein expression.\(^43,44\) McCullough et al.\(^46\) identified that continuous activation of AMPK increased neuronal injury during ischemia. Activation of AMPK has also been shown to potentiate neurodegeneration of striatal neurons in a mouse model of Huntington’s disease.\(^46\) On the other hand, AMPK activation has been shown to promote pro-survival signaling, and latrepirdine has recently been shown to stimulate autophagy and reduce the accumulation of \(\alpha\)-synuclein in vitro and in vivo.\(^47\) to enhance mTOR- and Atg5-dependent autophagy and to arrest progression of neuropathology in an AD mouse model.\(^48\) Our data suggest that AMPK activation by latrepirdine may underlie the reported effects of latrepirdine on autophagy-mediated clearance of protein aggregates in such disease models. Indeed, induction of autophagy through AMPK-activating compounds has been shown before to enhance the clearance of both soluble and aggregated forms of A\(\beta\) and tau proteins in vivo and in vitro.\(^49,50\) However, as AMPK may already be abnormally activated in symptomatic AD,\(^50\) effects of AMPK activators such as latrepirdine on AD pathogenesis may strongly depend on disease progression. The experimental paradigm employed in our study naturally differs from the chronic exposure paradigm used in earlier clinical trials. Nevertheless, our in vitro data carefully argue for a potentially beneficial effect of latrepirdine in early AD, rather than at an advanced disease stage. Likewise, latrepirdine may be effective in individuals at risk of developing neurodegenerative disorders when given pre-symptomatically, for example, in familial forms of neurodegenerative disorders.

AMPK is considered a key sensor of the cellular energy status. AMPK signaling regulates energy balance at the cellular, organ and whole-body level.\(^10\) Our findings that latrepirdine activates AMPK and that the activation of AMPK by latrepirdine requires the upstream kinases LKB1 and CaMKK\(\beta\), shed new light into the mechanism of action of latrepirdine. Knockdown of either LKB1 or CaMKK\(\beta\), the upstream kinases that activate AMPK, prevented the latrepirdine-induced increase in plasma membrane potential. This indicates latrepirdine may act upstream of both of these kinases to induce hyperpolarization. CaMKK\(\beta\) is thought to activate AMPK in response to increased levels of intracellular calcium concentration,\(^31\) whereas LKB1 is required for maintaining baseline AMPK phosphorylation levels.\(^51\) Although we show that latrepirdine reduced the amplitude of spontaneous calcium oscillations, we did not observe an increase in overall intracellular calcium per se on addition of latrepirdine. This suggests that the effects of latrepirdine on AMPK phosphorylation are independent of the elevation of calcium. Latrepirdine may result from latrepirdine activity at sites upstream of AMPK itself. In accordance with this hypothesis, treatment with latrepirdine progressively increased AMPK activity over 24 h, and hyperpolarization of \(\Delta \psi_p\), also continued to occur for the duration of the 4-h experiment. However, treatment with latrepirdine immediately attenuated \(\text{Ca}^{2+}\) oscillations, indicating that latrepirdine has direct effects on proteins involved in neuronal \(\text{Ca}^{2+}\) dynamics. Therefore, further molecular and structural studies will be required to determine precise targets and binding sites. Nevertheless, the observation that 0.1 \(\text{nM}\) latrepirdine activates AMPK indicates that this compound is one of the most potent pharmacological activators of AMPK described so far.
CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

39 Culmsee C, Monnig J, Kemp BE, Mattson MP. AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. J Mol Neurosci 2001; 17: 45–58.


Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)