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

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

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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 DisBAC2(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,1,12 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-SYSY neuroblastoma cells.5 Latrepirdine has also been shown to increase cellular ATP levels, to protect SH-SYSY cells against serum starvation-induced cell death, and to reduce Ca2+-induced swelling of rat brain mitochondria.5,9 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 20–25 min at 4 °C; 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 10^6 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 12-well plates at a density of 1 × 10^6 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 licenses 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, hysterecotomies of the uterus of pregnant female mice were performed using an abdominal injection of 40 mg kg^-1 pentobarbital (Dolethral) 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 neurons were then dissociated by gentle pipetting, and, after centrifugation (300g for 3 min), the medium containing trypsin was aspirated. Neocortical neurons were then resuspended in fresh plating medium (MEM containing 5% fetal calf serum, 5% horse serum, 100 U ml^-1 penicillin/streptomycin, 0.5 µM L-glutamine and 0.6% glucose). Cells were plated at 2 × 10^5 cells per cm² on poly-lysine-coated plates and incubated at 37 °C, 5% CO2. The plating medium was exchanged with 50% feeding medium (Neurobasal medium embryonic containing 100 U ml^-1 Pen/Strep, 2% B27 and 0.5 µM L-glutamine) and 50% plating medium with additional cytosine arabinofuranoside (600 µM).

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, 10 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 MgCl2-supplemented experimental buffer and returned to preconditioned media.

Determination of neuronal injury
Cells cultured on 24-well plates were stained alive with Hoechst 33258 (Sigma) at a final concentration of 1 µg ml^-1. Nuclear morphology was imaged using an Eclipse TE 300 inverted microscope (Nikon) and a × 20 dry objective. For each timepoint and treatment (glutamate/glycine, 100 µM/10 μM; latrepirdine 0.1–100 nM), cells were analyzed for apoptotic morphology in three subfields of each well (1000–2000 cells per well) in a blinded manner. All experiments were performed at least twice with similar results.

Automated epifluorescence analysis of Hoechst 33258 staining and propidium iodide (PI) uptake using the Cellomics high-content screening platform
To test the effects of a range of concentrations of latrepirdine against glutamate excitotoxicity on a single-cell level, we used a Cellomics ArrayScan VTI platform (Pittsburgh, PA, USA). The platform consists of an automated epifluorescence microscope connected to an automated plate reader with temperature (37 °C) and CO2 control. CGNs seeded at density 10^6 per well were grown on a 96-well plate for 7 days and either pretreated (for 24 h before glutamate treatment) or co-treated with a range of concentrations of latrepirdine (0.01 nM – 100 nM). For quantification of cell death, neurons were double stained with low concentrations of Hoechst 33342 (100 nM for 1 h before imaging) and PI (150 ng ml^-1 supplemented in culture media). Apoptotic and necrotic cells were determined based on the intensity of Hoechst staining and nuclear morphology. Hoechst-positive cells with large (or normal) nucleus and PI negative were considered as healthy neurons, Hoechst positive (high intensity) with condensed nuclei were considered as apoptotic and Hoechst and PI positive with large (or normal) were considered as necrotic. A × 10 dry objective was used and nine subfields within each well (5000–6000 cells) were imaged at 60-min intervals over 24 h. DynD concentration and image acquisition rate were optimized to reduce phototoxicity. A 120-W metal halide lamp was for activation of the fluorophores. PI was excited at 545–575 nm; emission was collected through a band pass of 590–625 nm. Hoechst was excited at 381–394 nm and emission light was collected through a 415–460 nm band pass filter. Images were registered using a Hamamatsu Orca AG CCD and digitized at 12-bit precision. Segmentation of cell nuclei was performed on the Hoechst channel using locally adaptive Otsu thresholding, implemented in Cell Profiler (http://www.cellprofiler.org/). Quantification of apoptotic, primary necrotic and healthy cells was executed using a CR&T classifier (validated by a human expert), with nuclear area and fluorescence intensity (Hoechst and PI, average, s.d., min and max) serving as the input.

shRNA and transfection of CGNs
Transfection of CGNs was performed at days in vitro 6 using the calcium-phosphate-based transfection method as previously described. Briefly, to produce the DNA/CaP coprecipitate, a mixture of CaCl2 solution, distilled H2O, DNA plasmid solution (equal of 3 µg DNA), and 2 × BBS (50 mM BES, pH 7.1 (N, N'-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na2HPO4) was used. The transfection mixture was added to the transfection medium (pH 7.65) in the culture dish. Cells were incubated in a humidified incubator (without CO2) at 36.5 °C until the formation of the DNA/CaP 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 si1/s2 (pFIV-AMPK-shRNA) and scrambled sequence (pFIV-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’-TGGTTGTCGTAGTCCTACGATGCTCTTG-3’ (Gene ID 25048, Origene) or camkkβ 5’-CCCTGGAATCTCCGACAGCAGATTAC-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.

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 bioluminescence detection kit (Promega, Southampton, UK) as per the manufacturer’s instructions. Luminescence was recorded using a Tescan GENios well plate reader (Männedorf, Switzerland) in luminescence mode. ATP content values were corrected for protein concentration determined using the Pierce BCA micro protein assay kit, and normalized to vehicle-treated control samples. The content of ATP was calculated by a concentration standard curve with ATP levels normalized to protein content in each sample and expressed as a percentage of the control.

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. 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, 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. 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,1,5,9,21,22 we next explored whether the protective effects of latrepirdine pretreatment against glutamate toxicity were related to changes in mitochondrial membrane potential (ΔΨm), 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.5 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 potential 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 mRNA levels of two transcription factors involved in mitochondrial biogenesis, the mitochondrial transcription factor A (tfrmA) and peroxisome proliferator-activated receptor γ coactivator 1α (pgc-1α), also remained unaltered in CGN cultures treated for 24 h with latrepirdine (Supplementary Figure 3C).

Latrepirdine hyperpolarizes the plasma membrane potential (ΔΨp)

As well as responding to changes in mitochondrial membrane potential (ΔΨm), TMRM fluorescence intensity is also affected by changes in plasma membrane potential (ΔΨp).11,17,23 To examine a possible contribution of plasma membrane potential (ΔΨp) changes to the increase in TMRM uptake, CGNs were loaded with the anionic, ΔΨp-sensitive probe DisBAC(2)(3) that is extruded from cells upon ΔΨp hyperpolarization.24 Interestingly, treatment with latrepirdine (0.1 nM) induced a significant decrease in DisBAC(3) fluorescence intensity, indicative of ΔΨp hyperpolarization (Figures 2c and d). Quantification of DisBAC(3) fluorescence intensities indicated a significant decrease after 90 min of latrepirdine exposure, and decreasing further up to 240 min after drug addition (Figure 2e). These results indicated that the increase in TMRM fluorescence intensity in response to latrepirdine (0.1 nM) may be attributable not only to changes in mitochondrial (ΔΨm) but also to changes in plasma membrane potential (ΔΨp).

To elucidate more precisely the contribution of plasma membrane potential changes to the TMRM signal kinetics, we fed ΔΨp changes, calculated from average DisBAC(3) fluorescence intensity changes (Figure 2f), into a modified Nernstian equation as described in Supplementary Materials (see also Ward et al.11). The resulting trace is shown in Figure 2g. This approach allowed us to correct TMRM kinetics for changes in ΔΨp kinetics (Figure 2h). This analysis showed that the increase in TMRM fluorescence intensity following treatment with latrepirdine was indeed attributable to changes in ΔΨp (Figure 2f).

Latrepirdine activates AMPK and affects neuronal bioenergetics

We have previously detected a direct link between increased TMRM uptake, pre-conditioning, and activation of an evolutionarily conserved metabolic sensor, AMPK.25,26,27 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...
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 Δψm 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 CaMKKβ...
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 mM) 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 depolarization,36 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 mM), 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 mM) 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
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 \(\mu M\)) 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 \(\beta\) amyloid itself by interfering with its synthesis, aggregation or...
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 Δψₘ and a strong inhibitory effect on neuronal excitability and glutamate-induced Ca²⁺ increases.

During conditions of increased energy demand, AMPK is activated as a protective response in an attempt to restore cellular homeostasis.⁴⁴ Our findings that latrepirdine activates AMPK is in agreement with previous studies that demonstrated that latrepirdine improves neuronal energy metabolism,¹⁵ and mitochondrial function.⁵,²² 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.⁴⁶ We also observed that latrepirdine treatment led to a pronounced increase in mitochondrial uptake of the cationic dye, TMRM, suggestive of hyperpolarization.¹⁷ This effect was abrogated in neurons in which AMPKα expression was silenced, demonstrating the requirement of AMPK for the effect of latrepirdine on mitochondrial TMRM uptake. However, TMRM uptake into cells is determined by the Nernst potential across both plasma and mitochondrial membranes.¹¹,²³ Subsequent experiments indicated that latrepirdine also induced a strong Δψₘ hyperpolarization. It is important to mention in this context that AMPK has previously been shown to hyperpolarize Δψₘ through the phosphorylation of a voltage-sensitive potassium channel.²⁹ Indeed, further quantitative analysis demonstrated that Δψₘ hyperpolarization largely contributed to the latrepirdine-induced increase in TMRM 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 Δψₘ, a Δψₘ 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 Ca²⁺ elevations, indicating that the latrepirdine-induced hyperpolarization of Δψₘ correlated with decreased neuronal excitability. We noted a strong inhibition of glutamate-induced Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ via plasma membrane ATPases or Na⁺/K⁺-ATPase-driven Na⁺/Ca²⁺ exchange. It is also possible that latrepirdine pretreatment alters NMDA receptor function or expression, however this requires further investigation. We also observed a potentiated reduced spontaneous Ca²⁺ spiking in cultured cortical neurons, findings that are compatible with the pronounced hyperpolarizing effect of latrepirdine on Δψₘ. Latrepirdine’s inhibition of glutamate-induced Ca²⁺ 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.¹²,⁴³,⁴⁴ 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 α-synuclein in vitro and in vivo.⁴⁷ to enhance mTOR- and Atg5-dependent autophagy and to arrest progression of neuroopathology in an AD mouse model.⁴⁶ 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 Aβ and tau proteins in vivo and in vitro.⁴⁸ 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 CaMKKβ, shed new light into the mechanism of action of latrepirdine. Knockdown of either LKB1 or CaMKKβ, 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. CaMKKbeta is thought to activate AMPK in response to increased levels of intracellular calcium concentration,⁵¹ whereas LKB1 is required for maintaining baseline AMPK phosphorylation levels.²⁸ 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 intracellular calcium. Latrepirdine AMPK 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 Δψₘ also continued to occur for the duration of the 4-h experiment. However, treatment with latrepirdine immediately attenuated Ca²⁺ oscillations, indicating that latrepirdine has direct effects on proteins involved in neuronal Ca²⁺ dynamics. Therefore, further molecular and structural studies will be required to determine precise targets and binding sites. Nevertheless, the observation that 0.1 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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