Chapter 6
SK channels differentially affect neurotoxicity in conditions of enhanced ER-mitochondrial coupling

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Abstract
Alterations in the strength and interface area of contact sites between the endoplasmic reticulum (ER) and mitochondria contributes to Ca\(^{2+}\) dysregulation and neuronal cell death, and has been implicated in the pathology of several neurodegenerative diseases. Weakening this physical linkage may reduce Ca\(^{2+}\) uptake into mitochondria, while boosting it may lead to mitochondrial Ca\(^{2+}\) overload and cell death. Small conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK) channels reduce mitochondrial respiration and attenuate oxidative stress-induced mitochondrial Ca\(^{2+}\) overload and mitochondrial superoxide formation, thereby protecting against mitochondrial dysfunction and neuronal cell death. In the present study, we enhanced ER-mitochondrial coupling and investigated the impact of SK channels on survival of neuronal HT22 cells in conditions of oxidative stress. Using genetically-encoded linkers, we show that mitochondrial respiration and the vulnerability of HT22 cells to oxidative stress was inversely linked to the strength of ER-mitochondrial contact points and the increase in mitochondrial Ca\(^{2+}\) uptake. Intriguingly, pharmacological activation of SK channels by CyPPA provided protection against glutamate-induced cell death while it potentiated auranofin-induced neurotoxicity and limited mitochondrial respiration in cells with increased ER-mitochondrial associations.

Oxidative cell death initiated by glutamate is elicited upon the specific inhibition of the glutamate/cystine antiporter while auranofin mediates cell death through inhibiting thioredoxin reductases, thereby directly inducing mitochondrial damage. The findings presented herein suggest that SK channel activation by CyPPA in conditions of enhanced ER-mitochondrial coupling diversely affected neuronal survival in the different paradigms of oxidative stress. Given that CyPPA still prevented glutamate toxicity highlights the therapeutic value for SK channel agonism in diseases associated with glutamate-mediated oxidative stress and mitochondrial dysfunction.

Introduction
Multiple research lines indicate that the etiology of neurodegenerative disorders such as Alzheimer’s disease (AD) or Parkinson’s disease (PD) are strongly associated with dysregulation of Ca\(^{2+}\) homeostasis and oxidative stress-induced neuronal damage\(^1\)–\(^8\). Altered Ca\(^{2+}\) homeostasis can lead to mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{m}}\)) overload, and subsequently to an impairment of mitochondrial metabolism and respiration\(^9\),\(^10\). However, under physiological conditions, [Ca\(^{2+}\)]\(_{\text{m}}\) stimulates oxidative phosphorylation (OXPHOS) and ATP production\(^11\),\(^12\). Close spatial interactions between the endoplasmic reticulum (ER) and mitochondria are essential for rapid and sustained [Ca\(^{2+}\)]\(_{\text{m}}\) uptake. These close contacts are established at the so called mitochondria-associated ER membrane (MAM), thereby facilitating Ca\(^{2+}\) transfer between ER and mitochondria through voltage-dependent anion channels (VDAC) on the outer mitochondrial membrane.
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(OMM) and ER-located inositol-1,4,5-trisphosphate receptors (IP₃R), physically connected by glucose-regulated protein 75 (GRP75)¹³⁻¹⁵. Mutations in MAM-associated proteins have been identified which either enhance or reduce ER-mitochondrial coupling (EMC), thereby leading to dysregulation of MAM interfaces and which have been associated with neurodegeneration as in AD and amyotrophic lateral sclerosis¹⁶⁻¹⁸.

In neuronal cells, activation of small conductance Ca²⁺-activated K⁺ (SK) channels regulated Ca²⁺ uptake and retention in the ER¹⁹, and controlled Ca²⁺ homeostasis and respiration of the mitochondria²⁰. Activation of SK channels in conditions of ER stress and glutamate-induced oxidative stress (oxytosis) preserved cell viability, and restored ER and mitochondrial function, respectively. Due to the fact that both organelles come into close contact at the MAM interface, we evaluated the ability of SK channels to confer protection following oxidative stress in conditions of enhanced EMC. We suggest that enhancing the organelle linkage accelerates mitochondrial Ca²⁺ uptake, thereby increasing the vulnerability of cells to undergo cell death, and we investigated whether SK channel activation will protect against mitochondrial damage and neuronal cell death.

Materials and methods

Cell culture

HT22 cells were cultured in Dulbecco’s modified Eagle Medium (DMEM; Sigma Aldrich, Munich, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Cölbe, Germany), 100U/mL penicillin, 100μg/mL streptomycin and 2mM L-glutamine (Invitrogen, Karlsruhe, Germany) at 37°C and 5% CO₂. Plasmid transfection was performed using the attractene transfection reagent according to the manufacturer’s fast-forward protocol (Qiagen, Hilden, Germany). HT22 cells were transfected with 1.2μg plasmid DNA and grown in a 6well plate for 48h followed by re-seeding into the appropriate plate format for subsequent experiments. HEK293T cells were transfected with 400ng plasmid DNA and grown in a white-walled 96well plate for 48h.

Cell viability

Cell viability was determined based on the metabolic activity using the MTT assay at a final concentration of 0.5g/L by incubation for 1h at 37 °C, followed by removal of the MTT and at least 1h incubation at -20 °C. After dissolving the resulting formazan in DMSO, the absorbance of each well was determined with the Synergy H1 Multi-Mode reader (Biotek, LA, USA) at 570nm and at 630nm. Alternatively, cell viability was monitored in real-time with cell impedance measurements, using the xCELLigence system (Roche Diagnostics, Penzberg, Germany). Cell impedance was normalized to the time of treatment (normalized cell index) which is defined as the starting point (t=0h) of the experiment.
**Visualization of ER-mitochondrial contacts in living cells**

To evaluate ER-mitochondrial contact formation upon transfection with TOM70-FKBP-mRFP and either ER-9xFRB-CFP (both linkers, depicted as EML) or ER-Flipper-GFP (depicted as FL) followed by the rapamycin-induced heterodimerization, widefield fluorescence microscopy (DeltaVision Elite) studies were performed. Transfected HT22 cells (2x10^5 cells/well) were grown on Ø25mm coverslips (Menzel-Gläser, Thermo Fisher, Landsmeer, The Netherlands). After image acquisition in untreated conditions, rapamycin (100nM) was applied, and images were taken again after 5min and 10min at 60x magnification, pixel size 6.5μmx6.5μm, 2560x2160 pixels, speed 400fps at 512x512 pixel. The software adopted for acquisition and integrated deconvolution was softWoRx (on Linux CentOS 6.3 platform). Image overlays were done using the ImageJ software.

**[Ca^{2+}]_m measurements**

HEK293T cells were transfected with wildtype and mutant mitochondrial GFP-aequorin (mtGA^wt, mtGA^mut) for 48h in a clear bottom, white-walled 96well plate (Greiner Bioscience, Frickenhausen, Germany), and the measurement was performed according to established protocols^{21}. MtGA constructs were reconstituted with native coelenterazine (Biotium, VWR Technologies, Darmstadt, Germany) in medium for 2h. Cells were washed with PBS followed by addition of 100μL internal buffer (140mM KCl, 1mM KH₂PO₄, 1mM MgCl₂, 10mM glucose, 0.1mM EGTA, 20mM HEPES, 8mM Na-succinate, 4mM Na-pyruvate,4mM MgATP). Ca^{2+} uptake was initiated by addition of 25-50mM CaCl₂ or 500μM carbachol in internal buffer supplemented with 2.5mM MgEDTA following background measurement for 5s. Final sensor saturation was performed by addition of lysis buffer (140mM KCl, 10mM CaCl₂, 1% TritonX100). Luminescence was recorded using the FluoStar OPTIMA plate reader (BMG Labtech, Offenbach, Germany). Each condition was measured at least in triplicate, and Ca^{2+} uptake was calculated as the total luminescent counts per second (L) from the time of stimulation (t=5sec) until the time of final cell lysis (t=30sec). Values were normalized to the initial value. Alternatively, changes in mitochondrial Ca^{2+} load were determined in HT22 cells by flow cytometry using the dye rhodamine-2-acetoxy methane ester (Invitrogen, Karlsruhe, Germany). Cells were harvested and incubated with 2μM dye in DMEM without serum for 30min followed by incubation in DMEM for 30min at room temperature in the dark. The fluorescence was excited at 552nm and detected at 581nm using the Guava Easy Cite 6-2L system (Merck Millipore, Darmstadt, Germany). Data were recorded from 1x10^4 cells in triplicate per condition.
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Fractionation of the mitochondria-associated membrane
Mitochondria-associated ER membranes (MAM) were fractionated according to established protocols. In brief, cells were collected homogenized using Teflon pestles. Crude mitochondrial fractions (CM) were obtained by centrifugation at 7,000xg for 10min. MAM and pure mitochondrial fractions (PM) were purified from the CM fractions using a Percoll gradient and high-speed centrifugation at 95,000xg for 30min. MAM were collected and again centrifuged at 100,000xg for 1h, and PM fractions were obtained after centrifugation at 6,300xg for 10min. The purity of the obtained fractions as well as SK2 expression was determined by Western blot using antibodies for rabbit polyclonal anti-SK2 (Abcam, Cambridge, UK), mouse monoclonal anti-TIM23 (BD Bioscience, Heidelberg, Germany), mouse monoclonal anti-OPA1 (BD), goat polyclonal anti-Calregulin (Santa Cruz, Dallas, TX, USA), rabbit polyclonal anti-IP3R (Abcam), mouse monoclonal anti-α-tubulin (Sigma Aldrich, Munich, Germany) and goat polyclonal anti-VDAC1 (Santa Cruz) following application of the respective secondary antibodies. Protein expression using the Chemidoc software (Bio-Rad, Hercules, CA, USA) and quantified using the Quantity One software (Bio-Rad).

Mitochondrial superoxide (ROS) formation
Mitochondrial superoxide (ROS) formation was assessed by the MitoSOX dye (Invitrogen, Karlsruhe, Germany). Cells were incubated with 2.5μM MitoSOX dye for 30min at 37°C and harvested afterwards. Fluorescence was excited at 488nm and detected at 690/50nm. Data were recorded from 1x10^4 cells in triplicate per condition.

Measurement of the mitochondrial membrane potential (Δψm)
Loss of the mitochondrial membrane potential (Δψm) was analyzed by staining with tetramethylrhodamine-ethyl ester (TMRE; Invitrogen, Karlsruhe, Germany) dye. Cells were harvested and incubated 30min with 0.2μM TMRE at 37°C. TMRE fluorescence was excited at 488nm and detected at 690/50nm. Data were recorded from 1x10^4 cells in triplicate per condition.

Seahorse XF analysis
HT22 cells were transfected with mitochondrial linkers and either ER-Flipper control plasmid or the ER linker in Seahorse XF 96well plates (Seahorse Biosystems, Agilent Technologies, Waldbronn, Germany). Before the measurement, the medium was removed and replaced by 180μL assay medium containing 4.5g/L glucose, 2mM L-glutamine, 1mM pyruvate (pH7.35) for 1h at 37 °C. Using the Seahorse XF Biosystem, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were analyzed. Three baseline measurements (3min mix, 0min delay, 3min measure = 3/0/3) were recorded followed by injection of rapamycin (50-200nM) or
medium and measurement for 2h (3/5/3). Following EMC, mitochondrial function
was assessed by injection of 3μM oligomycin (3/0/3), 50μM DNP (3/0/3), and
100nM rotenone, 1μM antimycin A and 50mM 2-DG (3/0/3). For the auranofin
experiment, auranofin (-/+CyPPA) and DNP were injected in port A for 6h (3/10/3).
After injection of each compound, OCR and ECAR were determined.

Statistical analysis
Statistical significance was assessed using the unpaired Student’s t-test or ANOVA
and Scheffé’s test for multiple comparisons, unless otherwise stated. P values
indicating statistically significant differences between the mean values are defined
as follows: *p<0.05, **p<0.01 and ***p<0.001.

Results
Inducing a physical linkage between the ER and mitochondria
ER and mitochondria temporarily and dynamically form close contacts at the MAM,
thereby allowing for the exchange of proteins, lipids and ions between the
organelles23. To study the structure and function of ER-mitochondrial connections,
we used genetically encoded bifunctional linkers24 that both tighten the contact
between ER and mitochondria, and expand the MAM interface area. These linkers
consist of OMM-FKBP12-mRFP and ER targeted-FRB-CFP fusion proteins which
heterodimerize in response to a very low concentration of 100nM rapamycin.
Upon application of rapamycin, we observed a time-dependent co-localization of
CFP-tagged ER with RFP-tagged mitochondria indicating the induction of EMC,
reaching full co-localization after 10min following rapamycin treatment (Figure 1). In
contrast, a GFP-tagged ER-Flipper control plasmid (FL), co-transfected with OMM-
FKBP12-mRFP, failed to co-localize with RFP-tagged mitochondria following
rapamycin treatment, which confirmed the specificity of the ER-mitochondrial
linkers (Supplementary figure 1).
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Figure 1. Inducing ER-mitochondrial associations in neuronal HT22 cells. Representative fluorescent images of an individual HT22 cell expressing ER-mitochondrial linkers (EML) prior to and 10min following rapamycin (100nM) addition. Fluorescent traces are shown individually (left and middle panel) and as an overlay of both fluorescent channels (right panel). Cyan: ER-CFP-9xFRB, red: TOM70-FKB12-RFP.

Strengthening EMC potentiates neuronal cell death
To study the consequences of increased EMC on neuronal cell death, we induced oxidative stress and mitochondrial damage in neuronal HT22 cells by glutamate (oxytosis)\textsuperscript{25}, and by the thioredoxin reductase inhibitor auranofin (1-Thio-β-D-glucopyranose atorriethylphosphine gold-2,3,4,6-tetraacetate)\textsuperscript{26,27}. Auranofin lowers the mitochondrial membrane potential and leads to formation of the mitochondrial permeability transition pore (mPTP)\textsuperscript{26}, thereby inducing cell death.

In HT22 cells, cell death was detectable approximately 12h following glutamate exposure with maximal damage detected after 16-18h, as assessed using the MTT assay (Supplementary figure 2A). In contrast, auranofin-induced cell death was detectable already after ~2h reaching its maximal effect at 6-8h (Supplementary figure 2B). By pharmacologically inducing heterodimerization of the linkers through the addition of rapamycin, we now investigated the consequences of increased EMC on cell viability using the MTT assay. As expected, glutamate reduced cell...
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viability in ER-transfected control and in linker-transfected cells. However, in linker-transfected cells, cell death was enhanced following rapamycin-induced linker heterodimerization (Figure 2A). In line with oxytosis, also toxic effects of auranofin on cell viability were potentiated in the presence of rapamycin (Figure 2B). Since rapamycin induces mammalian target of rapamycin (mTOR) signaling and could thereby induce autophagy, we also investigated effects of rapamycin on cell viability. Rapamycin alone neither affected glutamate nor auranofin toxicity (Supplementary figure 2C-D), and did not change proliferation of HT22 cells transfected with ER or linkers (Supplementary figure 2E). This indicates that rapamycin at low concentrations used in our study did not induce mTOR-dependent autophagy.

Figure 2. Enhancing EMC potentiates neuronal cell death.
(A-B) MTT Assay in HT22 cells transfected with ER-CFP (ER) or ER-FRB-CFP and TOM70-FKBP12-RFP (EML) following treatment with (A) glutamate (4mM) and rapamycin (100nM) or (B) auranofin (1.5μM) and rapamycin (30min pre-treatment, 100nM). Data are presented as mean ± SD, n=6, Student’s t-test ***p<0.0001 compared to glutamate in ER control.

Mitochondrial respiration is impaired by enhancing EMC
It has been reported that Ca²⁺ transfer to the mitochondria along the MAM interface is determined by EMC, and thereby modulates mitochondrial respiration and function.

Here, we determined the effects of strengthening EMC on mitochondrial bioenergetics by assessing mitochondrial respiration (OXPHOS) and glycolysis in HT22 cells using the Seahorse extracellular flux analysis. Analysis of the OXPHOS activity, assessed as the O₂ consumption rate (OCR), revealed that strengthening EMC did not influence basal OCR in linker-transfected cells as compared to control (FL) cells (Figure 3A and supplementary figure 3A). However, following uncoupling with dinitrophenol (DNP), the OCR was lower in linker-transfected as compared to FL-transfected cells. To evaluate mitochondrial function, OCR values were quantified following the application of rapamycin, oligomycin, DNP, and antimycin A/rotenone/2DG (Figure 3B-E). In linker-transfected cells, rapamycin application over a period of 2h did not reduce basal OXPHOS or ATP production, as assessed by oligomycin treatment. Interestingly, uncoupling of the respiratory chain using
DNP, indicating maximal OXPHOS activity, was decreased in linker-transfected cells in the presence of rapamycin compared to FL-transfected cells. Final inhibition of mitochondrial complexes I (rotenone) and III (antimycin A) and glycolysis (2DG) resulted in a reduction of the OCR to a similar extent in all cells. Regarding the extracellular acidification rate (ECAR), an indicator of glycolysis, there was no change in either FL- or linker-transfected cells following rapamycin application (Supplementary figure 3B). These results suggest that enhanced EMC reduced mitochondrial bioenergetics by attenuating the respiratory capacity and maximal respiration.

Figure 3. Augmenting EMC impairs mitochondrial bioenergetics in HT22 cells.
(A) Representative measurement of the O₂ consumption rate (OCR) in HT22 cells transfected with TOM70-FKBP12-mRFP and Flipper control plasmid (named FL) or together with CFP-FRB-ER (named EML) following the application of 100nM rapamycin. A:rapamycin, B:oligomycin, C:DNP, D:antimycin A, rotenone, 2DG. Data are presented as mean ± SD, n=3-6 per condition. Arrows indicate analyzed time points. (B-E) Quantification of OCR values at the indicated time points following the application of (B) rapamycin, (C) oligomycin, (D) DNP and (E) antimycin A, rotenone, 2DG. Student’s t-test, ***p<0.0001. (F) Representative OCR measurement following the application of 1.5μM auranofin (Aura). A:auranofin, B:oligomycin, C:DNP. Data are presented as mean ± SD, n=6-8 per condition. Arrows indicate analyzed time points. (G-I) Quantification of OCR values at the indicated time points following the application of (G) auranofin, (H) oligomycin and (I) DNP. Student’s t-test, ***p<0.0001.

Glutamate-induced mitochondrial dysfunction is characterized, in parts, by attenuated OXPHOS activity30, however, the effect of auranofin on mitochondrial respiration is still unclear. As shown in our previous experiments, glutamate induced cell death within approximately ~16h, while auranofin-induced cell death was detectable few hours following the onset of treatment. Therefore, we performed a real-time analysis of OXPHOS and glycolysis for a period of 6h
SK channels regulate neurotoxicity during enhanced ER-mitochondrial coupling following auranofin application. Analysis of the OCR revealed a decrease in basal respiration following auranofin application (Figure 3F). Subsequent oligomycin treatment to assess ATP production, revealed that auranofin treatment led to an impairment of energy production. Uncoupling with DNP induced maximal OXPHOS activity in untreated cells while there was no increase in auranofin-treated cells. Final application of antimycin A/rotenone/2DG to block mitochondrial respiration and glycolysis resulted in a strong decrease in OCR values to a similar extent in untreated and auranofin-treated cells. Quantification of OCR values revealed a decrease in basal respiration and maximal uncoupled respiration (Figure 3G-I). Interestingly, auranofin application also reduced glycolysis, measured and quantified as the ECAR (Supplementary figure 3C). These results suggest that auranofin attenuated the overall energy metabolism by inhibiting maximal respiration and decreasing the glycolytic activity.

To rule out the possibility that auranofin-induced toxicity was mediated by uncoupling the respiratory chain, we included DNP as an uncoupling compound. Unlike auranofin, DNP increased mitochondrial respiration and glycolysis, as assessed by OCR and ECAR values, immediately after application and maintained these high values over the full period of 6h (Supplementary figure 3D-E). OCR and ECAR values in DNP-treated cells following ATP synthase inhibition, uncoupling and final blockage of respiration and glycolysis were similar to untreated control cells. These findings indicate that auranofin did not act by uncoupling the respiratory chain, rather it attenuated respiration and reduced glycolysis.

**Figure 4.** EMC strengthening by rapamycin does not further promote the auranofin-mediated reduction of maximal uncoupled respiration or the effect on glycolysis.

(A) Representative OCR measurement of HT22 cells transfected with the ER-mitochondrial linkers (EML) following the application of 100nM rapamycin and 1.5μM auranofin. A:auranofin±rapamycin, B:oligomycin, C:DNP, D:antimycin A, rotenone, 2DG. Data are presented as mean±SD, n=6-8 per condition. Arrow indicates analyzed time point. (B) Quantification of OCR values at the indicated time point following the application of DNP. Data are presented as mean±SD, n=6-8 per condition, Student's t-test **p<0.01. (C) Representative ECAR measurement of HT22 cells transfected with the ER-mitochondrial linkers (EML) following the application of 100nM rapamycin and 1.5μM auranofin. A:auranofin±rapamycin, B:oligomycin, C:DNP, D:antimycin A, rotenone, 2DG. Data are presented as mean±SD, n=6-8 per condition.
In our previous experiments, we observed that auranofin-mediated cell death was enhanced in HT22 cells where EMC was amplified by transfection of the genetically-encoded linkers and upon rapamycin addition. Thus, we investigated if strengthening EMC had an additive effect on the auranofin-dependent reduction in mitochondrial uncoupled respiration. To this end, we applied auranofin to HT22 cells transfected with the linker plasmids (EML) in the presence or absence of rapamycin. However, although both, EMC and auranofin treatment reduced mitochondrial respiration, the combination of these treatments did not further reduce OCR values (Figure 4A-B) or ECAR values (Figure 4C), suggesting that auranofin-induced cell death may rely on additional pathways such as alterations of mitochondrial calcium homeostasis.

**Mitochondrial Ca\(^{2+}\) uptake is increased by EMC**

The rapamycin-induced heterodimerization of the genetically-encoded linkers tightens ER-mitochondrial contact points and increases the surface of the MAM interface\(^{24}\) which might amplify Ca\(^{2+}\) transfer into the mitochondrial matrix. To this end, we assessed [Ca\(^{2+}\)]\(_m\) uptake in the presence or absence of the linkers and their pharmacological inducer rapamycin. Glutamate (Figure 5A) and auranofin (Figure 5B) initiated [Ca\(^{2+}\)]\(_m\) influx as assessed using Rho2 fluorescence and flow cytometry. However, glutamate- and auranofin-induced cell death and associated [Ca\(^{2+}\)]\(_m\) overload is a long-term effect occurring in the range of 16h (glutamate) and 6h (auranofin). In order to study fast changes in [Ca\(^{2+}\)]\(_m\) uptake induced by strengthening EMC, we utilized a system to perform real-time [Ca\(^{2+}\)]\(_m\) uptake measurements in HEK293T cells transfected with the linkers and a mitochondrial GFP-aequorin\(^{21}\). Up to 80% of HEK293T cells on average were transfected with the wildtype (mtGA\(^{wt}\)) or a Ca\(^{2+}\)-binding deficient mutant aequorin version (mtGA\(^{mut}\)) serving as an internal control (Supplementary figure 4A). Calibration of the aequorin sensor revealed a dose-dependent increase in the luminescence (analyzed as counts per second) with increasing CaCl\(_2\) concentrations (Supplementary figure 4B). Stimulation of HEK293T cells transfected with the linkers and mtGA\(^{wt}\) with 50mM CaCl\(_2\) induced [Ca\(^{2+}\)]\(_m\) uptake as shown by an increase in the luminescence signal (Figure 5C) and in the total luminescence (sum of L; Figure 5D) compared to cells stimulated with control solution.
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Importantly, stimulation of linker-transfected HEK293T cells which were pre-treated with 100nM rapamycin during sensor reconstitution, resulted in higher [Ca^{2+}]_m uptake compared to control and untreated cells. [Ca^{2+}]_m uptake in cells transfected with the Ca^{2+}-binding deficient mutant (mtGA^{mut}) was unchanged, indicating the specific binding of Ca^{2+} to the mtGA^{wt} construct (Figure 5E). The treatment of transfected HEK293T cells with rapamycin did not affect cell viability as measured by the MTT Assay (Supplementary figure 4C). Taken together, we show that specifically [Ca^{2+}]_m influx was specifically amplified in cells where EMC was increased.

Small conductance Ca^{2+}-activated K^+ (SK) channels alter cell survival in conditions of enhanced EMC

Small conductance Ca^{2+}-activated K^+ (SK) channels have been identified in the ER membrane^{19} as well as in the IMM^{20} of neuronal cells where they contribute to cell survival in conditions of stress.

Here, we investigated the effect of pharmacological SK channel activation on mitochondrial function, and further on cell survival in conditions of strengthened EMC in neuronal HT22 cells. We identified SK channel expression in subcellular
fractions of the MAM as well as mitochondria, and confirmed the purity of the isolated MAM compartment by analysis of the corresponding ER, mitochondrial and MAM marker proteins (Figure 6A).

SK channel activation with the SK2/3 channel activator CyPPA\textsuperscript{31} reduced mitochondrial respiration which preconditioned mitochondria against following injuries\textsuperscript{20}. In our study, we found that prolonged exposure of HT22 cells to CyPPA dose-dependently increased mitochondrial superoxides (Figure 6B) and depolarized the mitochondrial membrane (Figure 6C), supporting the concept that mitochondrial preconditioning might be involved in the molecular mechanism of CyPPA-mediated protection.

In order to assess the effects of SK channel activation in conditions of strengthened EMC on neuronal toxicity induced by oxytosis or auranofin, we treated linker-transfected HT22 cells with rapamycin and challenged them with either glutamate or auranofin, in the presence or absence of CyPPA. To rule out the possibility that CyPPA impaired cell growth under basal conditions, we treated HT22 cells transfected with Flipper control or linkers with CyPPA, and did not observe a change in cell viability as assessed by cellular impedance measurements (Supplementary figure 5). In agreement with our previous study\textsuperscript{20}, CyPPA protected against oxytosis even in conditions of strengthened EMC (Figure 6D). Interestingly, SK channel activation further potentiated neuronal toxicity induced by auranofin rather than providing protection (Figure 6E). Analysis of metabolic flux measurements revealed that co-treatment of HT22 cells with auranofin and CyPPA slightly accelerated the auranofin-induced decrease in OXPHOS and particularly the decrease in glycolysis (Figure 7A-B). Quantification of the OCR revealed that CyPPA lowered basal respiration and ATP production following auranofin treatment which resulted in blockage of maximal respiration (Figure 7C-E). Although the ECAR values at the indicated time point were not further reduced in the presence of auranofin and CyPPA (Figure 7F), the auranofin-mediated decrease in glycolysis was strongly accelerated in the presence of CyPPA (Figure 7B). These results indicate that CyPPA had differential effects on neuronal cell death, and we suggest that this is due to the fact that the pathway leading to mitochondrial dysfunction is different between glutamate (oxytosis cascade) and auranofin (TRXR inhibition).
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Figure 6. SK channels are expressed in mitochondria-associated membranes and differentially affect neuronal toxicity in conditions of enhanced EMC.
(A) Subcellular fractionation of mitochondria-associated membranes from HT22 cells and detection of SK2 channels by Western blot. SK2 channel expression in the MAM fraction is assessed using SK2-specific antibodies. IP₃R, Calregulin, Tubulin, VDAC1 and TIM23 are used as fraction markers. HG: homogenate, CM: crude mitochondria, PM: pure mitochondria, MAM: mitochondria-associated membrane. (B-C) Mitochondrial superoxides and the mitochondrial membrane potential was assessed following pharmacological SK channel activation by CyPPA (10, 25, 50μM) for 24h by (B) MitoSOX staining, and (C) TMRE staining. CCCP was used as a positive control for TMRE staining. Data are shown as mean ± SD, n=3, **p<0.01, ***p<0.0001. (D-E) MTT Assay assessing cell death in HT22 cells transfected with ER-mitochondrial linkers following treatment with (D) glutamate (4mM) or (E) auranofin (1.5μM) in the presence or absence of rapamycin (100nM) and CyPPA (10μM). Data are presented as mean ± SD, n=6, ***p<0.0001.
Figure 7. CyPPA promotes auranofin-induced mitochondrial dysfunction.
(A-B) Representative measurement of \(O_2\) consumption (OCR; A) and glycolysis (ECAR; B) in HT22 cells treated with auranofin (1.5\(\mu\)M) in the presence or absence of CyPPA (10\(\mu\)M). A: rapamycin, B: oligomycin, C: DNP, D: antimycin A, rotenone, 2DG. Data are presented as mean ± SD, \(n=3-6\) per condition, unpaired Student’s t-test, \(\ast p<0.05\), \(\# p<0.01\) compared to auranofin. Arrows indicate analyzed time points. (C-E) Quantification of \(O_2\) consumption using extracellular flux analysis at the indicated time points following the injection of (C) auranofin±CyPPA, (D) oligomycin and (E) dinitrophenol (DNP). Data are presented as mean ± SD, \(n=5-8\), unpaired Student’s t-test \(**<0.01\), ***\(p<0.001\) compared to untreated control, \#p<0.05 compared to auranofin. (F) Quantification of glycolysis using extracellular flux analysis following the application of auranofin±CyPPA at the indicated time point. Data are presented as mean ± SD, \(n=5-8\), unpaired Student’s t-test \(\ast p<0.05\), \(**<0.01\) compared to untreated control.

Differential effect of SK channel overexpression on neuronal toxicity mediated by EMC

Recently, we reported that the overexpression and activation of SK channels in the IMM protected neuronal cells from oxidative glutamate toxicity\(^{20}\). Here, we showed that SK channel activation restored cell viability in conditions of strengthened EMC and oxytosis while cell death was potentiated during auranofin toxicity.

To this end, we challenged linker-transfected HT22 cells co-transfected with either wildtype SK2 channels (SK2) or mitochondria-targeted SK2 channels (mitoSK2) with glutamate in the presence of rapamycin to induce linkage formation. Overexpression of either SK2 (Figure 8A) or mitoSK2 (Figure 8B) combined with CyPPA preserved cell viability following oxytosis. Cell survival was enhanced in cells transfected with SK2 or mitoSK2 compared to the GFP/mitoGFP control.
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plasmids, indicating that overexpression of SK2 channels increased CyPPA-mediated protection against glutamate toxicity.

As we observed that CyPPA further potentiated auranofin-induced neuronal cell death in conditions of strengthened EMC, we investigated the effects of SK channel overexpression on auranofin-induced toxicity. HT22 cells co-transfected with either GFP/SK2 or mitoGFP/mitoSK2 in the presence of the linkers were challenged with auranofin plus rapamycin, and different concentrations of CyPPA. Cell viability analysis revealed that overexpression of either wildtype or mitochondria-targeted SK2 channels did not influence cell death induced by auranofin compared to the corresponding GFP/mitoGFP control plasmids (Figure 8C-D). Co-treatment with
CyPPA dose-dependently increased cell death in cells transfected with SK2 and mitoSK2 following auranofin treatment, yet this was not observed in GFP/mitoGFP-transfected cells. These findings indicate that activation of the transfected SK channels might enhance the toxic effects of auranofin on cell viability.

**Discussion**

Recent advances in the knowledge on the pathology of neurodegenerative diseases revealed a strong impact of EMC, MAM formation and associated mitochondrial Ca$^{2+}$ transfer on mitochondrial metabolism and neuronal cell survival, as Ca$^{2+}$-dependent processes are activated to drive mitochondrial respiration and the generation of ATP$^{32-34}$. Modulating the ER-mitochondrial interface, thereby controlling mitochondrial function, has therefore emerged as a strategy to prevent the pathological death of neurons$^{35,36}$. SK channels which control neuronal excitability and neuronal firing have been linked to neuroprotection in different disease paradigms *in vitro* and *in vivo*. In the present study, we provide evidence that increasing EMC in neuronal HT22 cells potentiated cell death in models of oxytosis and auranofin toxicity. In these paradigms, SK channel activation differentially affected neuronal survival as glutamate-induced cell death was prevented while auranofin toxicity was enhanced.

ER and mitochondria are physically linked mainly by a trimeric complex consisting of ER-bound IP$_3$R, VDAC on the OMM and the molecular chaperone GRP75$^{14,37,38}$. In addition, the mitochondrial tethering proteins mitofusin 1 and 2 (MFN1/2) heterodimerize$^{39,40}$, and mitochondrial fission 1 (FIS1) interacts with the ER membrane-integral protein BAP3$^{41}$ to establish this physical connection. Recently, it has been shown that ER-mitochondrial contact points can be formed using drug-inducible fluorescent organelle linkers at the ER membrane and OMM which bring ER and mitochondria into close proximity (<10nm), thereby facilitating Ca$^{2+}$ uptake into mitochondria$^{24}$. These linkers are based on dimerization partners from the mTOR signaling pathway which heterodimerize in the presence of rapamycin. In our current study, we expressed these drug-inducible ER-mitochondrial linkers in immortalized hippocampal HT22 cells, and confirmed the rearrangement of ER and mitochondria into the MAM interface using live-cell imaging. Similar to Csordás and colleagues$^{24}$, we observed full co-localization of the CFP-tagged ER plasmid and RFP-tagged mitochondrial plasmids, indicating a pronounced increase in EMC within minutes following addition of rapamycin.

Contact points between the ER and mitochondria determine organelle function through the transfer of proteins and Ca$^{2+}$ ions. Stress-induced Ca$^{2+}$ dysregulation in both organelles is detrimental *per se*, and ER stress was also shown to impair mitochondrial function and to induce cell death$^{42,43}$. In HT22 cells, brefeldin A triggered ER stress by inhibiting protein trafficking in the endomembrane system, thereby leading to [Ca$^{2+}$]$_{ER}$ dysregulation$^{19}$. Interestingly, subsequent cell death was
mediated by caspase activation, yet independent of the mitochondrial cell death axis. In contrast, oxytosis in HT22 involved oxidative stress and mitochondrial dysfunction through $[\text{Ca}^{2+}]_{\text{m}}$ overload and the accumulation of mitochondrial reactive oxygen species (ROS)$^{20}$ without affecting the ER. In this study, we sought to investigate mitochondrial function and cell survival following stress induction in HT22 cells expressing the bifunctional linkers to establish a stable platform for increased ER-mitochondrial interactions. We found that glutamate-induced cell death was increased in linker-transfected cells compared to control cells indicating that the detrimental effects of glutamate on mitochondrial integrity were further enhanced when EMC was increased.

In addition to the well-known paradigm of oxidative glutamate toxicity, we confirmed our findings in another cell death model using an inhibitor of the thioredoxin/thioredoxin reductase (TRX/TRXR) system$^{44,45}$. Here, we applied the gold(I)-phosphine derivative auranofin$^{27}$ to block TRXR, thereby impairing the generation of antioxidants which lowers the mitochondrial membrane potential, supports the formation of the mPTP and induces $\text{Ca}^{2+}$-dependent cell death$^{26}$. Following auranofin treatment, we also observed potentiated cell death in conditions of increased EMC.

The pathways underlying auranofin toxicity and oxytosis share $[\text{Ca}^{2+}]_{\text{m}}$ overload as well as ROS formation$^{46,47}$ as critical parameters. Since ROS and $\text{Ca}^{2+}$ can result from similar intracellular processes in either the cytosol or the mitochondria, an interplay between these parameters may potentiate the toxic outcome following cell death induction. For instance, ER oxidase 1α (ERO1α) activation increased IP$_3$R-dependent ER-mitochondrial $\text{Ca}^{2+}$ transfer by preventing the inhibitory association of the ER chaperone ERP44 with IP$_3$R, a process that was accompanied by H$_2$O$_2$ generation$^{48}$. Further, H$_2$O$_2$ generation in the mitochondrial matrix following ER-mitochondrial $\text{Ca}^{2+}$ transfer was shown to induce a positive feedback loop on IP$_3$R opening which amplified ER-$\text{Ca}^{2+}$ release$^{49}$. Thus, we suggest that introducing a stable interaction between ER and mitochondria served as a platform for sustained ER-mitochondrial $\text{Ca}^{2+}$ transfer that amplified $\text{Ca}^{2+}$-dependent cell death and may have enhanced oxidative stress.

Several lines of evidence suggest that EMC is essential to maintain the cellular bioenergetic state. In particular, this was shown for $\text{Ca}^{2+}$ release through IP$_3$R$^{29,50,51}$ and the interaction of MFN1 and MFN2$^{52}$ at the MAM interface. Bravo and colleagues$^{50}$ reported that ER stress led to an initial increase in EMC accompanied by an increase in OXPHOS activity before mitochondrial respiration was attenuated at the onset of cell death (20h). In our study, we observed a decrease, rather than an increase, in mitochondrial respiration upon establishing a fast and stable connection between ER and mitochondria. In particular, extracellular flux analysis revealed that enhanced EMC did not increase basal respiration or glycolysis, yet fully blocked uncoupled respiration. EMC induced by the bifunctional linkers was a
rather fast and highly efficient process occurring within minutes compared to the ER stress-induced increase in EMC (1-4h). Thus, we propose that the mitochondrial respiration was constantly decreased without an initial increase due to the speed and strength of the artificial linkage induced by the linkers. Matching the observations that mitochondrial respiration was impaired while there was no change in cell survival or in glycolysis following increased EMC further suggests that energy supply to maintain cell survival might have been exclusively covered by the glycolytic pathway.

Recently, it was shown that thioredoxin-related transmembrane protein 1 (TMX1) in the ER membrane was enriched in the MAM interface, a process blocking the activity of the sarcoplasmic/endoplasmic Ca\(^{2+}\) ATPase (SERCA) and enhancing mitochondrial respiration by increasing Ca\(^{2+}\) release from the ER. Further, in cancer cells reduced TMX1 levels were associated with decreased EMC and a metabolic shift from respiration towards glycolysis\(^5\). Similarly, treatment of immortalized dopaminergic N27 cells with auranofin or mitochondrial \(\text{TRXR2}\) silencing in these cells was found to decrease maximal mitochondrial respiration. In line with these findings, we show that auranofin-mediated TRXR inhibition blocked maximal respiration and attenuated glycolysis suggesting that auranofin impaired energy production from both pathways, mitochondrial respiration and glycolysis.

ER-mitochondrial Ca\(^{2+}\) transfer and [Ca\(^{2+}\)]\(_{\text{m}}\) load are critical factors to maintain mitochondrial function, and alterations in these processes promotes mitochondrial dysfunction. For instance, enhanced EMC provoked by the saturated fatty acid palmitate disrupted intracellular Ca\(^{2+}\) homeostasis, thereby promoting hepatotoxicity\(^5\), and enhanced VDAC-mediated [Ca\(^{2+}\)]\(_{\text{m}}\) influx amplified cell death induction in HeLa cells\(^13,55\). In HT22 cells, [Ca\(^{2+}\)]\(_{\text{m}}\) uptake induced by glutamate and auranofin occurred slow and at advanced stages following cell death induction. To detect rapid changes in [Ca\(^{2+}\)]\(_{\text{m}}\) induced by strengthened EMC, we performed real-time [Ca\(^{2+}\)]\(_{\text{m}}\) uptake measurements following the stimulation with CaCl\(_{2}\) in HEK293T cells transfected with the linkers and a mitochondria-targeted GFP-aequorin\(^21\). We found that rapamycin-induced EMC formation in HEK293T cells evoked an increase in [Ca\(^{2+}\)]\(_{\text{m}}\) uptake which was absent in cells stimulated with a solvent solution. As rapamycin treatment in the range of the applied concentrations did not reduce cell viability, this rise in [Ca\(^{2+}\)]\(_{\text{m}}\) was independent of cell death induction. Our results confirm that the use of the linkers specifically enhanced the transfer of Ca\(^{2+}\) from ER to mitochondria at the MAM interface.

SK channels which are involved in controlling synaptic plasticity and neuronal firing, preserve cell survival in multiple disease paradigms by regulating Ca\(^{2+}\) signaling and mitochondrial function\(^56,57\). Despite their localization at the plasma membrane, recent studies have identified intracellularly expressed SK channels in the ER membrane and the IMM\(^19,20,56,58\) where they provide protection against ER stress and/or mitochondrial demise. Here, we show that SK channels were enriched in
MAM fractions isolated from HT22 cells. Activation of SK channels by the pharmacological SK2/3 channel activator CyPPA induced mitochondrial ROS formation and mitochondrial membrane depolarization suggesting that SK channels and CyPPA-mediated SK channel activation may play a role in mitochondrial preconditioning. These findings are in agreement with a previous study where CyPPA reduced mitochondrial respiration, thereby protecting against oxytosis\textsuperscript{20}, and with another study investigating preconditioning by BK channels\textsuperscript{59} which belong to the same family of $K_{Ca}$ channels. In the present study, we found that CyPPA-mediated SK channel activation was still able to protect HT22 cells from glutamate-induced cell death, even in conditions of enhanced EMC. Interestingly, CyPPA failed to protect against auranofin-induced cell death, and rather increased neuronal damage. In addition to cell death potentiation, CyPPA accelerated the auranofin-induced impairment of both, mitochondrial respiration and glycolysis, respectively. The overexpression of mitoSK2 channels along with CyPPA enhanced mitochondrial resilience in HT22 cells during glutamate toxicity\textsuperscript{20}. In particular, CyPPA treatment consistently prevented major hallmarks of mitochondrial damage induced by glutamate including loss of cellular ATP and mitochondrial ROS production. The overexpression of mitochondrial SK2 channels had an additive effect on the observed protection in all analyzed parameters compared to overexpression of wildtype SK2 channel which localized only partially to the mitochondria. Interestingly, the overexpression of both, wildtype and mitoSK2 channels, provided an additive effect on CyPPA-mediated protection against oxytosis compared to the corresponding controls. Wildtype SK2 channels partially co-localized with the ER\textsuperscript{19} suggesting that in the MAM interface, SK channels from the ER and the mitochondria might contribute to the observed protection during increased EMC and impaired mitochondrial function. In contrast, overexpression of wildtype or mitoSK2 channels alone had no effect on auranofin-induced cell death. Yet, in line with our results showing that SK channel activation enhanced auranofin toxicity, co-treatment of SK2/mitoSK2-overexpressing cells with CyPPA further reduced cell viability compared to auranofin treatment alone in these cells. These unexpected findings lead us to the conclusion that CyPPA-mediated mitochondrial preconditioning did not apply to auranofin toxicity. The distinct effect of SK channel activation on auranofin toxicity might be due to the different signaling pathway involved. Oxytosis is initiated at the plasma membrane through inhibiting glutamate export from the cell which leads to loss of the antioxidant glutathione, and subsequently to mitochondrial demise within several hours\textsuperscript{25}. Mitochondrial damage is associated with $[Ca^{2+}]_{m}$ overload and ROS formation. In contrast, auranofin inhibits TRXR which are present in the cytosol and in the mitochondria\textsuperscript{60} and induces mitochondrial damage through ROS formation and mPTP opening. However, ROS-mediated damage may result from total ROS as well as mitochondrial ROS. In addition, TRX-related proteins such as ER-bound TMX1
might be affected by TRXR inhibition, thereby also causing ER stress. In plants and some bacterial species, it was shown that the NADPH-dependent TRXR is essential to cope with oxidative stress\(^{61-63}\). As NADPH is a substrate required for the redox reactions in mitochondrial complexes and the citric acid cycle, inhibition of TRXR might impair mitochondrial metabolism and ATP generation at multiple steps. Indeed, extracellular flux analysis showed that respiration and glycolysis were attenuated by auranofin in HT22 cells. Therefore, we suggest that CyPPA failed to protect against auranofin toxicity due to the multiple targets of auranofin that culminated in mitochondrial demise and neuronal cell death which exceeds the protective capacity of CyPPA. To follow up on these novel findings, more experiments are required to identify the exact pathway(s) involved in auranofin toxicity in HT22 cells, and to investigate the performance and function of individual mitochondrial complexes following auranofin treatment.

In conclusion, we show that strengthening EMC using the genetically encoded linkers potentiated neuronal cell death induced by glutamate and the TRXR inhibitor auranofin by attenuating mitochondrial respiration and promoting \([\text{Ca}^{2+}]_m\) overload. SK channel activation by CyPPA, known to provide protection in different \textit{in vitro} models of neurodegeneration, conferred protection against glutamate toxicity in conditions of increased EMC. Protection against oxytosis involved mitochondrial preconditioning, as CyPPA generated mitochondrial ROS and slightly depolarized the mitochondrial membrane. Interestingly, CyPPA failed to protect against auranofin toxicity and it accelerated the auranofin-induced decrease in mitochondrial respiration and glycolysis. In line, SK channel overexpression positively added on protection against glutamate-induced cell death while cell death following auranofin treatment was more pronounced in the presence of CyPPA. This novel differential effect of SK channel activation on neurotoxicity opens a new platform to study SK channel-mediated protection in yet unexplored mechanisms of oxidative stress.

\textbf{Acknowledgements}

We would like to thank Prof Györgyi Hajnoczky for kindly providing the ER-mitochondrial linker plasmids, Prof Ben N.G. Giepmans for providing the ER-Flipper-GFP plasmid, and Dr Maria Alonso for providing mitochondrial GFP-aequorin constructs. Furthermore, we would like to thank Prof Dr Moritz Bünemann and Prof Dr Cornelius Krasel for providing HEK293T cells, Prof Frank J Dekker and Dr Nick Eleftheriadis for providing CyPPA, and Klaas Sjollema for his technical support on live cell imaging. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, DFG (DO 1525/3-1). AMD is the recipient of a Rosalind Franklin Fellowship co-funded by the European Union and the University of Groningen.
Supplementary figure 1. Inducing ER-mitochondrial associations in neuronal HT22 cells. Representative fluorescent images of an individual HT22 cell expressing ER-Flipper-GFP control plasmid and TOM70-FKB12-RFP prior to and 10min following rapamycin (100nM) addition. Fluorescent traces are shown individually (left and middle panel) and as an overlay of both fluorescent channels (right panel). Green: ER-Flipper-GFP, red: TOM70-FKB12-RFP.
Supplementary figure 2. Effects of rapamycin on cell proliferation and cell viability of HT22 cells.
(A-B) MTT Assay following (A) glutamate treatment (8mM) for 12-18h or (B) auranofin treatment (1.5μM) for 0-8h. Data are presented as mean ± SD, n=8, Student t-test ***p<0.0001 compared to untreated control. (C-D) MTT Assay following treatment with (C) glutamate (16h) or (D) auranofin (6h) in the presence or absence of rapamycin (0, 50, 100, 150, 200nM). Data are presented as mean ± SD, n=6, Student t-test ***p<0.0001 compared to untreated control. (E) xCELLigence measurement of HT22 cells transfected with ER-Flipper-GFP (FL) or ER-FRB-CFP and TOM70-FKB12-RFP (EML) following addition of the indicated rapamycin concentrations. Data are presented as mean ± SD, n=6.
Supplementary figure 3. Extracellular flux analysis of mitochondrial function in HT22 cells.

(A-B) Representative measurement of (A) OCR and (B) ECAR following the application of rapamycin at the indicated concentrations (50, 100, 150, 200nM). A: rapamycin, B: oligomycin, C: DNP, D: antimycin A, rotenone, 2DG. Data are presented as mean ± SD, n=6-8. (C) Left panel: representative ECAR measurement following auranofin application. A: auranofin, B: oligomycin, C: DNP, D: antimycin A, rotenone, 2DG. Data are presented as mean ± SD, n=6-8. Arrow indicates analyzed time point. Right panel: quantification of ECAR values at the indicated time point following auranofin application, unpaired Student t-test, *p<0.05. (D-E) Representative (D) OCR and (E) ECAR measurement of following DNP application. A: DNP, B: oligomycin, C: DNP, D: antimycin A, rotenone, 2DG. Data are presented as mean ± SD, n=6-8.
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Supplementary figure 4. [Ca\(^{2+}\)]\(_{m}\) measurements in HEK293T cells.
(A) Transfection efficiency of HEK293T cells transfected with mtGA\(^{wt}\) or mtGA\(^{mut}\). Data are presented as mean ± SD, n=8-9, ***p<0.0001. (B) Representative measurement of [Ca\(^{2+}\)]\(_{m}\) uptake in HEK293T cells transfected with mtGA\(^{wt}\) stimulated with 0-100mM CaCl\(_2\). Data are presented as mean ± SD, n=3-4. (C) MTT assay in ER or linker-transfected HEK293T cells treated with the indicated rapamycin concentrations. Data are presented as mean ± SD, n=6-8.

Supplementary figure 5. CyPPA does not affect cell viability in linker-transfected cells.
xCELLigence measurement of HT22 cells transfected with Flipper control plasmid (FL) or linkers (EML) following treatment with CyPPA (10, 25, 50μM). Data are presented as mean ± SD, n=6.
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