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Resveratrol directly controls the activity of neuronal ryanodine receptors at the single channel level

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Abstract

Calcium ion dyshomeostasis contributes to the progression of many neurodegenerative diseases and represents a target for the development of neuroprotective therapies (1–3). Dysfunctional ryanodine receptors contribute to calcium ion dyshomeostasis and potentially to the pathogenesis of neurodegenerative diseases by generating abnormal calcium ion release from the endoplasmic reticulum (4–5). Since ryanodine receptors share functional and structural similarities with potassium channels (6), and small molecules with anti-oxidant properties, such as resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), directly control the activity of potassium channels (7–12), we hypothesized that *trans*-resveratrol can modulate intracellular calcium signaling through direct binding and functional regulation of ryanodine receptors. The goal of our study was to identify and measure the control of ryanodine receptor activity by *trans*-resveratrol. Mechanisms of calcium signaling mediated by the direct interaction between *trans*-resveratrol and ryanodine receptors, were identified and measured with single channel electrophysiology. Addition of *trans*-resveratrol to the cytoplasmic face of the ryanodine receptor increased single channel activity at physiological and elevated pathophysiological cytoplasmic calcium ion concentrations. The open probability of the channel increases after interacting with the small molecule in a dose-dependent manner, but remains also dependent on the concentration of its physiological ligand, cytoplasmic free calcium ions. This study provides the first evidence of a direct functional interaction between *trans*-resveratrol and ryanodine receptors. Such functional control of ryanodine receptors by *trans*-resveratrol as a novel mechanism of action could provide additional rationales for the development of novel therapeutic strategies to treat and prevent neurodegenerative diseases.

Keywords

3,5,4'-trihydroxy-*trans*-stilbene; calcium; Ca²⁺; electrophysiology; neurodegeneration; neuroprotection; neuroprotective therapies; pharmacology; RyR; *trans*-resveratrol

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Introduction

Calcium ions (Ca^{2+}) contribute to multiple signaling pathways in neurons, functioning as a universal secondary messenger with the activation of Ca^{2+} -controlled signaling pathways dependent on both intracellular Ca^{2+} stores and the rate of change in intracellular Ca^{2+} concentrations (13–14). Cellular Ca^{2+} entry is mediated through Voltage-Operated Ca^{2+} Channels (VOCC), Receptor-Operated Ca^{2+} Channels (ROCC), and Store-Operated Ca^{2+} Channels (SOCC) and is amplified by calcium-induced Ca^{2+} -release (CICR) channels in the endoplasmic reticulum (ER) and mitochondrial membranes mediating localized Ca^{2+} release from ER and mitochondrial Ca^{2+} stores (15–20). The termination of such intracellular Ca^{2+} transients and restoration of resting cytoplasmic Ca^{2+} concentrations is accomplished by the activity of transmembrane proteins of the plasma membrane (plasma membrane Ca^{2+} ATPase, PMCA; $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX), the ER membrane (Sarco-/endoplasmic reticulum Ca^{2+} -ATPase, SERCA), and the mitochondrial inner membrane (mitochondrial calcium uniporter, MCU) (21–22). Additionally, Ca^{2+} sequestration by Ca^{2+} -binding proteins and Ca^{2+} buffering molecules contributes to restoring resting cytoplasmic Ca^{2+} concentrations (17–18, 23–29). Ryanodine receptors (RyR) and inositol-1, 4, 5-trisphosphate receptors (IP_3R), ER transmembrane proteins, are clustered in micro-domains and respond to and amplify VOCC/ROCC/SOCC-mediated Ca^{2+} entry, thereby mediating CICR (30–31).

Ca^{2+} dyshomeostasis, a hallmark of several neurodegenerative diseases including Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) results in and is associated with altered Ca^{2+} buffering capacity, altered regulation of Ca^{2+} channels and pumps, and altered neuronal excitability (28, 32–33). Experimental models of neuronal aging have provided evidence for the "Calcium hypothesis of brain aging," which describes how aging is closely linked to dysregulation of Ca^{2+} homeostasis resulting in a chronically elevated level of cytosolic Ca^{2+} , which in turn contributes to age-related deficits in memory and learning through altered intracellular Ca^{2+} transients (1, 34). Similarly, pathogenic mechanisms disrupt Ca^{2+} homeostasis either in chronic (e.g. AD and ALS) or acute disease conditions (e.g. cerebral ischemia and traumatic brain injury) (32, 34–37). While the hypothesis focused initially on plasma membrane VOCC and decreased PMCA activity (40, 64–65), altered functional interactions between VOCC and RyR and abnormal Ca^{2+} buffering capacity of aged neurons were identified subsequently as contributing mechanisms (38–42).

As RyR and IP_3R CICR channels are responsible for amplifying Ca^{2+} signals under normal physiological conditions, but also during periods of intracellular Ca^{2+} dysregulation, they represent potential targets for neuroprotective therapy development. RyR2 and RyR3 are the two prominent isoforms found in the cerebellum, cortex, and hippocampus regions of the brain (1, 6, 43–44). Brain RyR activation is highly dependent on cytosolic Ca^{2+} concentrations; however, the receptor also contains a secondary, low affinity Ca^{2+} binding site that inhibits channel activity when high levels of free cytosolic Ca^{2+} are present (6, 32–33, 45). In addition to Ca^{2+} , other small molecules, both endogenous signaling substances as well as pharmacological agents, bind to the RyR and can modulate the release of Ca^{2+} from the ER (46–50). The goal of our study was to identify and measure the control of brain RyR activity by one such molecule with potential pharmacological benefits for the therapy of

neurodegenerative processes, *trans*-resveratrol. As a naturally occurring stilbenoid compound with multiple potential medicinal applications in cardiovascular, neurological and inflammatory disorders (51–55), *trans*-resveratrol affects several cellular signaling pathways involved in disease pathogenesis, most notably through its antioxidant properties by directly scavenging reactive oxygen species and by upregulating neuronal antioxidant proteins, such as quinone reductase 2 and heme oxygenase 1 (56–60), but also by a range of pleiotropic effects such as inhibiting pro-inflammatory signaling and enhancing NF- κ B signaling (54, 58, 61). While *trans*-resveratrol directly attenuates programmed cell death and calcium toxicity induced by oxygen-glucose deprivation in primary neurons (62), it also stimulates cell cycle arrest, caspase activation, cytochrome c release and apoptosis in a range of cancer cell lines (63–67). Some of these pharmacological properties of *trans*-resveratrol are likely caused by the control of cellular signaling pathways, specifically through interactions with ion channels and related signaling proteins. *Trans*-resveratrol regulates the biophysical properties of a variety of ion channels including the inhibition of the gamma-aminobutyric acid (GABA) receptor (68) and the activation of 5-hydroxytryptamine (5-HT) / serotonin receptors (69) and some transient receptor potential (TRP) channels, such as TRPA channels, but not TRPV1 receptors (70). *Trans*-resveratrol treatment reduces the activity of L- and T-type VOCCs and voltage-gated sodium channels in cardiomyocytes, neurons, and cell lines at the single channel level (7–8, 71–72). In addition, a range of studies document the control of potassium channels, including Ca^{2+} -activated K^+ (K_{Ca}), voltage-gated K^+ , and inwardly rectifying K^+ channels by *trans*-resveratrol. Such pharmacological activity includes the activation of large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) (7–10) and the inhibition of ATP-sensitive K^+ channels (K_{ATP}) (11–12) where specific underlying mechanisms of action, such as direct modulation of the ion channel versus effects mediated by posttranslational modifications for example, remain to be identified. While *trans*-resveratrol's direct interaction with L-Type VOCC channels, PMCA, and K_{ATP} channels are well documented, studies identifying *trans*-resveratrol-mediated modulation of intracellular Ca^{2+} signaling lack a clear mechanistic focus on proteins controlling CICR. The present study tests the hypothesis that direct binding of *trans*-resveratrol to intracellular calcium release channels (RyR) controls channel activity based on the rationale that RyRs and K^+ channels share significant structural homology in their pore forming transmembrane regions (12, 73–75).

In a potential coupling effect, *trans*-resveratrol directly regulates L- and T-type Ca^{2+} ion channels that can mediate CICR. *Trans*-resveratrol also attenuates expression and activity of Ca^{2+} handling proteins such as CaMKII and prevents downregulation of Ca^{2+} -sequestering protein pump levels in neuronal pathophysiology (76–78).

The pharmacological control of abnormal and potentially neurotoxic Ca^{2+} signaling as an approach for the treatment of or the protection against neurodegenerative diseases has been established through a variety of *in vitro* and *in vivo* models, but currently available data is focused on voltage-gated Ca^{2+} and storage-operated Ca^{2+} channels of the plasma membrane (1, 8). However, while the role of intracellular Ca^{2+} -induced Ca^{2+} -release channels in neuronal physiology and in neurodegenerative diseases has been studied, novel research into the regulation of these channels by *trans*-resveratrol has significant potential to identify innovative therapeutic approaches. In the present study, it is hypothesized that *trans*-

resveratrol directly binds neuronal RyRs and thereby controls their activity. To test this hypothesis, single channel electrophysiology was used to determine the change in biophysical properties of brain RyRs in the presence of *trans*-resveratrol.

Materials and Methods

Measurement of RyR single channel biophysical properties with planar lipid bilayer electrophysiology

Isolation and enrichment of mouse brain endoplasmic reticulum—Endoplasmic reticulum (ER) was isolated from brain tissue of C57BL/6 mice (7–9 weeks of age; The Jackson Laboratory, Sacramento, CA; approved by the Institutional Animal Care and Use Committee). Following dissection, tissue was washed in chilled (4°C), Ca²⁺- and Mg²⁺-free phosphate buffered saline and transferred to homogenization buffer [260mM sucrose, 5 mM HEPES, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 1 mM DTT (dichlorodiphenyltrichlorethane), and 10 µg/mL protease inhibitor cocktail consisting of PMSF (phenylmethylsulfonyl fluoride), aprotinin, pepstatin, trypsin inhibitor at pH 7.35]. The tissue was homogenized with a glass Dounce homogenizer in an excess of cold homogenization buffer and the homogenate was transferred to an ultracentrifuge tube to separate cell debris and the nuclear fraction by centrifugation at 1,000 × g for 10 min in a Sorvall™ TFT-80.2 rotor and WX ultracentrifuge (ThermoFisher Scientific, Waltham, MA). Supernatant between the debris pellet and floating lipid layer was transferred to a new chilled ultracentrifuge tube and spun down at 12,000 × g for 20 minutes to separate the ER from the mitochondrial fractions. One final spin at 100,000 × g for 75 min was performed after the supernatant (between the mitochondrial fraction pellet and lipid layer) was transferred to a new chilled tube to collect the ER fraction pellet. Storage buffer, mimicking the homogenization buffer with the omission of EGTA, was used to resuspend the ER pellet. The enriched ER microsome preparations were then quickly aliquoted, snap frozen in liquid nitrogen, and stored at –80°C until use.

Single channel planar lipid bilayer system configuration—The principle underlying planar lipid bilayer electrophysiology is the formation of an artificial lipid bilayer across a small aperture connecting two independent reservoirs (Suppl. Fig. 1) and the handling of two distinct buffers representing the cytosol (cis reservoir) and the ER lumen (trans reservoir). The planar lipid bilayer (Warner Instruments, Hamden, CT) chamber contains two reservoirs, cis and trans, with the trans side containing a polystyrene cuvette with an aperture size of 150–200 µm. The cis reservoir and trans-cuvette are filled with 1 mL of cis-buffer (250 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 110 mM Tris (2-Amino-2-(hydroxymethyl) propane-1,3-diol), pH of 7.35 ± 0.05) and trans-buffer (50 mM barium hydroxide (Ba(OH)₂) and 250 mM HEPES, pH of 7.5 ± 0.05), respectively. Two millimeter silver-silver chloride electrodes (Ag-AgCl) from the headstage were inserted into their respective salt buffer chambers where approximately 200 µL of a 3 M KCl solution was added in conjunction with salt bridges (5% agar in 1 M KCl) to effectively connect the cis or trans reservoirs to the electrical signal amplifier (Warner Instruments BCD525). Two lipid compositions were prepared by mixing phosphatidylcholine (PC) and phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL)

4:1 to a final concentration of 25 mg/mL or phosphatidylethanolamine (PE) and phosphatidylserine 3:1 to a final concentration of 40 mg/mL dissolved in decane. The aperture is initially primed with lipids using the PC:PS mixture to facilitate the collection and arrangement of the PE:PS lipid bilayer mixture. The thickness of the bilayer is visualized in a capacitance test to ensure the pore forming region of the channel can pass completely through the lipid bilayer. When needed, the bilayer thickness was manually adjusted with a small air bubble, protruding from a capillary tube, passing over the aperture to disperse the lipids. A final integrity test was performed with the trans reservoir grounded in the chamber and the cis side clamped at 0 mV. A stable and rigid seal of the aperture was monitored by the current movement between the cis and trans reservoirs with a measurement of 0 pA correlating to a closed membrane.

Measurement of brain ryanodine receptor biophysical activity, confirmation of channel orientation, and experimental protocol

Once optimal bilayer thickness was achieved (~ 80 pF) ER microsomes containing RyRs were added to the cis reservoir, a brief vortex distributed the microsomes followed by a 20–30 min incubation. Microsome fusion occurred when microsomes move in proximity to the artificial bilayer and spontaneously fuse into the lipid bilayer. This process can also be facilitated with the addition of small 3 M KCl volumes (50 – 100 μ L) to the cis reservoir, effectively forming an osmotic gradient, resulting in the swelling of the microsome and enhancing fusion. Fusion was visualized by the appearance of channel activity and confirmed with the absence of activity after the cis reservoir was washed with 10 mL of cis buffer. To ensure only cis facing RyRs (with their cytosolic portion in the cis reservoir) are measured the use of barium (Ba^{2+}) as a current carrying ion was used. The Ba^{2+} ion can pass through the RyR, replicating the movement of Ca^{2+} through RyRs, but does not affect channel activity as it does not bind to the Ca^{2+} binding sites; any RyRs facing the trans reservoir cannot be activated by Ba^{2+} . Activation of RyR currents was stimulated with increasing additions of Ca^{2+} and EGTA in proportions, calculated by MaxChelator software (Stanford University, Sanford, CA), to achieve a desired free cytosolic Ca^{2+} concentration. Ca^{2+} concentrations are expressed as pCa, defined as the negative decadic logarithm of the free Ca^{2+} concentration.

A baseline of RyR current activity was recorded for 3.5 min after observing activity at a desired Ca^{2+} concentration establishing the channel's baseline activity. An increasing concentration of *trans*-resveratrol (dissolved in 100% DMSO and diluted with cis buffer) was added to the cis reservoir, vortexed for ~ 10 sec, and allowed to incubate for 30 sec before a 3.5 min recording was collected. Each recording was performed at room temperature with the traces collected and saved individually. At the conclusion of each experiment 10 μ M ruthenium red (RR) was added to the cis reservoir as a selective inhibitor of RyRs as a secondary pharmacological tool to confirm the presence of RyRs.

Software configuration for data acquisition and analysis of single channels—

Amplifying the analog electrophysiological signals was performed using a BCD525 amplifier with filter parameters to reduce noise amplitudes at 500 Hz. The analog to digital conversion was performed at 10 kHz within a Molecular Devices (Sunnyvale, CA)

Digidata 1322A system and pClamp 10 software. Each individual single channel's recording baseline was set to 0 pA, representing a closed channel, and a Gaussian low pass (320 Hz) filter was applied to minimize the electrical noise generated by the digitalization process. A single-channel search was performed using ClampFit 10 software over a 3 min timeline with two identified sub-conductance states, S2 and S4. Four biophysical properties were calculated by the software in each sub-conductance level including the number of channel openings (number of events), duration of channel openings (dwell time), average current transferred (average amplitude), and the probability of the channel being open over the given time (open probability, P_o).

Biophysical data analysis and comparison of single channels in the absence or presence of trans-resveratrol

—To limit the impact of variance in RyR channel activity, because of scaffolding proteins being removed during ER enrichment process or different levels of posttranslational modifications, normalized biophysical values were reported as a percentage of the baseline. Each initial baseline, i.e. RyR activity after Ca^{2+} activation, was used to normalize subsequent *trans*-resveratrol dosage recordings to off-set the variable activity among single channels and allow comparison of replicates. *Trans*-resveratrol was applied to the cis reservoir, vortexed for 10 sec, and channel activity was recorded for 3.5 min. Subsequent *trans*-resveratrol volumes were added to the cis reservoir up to a final 100 μM *trans*-resveratrol concentration. Prism GraphPad software was used to calculate and plot the normalized values and express the three levels of significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$) using one-way ANOVA with Dunnett's post hoc test.

Results

***Trans*-resveratrol potentiates brain ryanodine receptor activity at elevated cytosolic calcium concentrations**

Figure 1 shows brain RyR single channel activity at three different cytosolic Ca^{2+} concentrations. The representative traces demonstrate more frequent channel openings with cytosolic Ca^{2+} concentrations increasing from 10 nM to 10 μM (pCa, defined as the negative decadic logarithm of the free Ca^{2+} concentration, 8 to 5; Fig. 1) without changes in single channel amplitude (Fig. 2). Three unique RyR sub-conductance states with distinct single channel amplitudes are indicated in Fig. 1, closed, partially open (S2), fully open (S4) subconductance states.

At high cytosolic Ca^{2+} concentrations (10 μM , pCa 5), 100 μM *trans*-resveratrol potentiates brain RyR activity by increasing channel open probability as indicated by the increased frequency of fully open channel events (bottom traces in Fig. 3A–B) when compared to baseline activity (top traces in Fig. 3A–B). The 2 pA subconductance state (S2) of the receptor is not significantly affected by addition of *trans*-resveratrol as both the number and dwell time of S2 opening events do not change significantly at any of the tested *trans*-resveratrol concentrations (Figs. 3 and 4A–B). However, 100 μM *trans*-resveratrol significantly increases the number of events and the dwell time of the fully open state (S4; Figs. 3 and 4C–D). The increase in the number of S4 events (Fig. 4C) and in the S4 dwell time (Fig. 4D) appears sufficient to increase brain RyR open probability (Fig. 4E) in the

absence of significant effects of *trans*-resveratrol on the S2 subconductance state (Fig. 4A–B).

Open probability of brain ryanodine receptors is elevated in the presence of *trans*-resveratrol at resting baseline cytosolic Ca²⁺ levels

At low intracellular Ca²⁺ concentrations (10 nM; pCa 8), *trans*-resveratrol potentiated RyR activity by increasing channel open probability. Representative 30 s (Fig. 5A) and 2 s (Fig. 5B) current traces show an increase in channel open frequency for both the S2 and S4 states in the presence of 10 pM *trans*-resveratrol as the underlying mechanism of action. The upward shift of the amplitude histogram curve (Fig. 5C) of 78% for the S2 and approximately 2100% for the S4 peaks, when compared to baseline (Figs. 5C, 6A and C), indicates that channel open frequency drives the *trans*-resveratrol-mediated increase in channel open probability. The rightward shift of the dwell time histogram curve (Fig. 5D) is indicative of an increase in channel open time. The S2 channel openings exhibit a 60% increase in duration (Fig. 6B), whereas the open dwell time of the fully open state S4 doubles when compared to baseline conditions (Fig. 6D). The combined increase in S2 and S4 opening frequency and dwell time results in an approximately fourfold increase in open probability of the channel in the presence of 10 pM *trans*-resveratrol (Fig. 6E).

Discussion

Nerve cells affected by neurodegenerative diseases display a decreased capacity to handle elevated ROS generation and Ca²⁺ signaling dyshomeostasis (28, 32–33, 79). The discovery of novel therapeutically active, pleiotropic agents, such as small molecules or biologics that have a dual function by regulating Ca²⁺ signaling and at the same time increasing neuronal resistance to oxidative stress, could lead to novel therapeutic approaches for neurodegenerative diseases. Here, we identified such a novel mechanism of action controlling neuronal Ca²⁺ signaling for the well-characterized antioxidant polyphenolic phytostilbene, *trans*-resveratrol.

Trans-resveratrol directly controls intracellular calcium signaling by modulating ryanodine receptor function

Trans-resveratrol directly interacts with K⁺ channels as determined previously in single channel electrophysiology planar lipid bilayer and patch-clamp studies (7–12). A *trans*-resveratrol binding site has been identified on the cytosolic sulfonylurea receptor subunit of K_{ATP} channels, while another binding site has been postulated to exist at or near the ion pore forming region of K⁺ channels (11–12). Structurally, K⁺ channels share direct homology with RyRs in the ion pore region (73–75). Taken together, these structural and sequence homologies among RyRs and K⁺ channels provide a rationale for *trans*-resveratrol to directly bind to brain RyRs and subsequently, to alter their activity. In the present study, we determined, using single channel electrophysiology, that *trans*-resveratrol increases the open probability of brain RyR channels in a dose- and Ca²⁺ dependent manner (Figs. 4 and 6). At elevated intracellular Ca²⁺ concentrations (pCa 5) and high, micromolar concentrations of *trans*-resveratrol, the open probability of RyRs increases due to a higher opening frequency and increased dwell time of the channel in the fully open S4 subconductance state (Figs. 3–

4). However, a higher open probability was already observed in the presence of 10 nM *trans*-resveratrol reaching significance ($p < 0.05$) at 100 μ M (Fig. 4E). This direct interaction implies that *trans*-resveratrol binding to brain RyRs increases the channel's open probability by favoring its fully open S4 subconductance state thus increasing the amount of Ca^{2+} released from the ER into the cytosol. At resting cytosolic Ca^{2+} concentrations (pCa 8), *trans*-resveratrol significantly potentiates RyR channel opening frequency (Figs. 5C, 6A and C) and dwell times (Figs. 5D, 6B and D) for both S2 and S4 subconductance states, unlike the pharmacological control of predominantly only the fully open S4 subconductance state at higher cytosolic Ca^{2+} concentrations (pCa 5). Consequently, a significantly increased open probability was already seen at low *trans*-resveratrol concentrations (10 pM), an effect that is attenuated as *trans*-resveratrol concentrations increase with intermediate *trans*-resveratrol concentrations (10 nM) being similar to activity measured at pCa 5 (Fig. 6E).

Our data provide evidence for two potential mechanisms of action of *trans*-resveratrol summarized and illustrated in figure 7. Assuming a single binding domain for *trans*-resveratrol on the RyR, such a site is accessible under low cytosolic Ca^{2+} concentrations when conformational changes of the ryanodine receptor due to Ca^{2+} binding on the cytosolic face of the channel are minimal (Fig. 7A, left panel). However, as cytosolic Ca^{2+} concentrations increase conformational changes of the ryanodine receptor become more extensive resulting in either reduced accessibility or reduced affinity of the *trans*-resveratrol binding domain for *trans*-resveratrol, thereby requiring higher *trans*-resveratrol concentrations to potentiate RyR activity (Fig. 7A, right panel).

A second potential mechanism of action of *trans*-resveratrol assumes two binding sites for *trans*-resveratrol on the RyR (Fig. 7B): At 10 pM *trans*-resveratrol concentrations, direct binding of *trans*-resveratrol to the RyR results in potentiation of channel activity through a single high affinity binding site that is accessible at resting, low cytosolic Ca^{2+} concentrations (pCa 8; Fig. 7B, left panel). At higher intracellular Ca^{2+} concentrations typically seen during high neuronal activity (pCa 5; Fig. 7B, right panel), the RyR undergoes conformational changes induced by Ca^{2+} binding (6, 80–83), one or more of these conformational changes thereby allowing *trans*-resveratrol to access a now exposed second low affinity binding site, but no longer the high affinity *trans*-resveratrol binding site. This potential pharmacological regulation of the RyR by *trans*-resveratrol assuming two different affinity-driven binding sites that cannot be accessed by *trans*-resveratrol simultaneously, a high affinity binding site that is only accessible at low Ca^{2+} concentrations and a low affinity binding site becoming accessible at higher Ca^{2+} concentrations, is illustrated in the summary diagram of Fig. 7B.

For both models of the newly discovered mechanism of action that direct binding of *trans*-resveratrol to the RyR potentiates channel activity presented in Fig. 7, *trans*-resveratrol increases Ca^{2+} release from the ER at the single channel level when cytoplasmic Ca^{2+} concentrations are clamped to specific levels when Ca^{2+} -mediated RyR inhibition resulting from the channel activity itself cannot be observed. As RyRs are CICR channels with inhibitory, low affinity Ca^{2+} binding sites, the highly localized increase in cytosolic Ca^{2+} release from the ER resulting from *trans*-resveratrol mediated potentiation of channel activity potentially results in Ca^{2+} transients with above average amplitudes and shorter

duration followed by a prolonged period of reduced Ca^{2+} leakage from the ER, all of which are hallmarks of healthy neuronal signaling (34). The total amount of Ca^{2+} released from the ER would be lower after *trans*-resveratrol treatment giving rise to potential therapeutic applications to attenuate Ca^{2+} homeostasis in chronic or acute neurodegenerative diseases (34).

Therapeutic limitations of *trans*-resveratrol lie in its limited oral bioavailability and short half-life *in vivo*. Single 25 mg oral doses of *trans*-resveratrol in healthy male subjects achieved low concentrations (~30 nM) of free *trans*-resveratrol in plasma between 0.5 – 2 h after dosing while conjugated *trans*-resveratrol peaked at 2 μM independent of the route of administration (oral or intraperitoneal; 51, 84–85). Continuous oral administration or an increased dosage improved free *trans*-resveratrol plasma levels in human subjects (86–88). Synthesizing methylated analogs of *trans*-resveratrol has been an approach to increase bioavailability. Rodent studies with pterostilbene and 3,5,4'-trimethoxy-resveratrol report plasma levels 60% and 90% greater than the *trans*-resveratrol control respectively, concluding that chemical modification of *trans*-resveratrol is a viable option for improving its bioavailability (89–90). Improving bioavailability or delivery techniques by reducing its clearance rate, increasing transport across the blood brain barrier, and/or establishing targeted delivery could enhance *trans*-resveratrol's neuroprotective potential even further, ultimately contributing to a novel therapeutic approach or supplementary treatment approach for diseases affecting the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Duncan RS; Goad DL; Grillo MA; Kaja S; Payne AJ; Koulen P, Control of Intracellular Calcium Signaling as a Neuroprotective Strategy. *Molecules* 2010, 15 (3), 1168–95. [PubMed: 20335972]
2. LaFerla FM, Calcium Dyshomeostasis and Intracellular Signalling in Alzheimer's Disease. *Nat. Rev. Neurosci* 2002, 3 (11), 862–72. [PubMed: 12415294]
3. Niittykoski M; Kalesnykas G; Larsson KP; Kaarniranta K; Akerman KE; Uusitalo H, Altered Calcium Signaling in an Experimental Model of Glaucoma. *Invest. Ophthalmol. Vis. Sci* 2010, 51 (12), 6387–93. [PubMed: 20592232]
4. Bruno AM; Huang JY; Bennett DA; Marr RA; Hastings ML; Stutzmann GE, Altered Ryanodine Receptor Expression in Mild Cognitive Impairment and Alzheimer's Disease. *Neurobiol. Aging* 2012, 33 (5), 1001 e1–6.
5. Stutzmann GE; Caccamo A; LaFerla FM; Parker I, Dysregulated Ip_3 Signaling in Cortical Neurons of Knock-in Mice Expressing an Alzheimer's-Linked Mutation in Presenilin1 Results in Exaggerated Ca^{2+} Signals and Altered Membrane Excitability. *J. Neurosci* 2004, 24 (2), 508–13. [PubMed: 14724250]

6. Lanner JT; Georgiou DK; Joshi AD; Hamilton SL, Ryanodine Receptors: Structure, Expression, Molecular Details, and Function in Calcium Release. *Cold Spring Harb. Perspect. Biol* 2010, 2 (11), a003996. [PubMed: 20961976]
7. Wang YJ; Chan MH; Chen L; Wu SN; Chen HH, Resveratrol Attenuates Cortical Neuron Activity: Roles of Large Conductance Calcium-Activated Potassium Channels and Voltage-Gated Sodium Channels. *J. Biomed. Sci* 2016, 23 (1), 47. [PubMed: 27209372]
8. McCalley AE; Kaja S; Payne AJ; Koulen P, Resveratrol and Calcium Signaling: Molecular Mechanisms and Clinical Relevance. *Molecules* 2014, 19 (6), 7327–40. [PubMed: 24905603]
9. Novakovic R; Radunovic N; Markovic-Lipkovski J; Cirovic S; Beleslin-Cokic B; Ilic B; Ivkovic B; Heinle H; Zivanovic V; Gojkovic-Bukarica LJ, Effects of the Polyphenol Resveratrol on Contractility of Human Term Pregnant Myometrium. *Mol. Hum. Reprod* 2015, 21 (6), 545–51. [PubMed: 25750187]
10. Li HF; Chen SA; Wu SN, Evidence for the Stimulatory Effect of Resveratrol on Ca(2+)-Activated K⁺ Current in Vascular Endothelial Cells. *Cardiovasc. Res* 2000, 45 (4), 1035–45. [PubMed: 10728430]
11. Gopalakrishnan M; Molinari EJ; Shieh CC; Monteggia LM; Roch JM; Whiteaker KL; Scott VE; Sullivan JP; Brioni JD, Pharmacology of Human Sulphonylurea Receptor Sur1 and Inward Rectifier K(+) Channel Kir6.2 Combination Expressed in Hek-293 Cells. *Br. J. Pharmacol* 2000, 129 (7), 1323–32. [PubMed: 10742287]
12. Hambrock A; de Oliveira Franz CB; Hiller S; Grenz A; Ackermann S; Schulze DU; Drews G; Osswald H, Resveratrol Binds to the Sulfonylurea Receptor (Sur) and Induces Apoptosis in a Sur Subtype-Specific Manner. *J. Biol. Chem* 2007, 282 (5), 3347–56. [PubMed: 17138562]
13. Brini M; Cali T; Ottolini D; Carafoli E, Neuronal Calcium Signaling: Function and Dysfunction. *Cell. Mol. Life Sci* 2014, 71 (15), 2787–814. [PubMed: 24442513]
14. Berridge MJ, Neuronal Calcium Signaling. *Neuron* 1998, 21 (1), 13–26. [PubMed: 9697848]
15. Contreras L; Drago I; Zampese E; Pozzan T, Mitochondria: The Calcium Connection. *Biochim. Biophys. Acta* 2010, 1797 (6–7), 607–18. [PubMed: 20470749]
16. Rizzuto R; De Stefani D; Raffaello A; Mammucari C, Mitochondria as Sensors and Regulators of Calcium Signalling. *Nat. Rev. Mol. Cell Biol* 2012, 13 (9), 566–78. [PubMed: 22850819]
17. Berridge MJ; Bootman MD; Roderick HL, Calcium Signalling: Dynamics, Homeostasis and Remodelling. *Nat. Rev. Mol. Cell Biol* 2003, 4 (7), 517–29. [PubMed: 12838335]
18. Clapham DE, Calcium Signaling. *Cell* 2007, 131 (6), 1047–58. [PubMed: 18083096]
19. Altschafel BA; Beutner G; Sharma VK; Sheu SS; Valdivia HH, The Mitochondrial Ryanodine Receptor in Rat Heart: A Pharmacokinetic Profile. *Biochim. Biophys. Acta* 2007, 1768 (7), 1784–95. [PubMed: 17499575]
20. Beutner D; Voets T; Neher E; Moser T, Calcium Dependence of Exocytosis and Endocytosis at the Cochlear Inner Hair Cell Afferent Synapse. *Neuron* 2001, 29 (3), 681–90. [PubMed: 11301027]
21. Blaustein MP; Juhaszova M; Golovina VA; Church PJ; Stanley EF, Na/Ca Exchanger and Pmca Localization in Neurons and Astrocytes: Functional Implications. *Ann. N. Y. Acad. Sci* 2002, 976, 356–66. [PubMed: 12502582]
22. De Stefani D; Patron M; Rizzuto R, Structure and Function of the Mitochondrial Calcium Uniporter Complex. *Biochim. Biophys. Acta* 2015, 1853 (9), 2006–11. [PubMed: 25896525]
23. Hashambhoy YL; Greenstein JL; Winslow RL, Role of Camkii in Ryr Leak, Ec Coupling and Action Potential Duration: A Computational Model. *J. Mol. Cell. Cardiol* 2010, 49 (4), 617–24. [PubMed: 20655925]
24. Chelu MG; Sarma S; Sood S; Wang S; van Oort RJ; Skapura DG; Li N; Santonastasi M; Muller FU; Schmitz W; Schotten U; Anderson ME; Valderrabano M; Dobrev D; Wehrens XH, Calmodulin Kinase II-Mediated Sarcoplasmic Reticulum Ca²⁺ Leak Promotes Atrial Fibrillation in Mice. *J. Clin. Invest* 2009, 119 (7), 1940–51. [PubMed: 19603549]
25. Kook SY; Jeong H; Kang MJ; Park R; Shin HJ; Han SH; Son SM; Song H; Baik SH; Moon M; Yi EC; Hwang D; Mook-Jung I, Crucial Role of Calbindin-D28k in the Pathogenesis of Alzheimer's Disease Mouse Model. *Cell Death Differ* 2014, 21 (10), 1575–87. [PubMed: 24853300]
26. Baimbridge KG; Celio MR; Rogers JH, Calcium-Binding Proteins in the Nervous System. *Trends Neurosci* 1992, 15 (8), 303–8. [PubMed: 1384200]

27. Heizmann CW; Braun K, Changes in Ca(2+)-Binding Proteins in Human Neurodegenerative Disorders. *Trends Neurosci* 1992, 15 (7), 259–64. [PubMed: 1381122]
28. Wojda U; Salinska E; Kuznicki J, Calcium Ions in Neuronal Degeneration. *IUBMB Life* 2008, 60 (9), 575–90. [PubMed: 18478527]
29. Stull JT, Ca2+-Dependent Cell Signaling through Calmodulin-Activated Protein Phosphatase and Protein Kinases Minireview Series. *J. Biol. Chem* 2001, 276 (4), 2311–2. [PubMed: 11096124]
30. Verkhratsky A; Petersen OH, The Endoplasmic Reticulum as an Integrating Signalling Organelle: From Neuronal Signalling to Neuronal Death. *Eur. J. Pharmacol* 2002, 447 (2–3), 141–54. [PubMed: 12151006]
31. Koulen P; Janowitz T; Jochenning FW; Ehrlich BE, Characterization of the Calcium-Release Channel/Ryanodine Receptor from Zebrafish Skeletal Muscle. *J. Membr. Biol* 2001, 183 (3), 155–63. [PubMed: 11696857]
32. Marambaud P; Dreses-Werringloer U; Vingtdeux V, Calcium Signaling in Neurodegeneration. *Mol. Neurodegener* 2009, 4, 20. [PubMed: 19419557]
33. Mattson MP, Calcium and Neurodegeneration. *Aging Cell* 2007, 6 (3), 337–50. [PubMed: 17328689]
34. Thibault O; Gant JC; Landfield PW, Expansion of the Calcium Hypothesis of Brain Aging and Alzheimer's Disease: Minding the Store. *Aging Cell* 2007, 6 (3), 307–17. [PubMed: 17465978]
35. Gennarelli TA; Graham DI, Neuropathology of the Head Injuries. *Semin. Clin. Neuropsychiatry* 1998, 3 (3), 160–175. [PubMed: 10085204]
36. Foster TC, Calcium Homeostasis and Modulation of Synaptic Plasticity in the Aged Brain. *Aging Cell* 2007, 6 (3), 319–25. [PubMed: 17517041]
37. Brewer LD; Thibault O; Staton J; Thibault V; Rogers JT; Garcia-Ramos G; Kraner S; Landfield PW; Porter NM, Increased Vulnerability of Hippocampal Neurons with Age in Culture: Temporal Association with Increases in Nmda Receptor Current, Nr2a Subunit Expression and Recruitment of L-Type Calcium Channels. *Brain Res* 2007, 1151, 20–31. [PubMed: 17433272]
38. Murchison D; Griffith WH, Calcium Buffering Systems and Calcium Signaling in Aged Rat Basal Forebrain Neurons. *Aging Cell* 2007, 6 (3), 297–305. [PubMed: 17517040]
39. Toescu EC; Verkhratsky A, The Importance of Being Subtle: Small Changes in Calcium Homeostasis Control Cognitive Decline in Normal Aging. *Aging Cell* 2007, 6 (3), 267–73. [PubMed: 17517038]
40. Buchholz JN; Behringer EJ; Pottorf WJ; Pearce WJ; Vanterpool CK, Age-Dependent Changes in Ca2+ Homeostasis in Peripheral Neurones: Implications for Changes in Function. *Aging Cell* 2007, 6 (3), 285–96. [PubMed: 17517039]
41. Alexianu ME; Ho BK; Mohamed AH; La Bella V; Smith RG; Appel SH, The Role of Calcium-Binding Proteins in Selective Motoneuron Vulnerability in Amyotrophic Lateral Sclerosis. *Ann. Neurol* 1994, 36 (6), 846–58. [PubMed: 7998770]
42. Iacopino AM; Christakos S, Corticosterone Regulates Calbindin-D28k Mrna and Protein Levels in Rat Hippocampus. *J. Biol. Chem* 1990, 265 (18), 10177–80. [PubMed: 2354995]
43. Hakamata Y; Nakai J; Takeshima H; Imoto K, Primary Structure and Distribution of a Novel Ryanodine Receptor/Calcium Release Channel from Rabbit Brain. *FEBS Lett* 1992, 312 (2–3), 229–35. [PubMed: 1330694]
44. Rybalchenko V; Grillo MA; Gasting MJ; Rybalchenko N; Payne AJ; Koulen P, The Unliganded Long Isoform of Estrogen Receptor Beta Stimulates Brain Ryanodine Receptor Single Channel Activity Alongside with Cytosolic Ca2+. *J. Recept. Signal Transduct. Res* 2009, 29 (6), 326–41. [PubMed: 19899956]
45. Koulen P; Thrower EC, Pharmacological Modulation of Intracellular Ca(2+) Channels at the Single-Channel Level. *Mol. Neurobiol* 2001, 24 (1–3), 65–86. [PubMed: 11831555]
46. Zhang L; Kelley J; Schmeisser G; Kobayashi YM; Jones LR, Complex Formation between Junctin, Triadin, Calsequestrin, and the Ryanodine Receptor. *Proteins of the Cardiac Junctional Sarcoplasmic Reticulum Membrane. J. Biol. Chem* 1997, 272 (37), 23389–97. [PubMed: 9287354]
47. Fill M; Copello JA, Ryanodine Receptor Calcium Release Channels. *Physiol. Rev* 2002, 82 (4), 893–922. [PubMed: 12270947]

48. Copello JA; Barg S; Sonnleitner A; Porta M; Diaz-Sylvester P; Fill M; Schindler H; Fleischer S, Differential Activation by Ca^{2+} , Atp and Caffeine of Cardiac and Skeletal Muscle Ryanodine Receptors after Block by Mg^{2+} . *J. Membr. Biol* 2002, 187 (1), 51–64. [PubMed: 12029377]
49. Sonnleitner A; Fleischer S; Schindler H, Gating of the Skeletal Calcium Release Channel by Atp Is Inhibited by Protein Phosphatase 1 but Not by Mg^{2+} . *Cell Calcium* 1997, 21 (4), 283–90. [PubMed: 9160164]
50. Xu L; Mann G; Meissner G, Regulation of Cardiac Ca^{2+} Release Channel (Ryanodine Receptor) by Ca^{2+} , H^{+} , Mg^{2+} , and Adenine Nucleotides under Normal and Simulated Ischemic Conditions. *Circ. Res* 1996, 79 (6), 1100–9. [PubMed: 8943948]
51. Bastianetto S; Menard C; Quirion R, Neuroprotective Action of Resveratrol. *Biochim. Biophys. Acta* 2015, 1852 (6), 1195–201. [PubMed: 25281824]
52. Marambaud P; Zhao H; Davies P, Resveratrol Promotes Clearance of Alzheimer's Disease Amyloid-Beta Peptides. *J. Biol. Chem* 2005, 280 (45), 37377–82. [PubMed: 16162502]
53. Huang TC; Lu KT; Wo YY; Wu YJ; Yang YL, Resveratrol Protects Rats from A β -Induced Neurotoxicity by the Reduction of Inos Expression and Lipid Peroxidation. *PLoS One* 2011, 6 (12), e29102. [PubMed: 22220203]
54. Rege SD; Geetha T; Griffin GD; Broderick TL; Babu JR, Neuroprotective Effects of Resveratrol in Alzheimer Disease Pathology. *Front. Aging Neurosci* 2014, 6, 218. [PubMed: 25309423]
55. Renaud S; de Lorgeril M, Wine, Alcohol, Platelets, and the French Paradox for Coronary Heart Disease. *Lancet* 1992, 339 (8808), 1523–6. [PubMed: 1351198]
56. Wu JM; Hsieh TC; Wang Z, Cardioprotection by Resveratrol: A Review of Effects/Targets in Cultured Cells and Animal Tissues. *Am. J. Cardiovasc. Dis* 2011, 1 (1), 38–47. [PubMed: 22254184]
57. Liu BL; Zhang X; Zhang W; Zhen HN, New Enlightenment of French Paradox: Resveratrol's Potential for Cancer Chemoprevention and Anti-Cancer Therapy. *Cancer Biol. Ther* 2007, 6 (12), 1833–6. [PubMed: 18087218]
58. Bastianetto S; Zheng WH; Quirion R, Neuroprotective Abilities of Resveratrol and Other Red Wine Constituents against Nitric Oxide-Related Toxicity in Cultured Hippocampal Neurons. *Br. J. Pharmacol* 2000, 131 (4), 711–20. [PubMed: 11030720]
59. Buryanovskyy L; Fu Y; Boyd M; Ma Y; Hsieh TC; Wu JM; Zhang Z, Crystal Structure of Quinone Reductase 2 in Complex with Resveratrol. *Biochemistry* 2004, 43 (36), 11417–26. [PubMed: 15350128]
60. Sakata Y; Zhuang H; Kwansa H; Koehler RC; Dore S, Resveratrol Protects against Experimental Stroke: Putative Neuroprotective Role of Heme Oxygenase 1. *Exp. Neurol* 2010, 224 (1), 325–9. [PubMed: 20381489]
61. Zhang F; Liu J; Shi JS, Anti-Inflammatory Activities of Resveratrol in the Brain: Role of Resveratrol in Microglial Activation. *Eur. J. Pharmacol* 2010, 636 (1–3), 1–7. [PubMed: 20361959]
62. Gong QH; Wang Q; Shi JS; Huang XN; Liu Q; Ma H, Inhibition of Caspases and Intracellular Free Ca^{2+} Concentrations Are Involved in Resveratrol Protection against Apoptosis in Rat Primary Neuron Cultures. *Acta Pharmacol. Sin* 2007, 28 (11), 1724–30. [PubMed: 17959022]
63. Wu XP; Xiong M; Xu CS; Duan LN; Dong YQ; Luo Y; Niu TH; Lu CR, Resveratrol Induces Apoptosis of Human Chronic Myelogenous Leukemia Cells in Vitro through P38 and Jnk-Regulated H2ax Phosphorylation. *Acta Pharmacol. Sin* 2015, 36 (3), 353–61. [PubMed: 25619392]
64. Huang T; Gao D; Jiang X; Hu S; Zhang L; Fei Z, Resveratrol Inhibits Oxygen-Glucose Deprivation-Induced Mmp-3 Expression and Cell Apoptosis in Primary Cortical Cells Via the Nf-Kappab Pathway. *Mol Med Rep* 2014, 10 (2), 1065–71. [PubMed: 24840287]
65. Hogg SJ; Chitcholtan K; Hassan W; Sykes PH; Garrill A, Resveratrol, Acetyl-Resveratrol, and Polydatin Exhibit Antigrowth Activity against 3d Cell Aggregates of the Skov-3 and Ovar-8 Ovarian Cancer Cell Lines. *Obstet. Gynecol. Int* 2015, 2015, 279591. [PubMed: 26617640]
66. Liao PC; Ng LT; Lin LT; Richardson CD; Wang GH; Lin CC, Resveratrol Arrests Cell Cycle and Induces Apoptosis in Human Hepatocellular Carcinoma Huh-7 Cells. *J. Med. Food* 2010, 13 (6), 1415–23. [PubMed: 20946021]

67. Varoni EM; Lo Faro AF; Sharifi-Rad J; Iriti M, Anticancer Molecular Mechanisms of Resveratrol. *Front Nutr* 2016, 3, 8. [PubMed: 27148534]
68. Lee BH; Choi SH; Hwang SH; Kim HJ; Lee JH; Nah SY, Resveratrol Inhibits Gabac Rho Receptor-Mediated Ion Currents Expressed in *Xenopus* Oocytes. *Korean J. Physiol. Pharmacol* 2013, 17 (2), 175–80. [PubMed: 23626481]
69. Lee BH; Hwang SH; Choi SH; Shin TJ; Kang J; Lee SM; Nah SY, Resveratrol Enhances 5-Hydroxytryptamine Type 3a Receptor-Mediated Ion Currents: The Role of Arginine 222 Residue in Pre-Transmembrane Domain I. *Biol. Pharm. Bull* 2011, 34 (4), 523–7. [PubMed: 21467640]
70. Yu L; Wang S; Kogure Y; Yamamoto S; Noguchi K; Dai Y, Modulation of Trp Channels by Resveratrol and Other Stilbenoids. *Mol Pain* 2013, 9, 3. [PubMed: 23413875]
71. Zhang LP; Yin JX; Liu Z; Zhang Y; Wang QS; Zhao J, Effect of Resveratrol on L-Type Calcium Current in Rat Ventricular Myocytes. *Acta Pharmacol. Sin* 2006, 27 (2), 179–83. [PubMed: 16412267]
72. Jakab M; Lach S; Bacova Z; Langeluddecke C; Strbak V; Schmidt S; Iglseder E; Paulmichl M; Geibel J; Ritter M, Resveratrol Inhibits Electrical Activity and Insulin Release from Insulinoma Cells by Block of Voltage-Gated Ca⁺ Channels and Swelling-Dependent Cl⁻ Currents. *Cell. Physiol. Biochem* 2008, 22 (5–6), 567–78. [PubMed: 19088439]
73. Samso M; Wagenknecht T; Allen PD, Internal Structure and Visualization of Transmembrane Domains of the Ryr1 Calcium Release Channel by Cryo-Em. *Nat. Struct. Mol. Biol* 2005, 12 (6), 539–44. [PubMed: 15908964]
74. Welch W; Rheault S; West DJ; Williams AJ, A Model of the Putative Pore Region of the Cardiac Ryanodine Receptor Channel. *Biophys. J* 2004, 87 (4), 2335–51. [PubMed: 15454434]
75. Ramachandran S; Serohijos AW; Xu L; Meissner G; Dokholyan NV, A Structural Model of the Pore-Forming Region of the Skeletal Muscle Ryanodine Receptor (Ryr1). *PLoS Comput. Biol* 2009, 5 (4), e1000367. [PubMed: 19390614]
76. Kim YH; Kim YS; Kang SS; Cho GJ; Choi WS, Resveratrol Inhibits Neuronal Apoptosis and Elevated Ca²⁺/Calmodulin-Dependent Protein Kinase II Activity in Diabetic Mouse Retina. *Diabetes* 2010, 59 (7), 1825–35. [PubMed: 20424226]
77. Li W; Wang YP; Gao L; Zhang PP; Zhou Q; Xu QF; Zhou ZW; Guo K; Chen RH; Yang HT; Li YG, Resveratrol Protects Rabbit Ventricular Myocytes against Oxidative Stress-Induced Arrhythmogenic Activity and Ca²⁺ Overload. *Acta Pharmacol. Sin* 2013, 34 (9), 1164–73. [PubMed: 23912472]
78. Dong Q; Wu Z; Li X; Yan J; Zhao L; Yang C; Lu J; Deng J; Chen M, Resveratrol Ameliorates Cardiac Dysfunction Induced by Pressure Overload in Rats Via Structural Protection and Modulation of Ca(2+) Cycling Proteins. *J. Transl. Med* 2014, 12, 323. [PubMed: 25425099]
79. Kim GH; Kim JE; Rhie SJ; Yoon S, The Role of Oxidative Stress in Neurodegenerative Diseases. *Exp. Neurobiol* 2015, 24 (4), 325–40. [PubMed: 26713080]
80. Efremov RG; Leitner A; Aebersold R; Raunser S, Architecture and Conformational Switch Mechanism of the Ryanodine Receptor. *Nature* 2015, 517 (7532), 39–43. [PubMed: 25470059]
81. Song DW; Lee JG; Youn HS; Eom SH; Kim DH, Ryanodine Receptor Assembly: A Novel Systems Biology Approach to 3d Mapping. *Prog. Biophys. Mol. Biol* 2011, 105 (3), 145–61. [PubMed: 20934451]
82. Ozawa T, Modulation of Ryanodine Receptor Ca²⁺ Channels (Review). *Mol Med Rep* 2010, 3 (2), 199–204. [PubMed: 21472222]
83. Zalk R; Clarke OB; des Georges A; Grassucci RA; Reiken S; Mancina F; Hendrickson WA; Frank J; Marks AR, Structure of a Mammalian Ryanodine Receptor. *Nature* 2015, 517 (7532), 44–9. [PubMed: 25470061]
84. Goldberg DM; Yan J; Soleas GJ, Absorption of Three Wine-Related Polyphenols in Three Different Matrices by Healthy Subjects. *Clin. Biochem* 2003, 36 (1), 79–87. [PubMed: 12554065]
85. Walle T; Hsieh F; DeLegge MH; Oatis JE Jr.; Walle UK, High Absorption but Very Low Bioavailability of Oral Resveratrol in Humans. *Drug Metab. Dispos* 2004, 32 (12), 1377–82. [PubMed: 15333514]
86. Almeida L; Vaz-da-Silva M; Falcao A; Soares E; Costa R; Loureiro AI; Fernandes-Lopes C; Rocha JF; Nunes T; Wright L; Soares-da-Silva P, Pharmacokinetic and Safety Profile of Trans-

- Resveratrol in a Rising Multiple-Dose Study in Healthy Volunteers. *Mol Nutr Food Res* 2009, 53 Suppl 1, S7–15. [PubMed: 19194969]
87. Walle T, Bioavailability of Resveratrol. *Ann. N. Y. Acad. Sci* 2011, 1215, 9–15. [PubMed: 21261636]
88. Vitaglione P; Sforza S; Galaverna G; Ghidini C; Caporaso N; Vescovi PP; Fogliano V; Marchelli R, Bioavailability of Trans-Resveratrol from Red Wine in Humans. *Mol Nutr Food Res* 2005, 49 (5), 495–504. [PubMed: 15830336]
89. Kapetanovic IM; Muzzio M; Huang Z; Thompson TN; McCormick DL, Pharmacokinetics, Oral Bioavailability, and Metabolic Profile of Resveratrol and Its Dimethylether Analog, Pterostilbene, in Rats. *Cancer Chemother. Pharmacol* 2011, 68 (3), 593–601. [PubMed: 21116625]
90. Lin HS; Ho PC, A Rapid Hplc Method for the Quantification of 3,5,4'-Trimethoxy-Trans-Stilbene (Tms) in Rat Plasma and Its Application in Pharmacokinetic Study. *J. Pharm. Biomed. Anal* 2009, 49 (2), 387–92. [PubMed: 19081693]

Figure 1. Representative traces of mouse brain ryanodine receptor activity. Sample 30 s current traces at resting (A, 10 nM, pCa 8) and elevated cytosolic Ca^{2+} concentrations (B, 1 μM , pCa 6; C, 10 μM , pCa 5). Channel openings are represented by downward deflections from the closed state at zero current baseline to a 2 pA subconductance state (S2) and to the fully open state (S4) at 4 pA.

Figure 2. Channel amplitude histograms from mouse brain ryanodine receptor single channel activity recorded at varying cytosolic Ca^{2+} concentrations. Channels were activated by 10 μM (pCa 5; black), 1 μM (pCa 6; red), and 10 nM (pCa 8; grey) free intracellular calcium concentrations. In response to increased cytosolic Ca^{2+} concentrations, the frequency of events at both the 2 pA subconductance state (S2) and the 4 pA fully open state (S4) is increased.

Figure 3. Channel open probability of mouse brain ryanodine receptors increases in the presence of *trans*-resveratrol at elevated cytosolic Ca^{2+} concentrations.

(A) Representative current traces (30 s) illustrating channel openings before (top trace) and after (bottom trace) the addition of 100 μM *trans*-resveratrol. (B) Short (2 sec) current traces illustrate channel dwell time at baseline (top) and in the presence of 100 μM *trans*-resveratrol (bottom). (C) Representative amplitude histogram showing a broadening of the S2 subconductance level and an increase in fully open S4 events, indicating an increase in the number of ryanodine receptor openings in the presence of 100 μM *trans*-resveratrol (red) compared to baseline (black) at 10 μM Ca^{2+} / pCa 5. A fifth order polynomial distribution

curve was fitted for baseline conditions ($R^2 = 0.899$; pCa 5) and for channels recorded in the presence of 100 μM *trans*-resveratrol ($R^2 = 0.952$; pCa 5) showing an upward shift indicative of elevated channel opening frequency. (D) Gaussian fitting (baseline, $R^2 = 0.937$; 100 μM *trans*-resveratrol, $R^2 = 0.928$) of the channel dwell time data from the same representative data set as in C indicates that average channel dwell time remains unchanged in the presence of *trans*-resveratrol (red).

Figure 4. Quantification of brain ryanodine receptor channel open frequency, dwell time and open probability in the presence of varying concentrations of *trans*-resveratrol at pCa 5 / 10 μ M Ca^{2+} .

Normalized channel open frequency (A) and normalized average dwell time (B) for subconductance level S2 indicating no change over baseline at all concentrations of *trans*-resveratrol tested. 100 μ M *trans*-resveratrol increases the channel open frequency (C) and average dwell time (D) of the fully open conductance state S4 by 134.9% and 90.2% respectively. (E) The open probability of the brain ryanodine receptor increases by 49.3% in the presence of 100 μ M *trans*-resveratrol. All values were normalized to baseline and are

averages $\pm$ standard error of the mean. One-way ANOVA with Dunnett's post hoc test; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; $n=3$.

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Figure 5. *Trans*-resveratrol potentiates brain ryanodine receptor activity through increased channel opening frequency and dwell time at low cytosolic Ca^{2+} levels. Representative 30 s (A) and 2 s (B) ryanodine receptor channel recordings at baseline conditions (10 nM Ca^{2+} , pCa 8, top) and after addition of 10 pM *trans*-resveratrol (bottom). (C) Representative amplitude histograms at pCa 8 (black) and pCa 8 in the presence of 10 pM *trans*-resveratrol (red) indicating that *trans*-resveratrol increases the number of fully open S4 events and 2 pA subconductance state S2 events. A fourth order polynomial distribution was fitted for baseline conditions (pCa 8; $R^2 = 0.782$) and for channels recorded in the presence of 10 pM *trans*-resveratrol (pCa 8; $R^2 = 10.922$) displaying an upward shift

for both S2 and S4 states indicative of elevated channel opening frequency. (D) Gaussian fitting (baseline, $R^2 = 0.946$; 10 pM *trans*-resveratrol, $R^2 = 0.895$) of the channel dwell time data from the same representative data set as in C shows that average channel dwell time increases significantly after *trans*-resveratrol administration as indicated by a rightward shift of the curve.

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Figure 6. Quantification of brain ryanodine receptor channel open frequency, dwell time and open probability in the presence of varying concentrations of *trans*-resveratrol at pCa 8 / 10 nM Ca^{2+} .

Normalized channel open frequency (A) and normalized average dwell time (B) for subconductance level S2 indicating potentiation by 10 pM *trans*-resveratrol (78% and 60% respectively) of a representative response. 10 pM *trans*-resveratrol significantly increases the channel open frequency (C) and average dwell time (D) of the fully open conductance state, S4, of a representative channel by approximately 2100% and 1200% respectively. (E) The

open probability of a representative brain ryanodine receptor increases by approximately 400% in the presence of 10 pM *trans*-resveratrol.

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Figure 7. Potential mechanisms of action underlying *trans*-resveratrol binding to and control of brain ryanodine receptors.

(A) A single *trans*-resveratrol (RV) binding domain is accessible at low cytosolic Ca^{2+} concentrations (*left*) resulting in minimal RyR conformational change (dark blue subunit) allowing low concentrations of *trans*-resveratrol to potentiate RyR activity. Higher concentrations of *trans*-resveratrol are needed to potentiate RyR activity when significant conformational changes due to Ca^{2+} binding at high cytosolic Ca^{2+} concentrations (*right*).

(B) The bottom panel illustrates regulation of the RyR by two separate binding domains for *trans*-resveratrol. The high affinity site (red, *left*) is accessible at resting Ca^{2+} concentrations, but is blocked when Ca^{2+} binds the receptor and induces a conformational change. The conformational change of the RyR at high cytosolic Ca^{2+} concentrations uncovers a low affinity binding site for *trans*-resveratrol (pink, *right*), requiring higher concentrations of *trans*-resveratrol for binding and subsequent potentiation of RyR activity. The locations of each site are hypothetical and do not imply identified structural domains.