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Resveratrol attenuates early pyramidal neuron excitability impairment and death in acute rat hippocampal slices caused by oxygen-glucose deprivation

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Abstract

Accumulating evidence indicates that the polyphenol resveratrol (trans-3, 5, 4'-trihydroxystibene, RVT) potently protects against cerebral ischemia neuronal damage due to its oxygen free radicals scavenging and antioxidant properties. However, it is unknown whether RVT can attenuate ischemia-induced early impairment of neuronal excitability. To address this question, we simulated ischemic conditions by applying oxygen-glucose deprivation (OGD) to acute rat hippocampal slices and examined the effect of RVT on OGD-induced pyramidal neuron excitability impairment using whole-cell patch clamp recording. 100 μ M RVT largely inhibited the 15 min OGD-induced progressive membrane potential (V_m) depolarization and the reduction in evoked action potential frequency and amplitude in pyramidal neurons. In the parallel neuronal viability study using TO-PRO-3 iodide staining, 20 min OGD induced irreversible CA1 pyramidal neuronal death which was significantly reduced by 100 μ M RVT. No similar effects were found with PQQ treatment, an antioxidant also showing potent neuroprotection in the rat rMCAO ischemia model. This suggests that antioxidant action *per se*, is unlikely accounting for the observed early effects of RVT. RVT also markedly reduced the frequency and amplitude of AMPA mediated spontaneous excitatory postsynaptic currents (sEPSCs) in pyramidal neurons, which is also an early consequence of OGD. RVT effects on neuronal excitability were inhibited by the large conductance potassium channel (BK channel) inhibitor paxilline. Together, these studies demonstrate that RVT attenuates OGD induced neuronal impairment occurring early in the simulated ischemia slice model by enhancing the activation of BK channel and reducing the OGD-enhanced AMPA/NMDA receptor mediated neuronal EPSCs.

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Keywords

Resveratrol; Oxygen-glucose deprivation; Antioxidant; sEPSC; BK channels; TO-PRO-3 iodide; AMPA/NMDA

INTRODUCTION

Recent studies have shown that resveratrol (RVT) can protect ischemia damage in a variety of organs, such as brain, kidney and heart (Ray, et al., 1999; Bastianetto et al., 2000; Chander and Chopra, 2006). RVT is one of the primary active polyphenol compounds identified in red wine (Jang et al., 1997; Bastianetto et al., 2000; Oak et al., 2005), which has attracted increasing research interest on how this compound could offer such a miracle protection. Mechanistically, RVT acts as a potent antioxidant and scavenger of hydroperoxyl free radicals (Pace-Asciak et al., 1996; Mokni et al., 2006; Das et al., 2006). These protective actions of RVT in cerebral ischemia have so far been identified from studies *in vivo* and in cultured neurons (Ray et al., 1999; Raval et al., 2006; Han et al., 2004). In the latter case it is unclear whether the same mechanisms occur *in situ* as *in vitro*. The *in vivo* studies usually only measure protective effects after several days which are important to assess potential therapeutic effects. However, cerebral ischemia induced ischemic cell death and the accompanying neuronal dysfunction is complex process with multiple time-dependent processes (Lipton, 1999). While very early effects may be of little relevance to treating clinical stroke they are necessary for understanding all the mechanisms of action of any potential therapeutic agent and may be beneficial if agents are being developed for prophylactic use.

It has been shown previously that a transient oxygen-glucose-deprivation (OGD) treatment of adult rat hippocampal slices leads to a rapid membrane potential (V_m) depolarization of CA1 pyramidal neurons (Bures et al., 1957; Hansen, 1980), excitability degeneration and enhancement of excitatory and inhibitory synaptic currents (EPSC and IPSC) (Allen and Attwell, 2004; Katchman and Hershkowitz, 1994). These neuronal dysfunctions occur in the early phase of the simulated ischemia conditions and are caused by breakdown of physiological transmembrane ion gradients and massive excitatory amino acid release (Hansen, 1980; Somjen and Aitken, 1984; Calabresi, et al., 1995).

In the present study, we applied OGD solution to acute rat hippocampal slices to simulate ischemia conditions *in vitro*, and performed whole-cell electrophysiological recording from CA1 pyramidal neurons to examine the acute effect of RVT on OGD-induced pyramidal neuron electrophysiology impairment and to study the underlying mechanisms. TO-PRO-3 iodide staining was also applied to directly measure OGD induced pyramidal neuron death and RVT protection.

MATERIALS AND METHODS

Hippocampal slice preparation

Acute hippocampal slices were prepared from 3–4 week old Sprague Dawley rats. The procedure was performed in accordance with a protocol approved by the Wadsworth Center, New York State Department of Health Institutional Animal Care and Use Committee. Animals were anesthetized with isoflurane before decapitation, and their brains were removed from the skull and placed in an ice-cold, oxygenated (5% CO_2 –95% O_2 , pH = 7.35) slice preparation solution containing (in mM) 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2.5 KCl, 10 MgCl_2 , 10 glucose, 0.5 CaCl_2 , and 240 sucrose. Final osmolarity was 350 ± 2 mOsm. Coronal slices of 300 μm thickness were cut with a Vibratome (Pelco 1500) and transferred to a nylon-basket slice holder in 20–22°C artificial cerebral spinal fluid (aCSF) containing (in mM) 125 NaCl, 25

NaHCO₃, 10 glucose, 3.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, and 1 MgCl₂ (osmolarity, 300 ± 5 mOsm). The slices were allowed to recover in aCSF with continuous oxygenation for at least 60 min before staining or recording.

Oxygen–glucose deprivation (OGD) procedure

Glucose deprivation was achieved by substituting *D*-glucose with equimolar sucrose in the standard aCSF (see above). Oxygen deprivation was achieved by bubbling *D*-glucose – deficient aCSF with 95% N₂ and 5% CO₂ for 30 min to deplete O₂ content in the aCSF, maintaining the same pH at 7.3–7.4 as in the control normal aCSF solution.

TO-PRO-3 iodide staining and quantification of neuronal viability

The cell viability of acute hippocampal slices under control, OGD treatment and upon addition of RVT conditions were also determined by TO-PRO-3 iodide staining. The level of TO-PRO-3 incorporation into cellular DNA is indicative of significant membrane injury and has been widely used to distinguish dead from living cells (Van Hooijdonk et al, 1994). Our previous results showed that pyramidal neuron survival was variable after a 15 min. OGD treatment. The number of TO-PRO-3-I positive neurons only consistently increased with 20 min OGD treatment. This was also consistent with the electrophysiological results, where the excitability of pyramidal neurons was functionally recoverable after 15 min OGD treatment but not after 20 min. treatment. Therefore, we used 20 min OGD exposure to examine the effects of RVT. To reliably determine OGD induced neuronal damage measured with TO-PRO-3 iodide staining, acute hippocampal slices were prepared according to the same procedure as for electrophysiological recording (n=5, P21 male rats). In each preparation, 6–8 hippocampal slices obtained were first allowed to recover in aCSF for one hour and were then randomly divided into control (normal aCSF), OGD, OGD + 0.1% DMSO, OGD + 100 μM RVT in < 0.1% DMSO and OGD + 100 μM PQQ groups. The slices were then placed in Petri dishes for 20 min under the above mentioned conditions, and then transferred into normal aCSF solution containing a 1:2000 dilution of TO-PRO-3 from a 1 mM stock solution purchased from Molecular Probes (Eugene, OR). The slices were incubated at room temperature for 20 min. The TO-PRO-3 staining in slices was then examined with a Carl Zeiss LSM510 META confocal microscope using the 630 nm line of the HeNe laser. Emission was filtered through a long pass 650 nm filter. Image stacks were acquired from the slice surface to a depth of 20 μm, with the same acquisition settings used for all conditions. TO-PRO-3 fluorescence above background in the CA1 pyramidal layer was determined using the “density slice” option of the National Institutes of Health Image J program. In each experiment with slices obtained from the same preparation, the TO-PRO-3 fluorescence densities from the control slices were defined as 100%, and TO-PRO-3 fluorescence densities from other groups were normalized to the control densities to calculate the percentage changes, as shown in Fig. 1.

Electrophysiology

For *in situ* recording, individual hippocampal slices were gently transferred into a recording chamber that was constantly perfused with oxygenated aCSF (2.5 ml/min). Whole cell membrane currents were amplified by a MultiClamp 700A amplifier, sampled by a DIGIDADA 1322A Interface, and data acquisition was controlled by PClamp 9.0 software (Axon Instruments, Foster City, CA) installed on a Dell personal computer. Low resistance patch pipettes were fabricated from borosilicate capillaries (OD: 1.5 mm; Warner Instrument Corporation, Hamden, CT) using a Flaming/Brown Micropipette Puller (Model P-87, Sutter Instrument Co., Novato, CA). When filled with K-gluconate-based electrode solution (see below), the electrode resistance range was 3–5 MΩ. Whole-cell recordings were made by rupturing the membrane with negative pressure pulses when the seal resistance in the initial cell- attached mode reached at least 3–4 GΩ. Membrane potential was read in “I= 0” mode

when recording was performed in voltage clamp mode, or continuously monitored in current clamp mode without current delivery. Membrane capacitance (C_m) and series resistances (R_s) were measured using the “Membrane test” protocol built into the PClamp 9.0. Patch pipettes were filled with a solution containing (mM) 3 KCl, 137 K-gluconate, 0.5 CaCl₂, 1 MgCl₂, 5 EGTA, 10 HEPES, 3 Mg-ATP and 0.3 Na-GTP (pH was adjusted to 7.25–7.27 at 20 °C with KOH, 280 ± 5 mOsm). All the experiments were conducted at room temperature (24–26°C).

Solutions and reagents

NBQX, Paxilline, bicuculline, QX-314 were all purchased from Tocris (Tocris Bioscience, Avonmouth, UK). Resveratrol and PQQ were purchased from Sigma (St. Louis, MO, USA). For stock solution preparation, NBQX, bicuculline and QX-314 were dissolved in water and the others were dissolved in DMSO and stored at –20 °C. RVT in DMSO, was freshly diluted from stock solution to 100 μ M concentration in OGD solution before each experiment (final DMSO less than 0.1%). For the in vivo neuroprotection study, typically 30 to 100 mg/Kg of RVT were intraperitoneally administrated (Sonmez et al, 2007; Wang et al, 2002). According to a HPLC analysis by Wang et al (2002), around 10 to 33 μ M concentration of RVT could be achieved in the brain extracellular space that is close to the 30–50 μ M commonly used for cultured or freshly isolated cell studies (Li et al., 2000; Gao et al., 2006). In the same study by Li et al, (2000), the maximal effect of RVT on BK type K⁺ channels was determined at 100 μ M. Since 100 μ M RVT was found effective in a comparable study in organotypic brain slice cultures for determining RVT neuroprotective or anti-apoptotic effects in organotypic brain slice cultures (Raval et al., 2006; Nicolini et al., 2001), we therefore used the same concentration of RVT in our study. 0.1% DMSO was also included in control solutions to rule out any potential effects of DMSO alone. DMSO and all the other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

Data analysis

The frequency and the cumulative fraction of the amplitudes of the spontaneous excitatory postsynaptic currents (sEPSCs) were analyzed using Synaptosoft software (Synaptosoft, Inc, GA, USA).

Data are presented as means $\pm$ S.E.M. Student's *t*-test was performed to assess the statistical significance of the difference before and after treatment in the same experimental group. One-way ANOVA test was performed to determine the statistical significance of the differences between 2–3 experimental groups. Differences were considered significant at $p < 0.05$.

RESULTS

OGD induced acute neuronal death was largely attenuated by RVT

TO-PRO-3-I staining was used to determine pyramidal neuron death in hippocampal CA1 pyramidal neuron layer. It labels DNA in cells only with compromised cell membranes (Van Hooijdonk et al, 1994). Exposure to OGD for 20 min. caused a marked 4.2 ± 0.6 fold increase in TO-PRO-3-I staining density in the hippocampal CA1 pyramidal neurons layer, which in the presence of 100 μ M RVT in the OGD solution was reduced to 1.6 ± 0.3 fold, as compared to control). No significant protection was shown by 0.1% DMSO (4.0 ± 0.6) or 100 μ M PQQ (3.5 ± 0.1 , dissolved in 0.1% DMSO). Although OGD also caused a noticeable TO-PRO-3-I staining in *stratum radiatum*, we found we were unable to correlate these staining signals to interneurons and astrocytes specifically, so that we focused on the pyramidal neurons as this allowed us to assess OGD caused neuronal damage and TAM effects unambiguously. To determine if RVT neuroprotection was mediated through its antioxidant function, we also used another antioxidant, PQQ (100 μ M), which we have shown is neuroprotective in rMCAo (Zhang et al., 2006). However, PQQ failed to show any protective effect with 20 min OGD

treatment (3.5 ± 0.1 , $n=6$). 0.1% DMSO had no effect on pyramidal neuron viability. Quantification of TO-PRO-3-I staining after 20 min OGD treatment in the presence and absence of RVT are shown in Fig. 1B.

Resveratrol attenuates OGD-induced impaired pyramidal neuron excitability

In our previous study, we showed that hippocampal CA 1 pyramidal neurons could be easily identified in hippocampal slices according to their location and characteristic soma and associated apical dendrite morphology (Zhang et al, 2007). In the same study, we reported that a 15 min OGD exposure is the maximal time window that mimics a reversible ischemic model and allows determining of neuroprotection by tamoxifen of the impaired neuronal excitability. In this study, we followed the same experimental protocol for identifying CA 1 pyramidal neurons and for determining RVT effects on OGD induced impaired neuronal excitability changes.

Changes in neuronal excitability were studied by monitoring evoked spikes every 20 s from stably established pyramidal neuron whole-cell recordings (Fig. 2A). As we established before that pyramidal neurons showed an average resting membrane potential (RMP) of -60 mV and an average membrane resistance (R_M) of ~ 200 M Ω (Zhang et al., 2007), so that only the cells that required less than 100 pA/5 ms for single spike induction were used, as this was the current necessary to bring the membrane potential (V_m) from RMP, ~ -60 mV, to the Na^+ channel threshold (-40 mV). Needing the greater currents might indicate a deteriorated seal resistance between the recording electrode and attached membrane during formation of the whole-cell configuration. In such cases the recordings were rejected. When the recording satisfied this condition, the amount of positive current was increased to evoke 3–4 spikes within a 100 ms pulse duration, so that the changes in neuronal excitability could be compared (Fig. 2A–a). The cell-to-cell variation in required current injections was between 200 pA to 300 pA ($n=24$).

For OGD, after a stable control recording for at least 5 min in normal aCSF (95% O_2 /5% CO_2), the perfusion fluid was switched to OGD solution bubbled with 95% N_2 /5% CO_2 (see Methods for further details). As noted above, a 15 min OGD mimics reversible ischemia and is suitable for testing RVT effects on OGD induced neuronal excitability changes, so we used 15 min. OGD for all the RVT electrophysiological experiments. The following electrophysiological changes were observed: 1. OGD induced a time-dependent membrane potential (V_m) depolarization and by the end of 15 min OGD, an average 10.2 ± 0.25 mV positive shift in V_m was observed (Fig. 3A). 2. The number of spikes evoked by positive current injection was reduced and the first spike amplitude was decreased (Fig. 2A, a). See Fig. 3A. for the complete time courses of these changes.

In the presence of 100 μ M RVT, the OGD effect on pyramidal neuron electrophysiology was reduced (Fig. 2 B). Only a 6 ± 0.6 mV depolarization was produced by the end of 15 min OGD (-59.8 ± 0.2 mV in control vs. -54.1 ± 0.9 mV in OGD) (Fig. 3A). RVT also blocked the OGD induced suppression of spike number. In both control and RVT groups, the membrane potential and spike number was almost recovered 5 min after OGD withdrawal (-59.1 ± 0.7 mV) in normal aCSF. DMSO had no effect. As the TO-PRO-3-I staining showed a marked protection of OGD induced neuronal damage by RVT, we also examined pyramidal neuronal excitability changes with a 20 min. OGD treatment. Of these tested neurons, all of them recovered from OGD exposure upon OGD withdrawal in the presence of 100 μ M RVT (data not shown). This was consistent with neuronal viability test result shown in the last section.

PQQ showed no effect on OGD-induced pyramidal neurons depolarization

PQQ was unable to protect OGD induced neuronal death after a 20 min OGD treatment. We also tested the PQQ effect on 15 min. OGD induced neuronal excitability impairment. As

summarized in Fig. 3C–D, unlike RVT, PQQ produced no protection on OGD induced neuronal excitability damage. This implies that the early acute RVT effect was unlikely mediated through its antioxidant action.

Resveratrol suppresses the OGD induced increases in spontaneous EPSCs

Cerebral ischemia induces massive glutamate release that can be measured as an enhanced spontaneous excitatory postsynaptic current (sEPSCs) in the OGD slice model (Katchman and Hershkowitz, 1994). Inhibition of these sEPSC events should attenuate neuronal over excitation, thereby reducing neuronal damage. To explore whether this could be a mechanism accounting for RVT protection, we next sought to determine RVT's effect on the OGD-induced increase in pyramidal neuron sEPSCs. For sEPSC measurement, the neuronal Na^+ channel currents were inhibited by adding QX-314 in the pipette solution, and the GABA_A receptor mediated inhibitory postsynaptic currents (IPSCs) were blocked by adding 25 μM bicuculline to the bath solution. When the pyramidal neurons were voltage-clamped close to their resting membrane potential (-60 mV), OGD induced a time-dependent downward shift in holding currents with an enhancement in the frequency and amplitudes of sEPSCs events. The representative segments of changes in sEPSCs taken from control, 10 min. and 15 min. of OGD treatment, respectively, are presented in Fig 4A. Since extracellular Mg^{2+} was present in the bath and the cells were clamped at -60 mV, the recorded sEPSCs were mediated by AMPA-R and not NMDA-R. We confirmed this by showing that these events could be completely inhibited by the AMPA receptor inhibitor NBQX at 10 μM concentration (data not shown). In the presence of 100 μM RVT the OGD-mediated increase in sEPSCs was largely attenuated (Fig 4 B). The detailed statistical analyses of these events are presented on Fig. 4 C and D. These results suggest that at least part of the RVT protection is mediated by attenuating OGD induced excitotoxicity due to increased AMPA-R mediated sEPSCs.

The protective effect of Resveratrol can be almost completely inhibited by the BK channel blocker Paxilline

The RVT acute neuroprotection appears, at least in part, to be due to the maintenance of the membrane potential in pyramidal neurons during OGD insult. To explore the mechanism underlying this, we asked whether the RVT effect on membrane potential could be mediated through potentiation of the large-conductance, calcium-activated K^+ channels (BK channels) as BK channel activation has been shown to maintaining neuronal V_m under ischemic conditions (Gong et al., 2000; Runden-Pran et al., 2002). Activation of BK channels by RVT has been identified in several cell types (Wu 2003; Wang et al., 2007). Using the same voltage-clamp recording protocol as shown in Fig. 4, we measured the effects of the BK channel blocker, paxilline (PAX), on the downward shift of the holding currents at the end of 15 min OGD treatment (Fig. 5). The downward shift in holding currents is proportional to neuronal membrane potential depolarization. In the control experiment with normal bath solution, the holding current remained unchanged over a more than 30 min recording period. In the presence of the BK channel inhibitor, 10 μM PAX, only an average -10 pA downward shift in holding currents was observed in the pyramidal neuron recordings (Fig. 5), consistent with the notion that the activity of BK channels is low under physiological conditions. However, in OGD solution, PAX further enhanced the downward holding current from -71.6 ± 9.0 pA ($n=9$) in OGD alone to -129.3 ± 20.6 pA ($n=6$), indicating that BK channels were activated in OGD to oppose the V_m depolarization. All the holding current shift values noted before and hereafter were measured at the end of 15 min OGD treatment. With the addition of 100 μM RVT, the downward shift in holding current declined from the control value of -71.6 ± 9.0 pA ($n=9$) to -50.6 ± 4.0 pA ($n=5$), and this RVT effect was blocked by PAX (-128.6 ± 22.7 pA, $n=5$) (see Fig. 5C.). Concomitantly, the RVT mediated inhibition of OGD induced sEPSC frequency was also blocked by PAX (Fig. 5B). We conclude from these results that 1) at least part of RVT

action is mediated through BK channel potentiation and 2) this BK potentiation contributes to the suppression of OGD induced excessive sEPSCs.

DISCUSSION

Our results demonstrate, for the first time, that RVT can protect the OGD-induced early impairment of neuronal excitability in hippocampal slices. This protection appeared, at least in part, to be due to potentiation of BK channels that opposed the depolarization of Vm otherwise seen and thereby suppressed OGD induced increased sEPSCs. The results also suggest that the antioxidant action of RVT was not primarily responsible for the observed effects.

RVT attenuates OGD induced neuronal excitability impairment and death

Cerebral ischemia can quickly lead to an abrupt neuronal Vm depolarization starting around 10 min., a phenomenon termed anoxic depolarization (AD) (Bures et al., 1957; Hansen, 1980). As the AD occurs soon after the beginning of severe hypoxia or cerebral ischemia, neuroprotectants that are able to delay this process should be beneficial for rescuing neurons if given very early. Although the mechanism underlying AD is still not fully understood, the massive Ca^{2+} influx via activated NMDA receptors leading to irreversible neuronal damage is an essential contributing mechanism (Balestrino et al., 1995; Kaminogo et al., 1998).

According to the report by Onitsuka et al (1998) the reversible neuronal Vm depolarization phase can last for 14.4 min at 27 °C. In order to determine RVT neuroprotection, we used room temperature so that the RVT effects on neuronal excitability can be reliably measured in this reversible ischemia in vitro model, which is consistent with our observation that the 15 min. OGD induced electrophysiological changes in hippocampal pyramidal neurons were readily reversible. This time window is also consistent with the availability of stored energy in the slice that can last for ~ 11 min under OGD conditions (Xie et al., 2007). We should note that other factors also contribute to the delayed occurrence of AD. These include that 1) the slices are submerged in perfusion chamber that prevents a rapid accumulation of extracellular K^+ , and 2) the pH was held at 7.3–7.4 by 5% CO_2 and 25 mM sodium bicarbonate.

In the present study, we identified RVT as a compound that can substantially sustain neuronal Vm during the early 15 min. of OGD, which should delay the occurrence of AD. Concomitantly, TO-PRO-3-I staining also showed RVT increased neuronal viability.

Antioxidant action does not appear to contribute to the acute RVT protective effects

Previous studies have discovered several mechanisms accounting for RVT protection under ischemic conditions. These include 1) antioxidant effects; 2) scavenging lipid hydroperoxyl free radicals, and 3) reducing nitric oxide-related toxicity (Mokni et al., 2006; Das et al., 2006; Han et al., 2004). To determine whether the acute RVT effects observed in the present studies was due to its antioxidant activities, we also tested PQQ. PQQ is a naturally occurring redox cofactor that acts as an essential nutrient, antioxidant, redox modulator and free radical scavenger. Those functions contribute to the protective effects in experimental stroke models (Zhang and Rosenberg, 2002; Zhu et al., 2005; Zhang et al., 2006). However, in the present study, we found no significant protection by PQQ of the early changes in the OGD slice model either by dye-exclusion or the electrophysiological changes. This indicates that the acute neuroprotection of resveratrol is probably not through its antioxidant pathway.

The RVT effects are mediated in appt through potentiation of BK channels leading to inhibition of OGD induced sEPSCs

Neurotoxicity due to excessive release of excitatory neurotransmitters is a major reason for ischemic damage. During the early stage of OGD, excessive glutamate release causes postsynaptic activation of AMPA and NMDA receptors causing a time dependent increase of spontaneous EPSCs (Liu B et al., 2006; Katchman et al., 1994). This increased sEPSCs can be effectively suppressed by 100 μ M RVT. Although sEPSCs are mostly mediated by AMPA-receptors at this holding potential (-60 mV), it does not rule out RVT's ability to directly attenuate Ca^{2+} influx through NMDA receptor, which is activated subsequent to AMPA-R activation.

Besides its function as an antioxidant, RVT also modulates the activation of several ion channels, such as Ca^{2+} -activated K^{+} channels (Li et al., 2000). These large-conductance, calcium-activated potassium channels (BK, also termed BKCa, Slo, or MaxiK) are a group of widely expressed potassium channels found widely in the brain, including hippocampal CA1 pyramidal neurons (Hu et al., 2001). Although BK channels are largely inactivated under normal conditions (Ghatta et al., 2006), channels when activated these stabilize the membrane potential by increasing K^{+} efflux and thereby decrease neuronal excitability (Ghatta et al., 2006). BK channels also influence neuronal signaling and neurotransmitter release during emergency conditions (Li et al., 2000; Wu 2003; Wang et al., 2007). Our experiments are consistent with these reports by showing that PAX has little effect on the pyramidal neuron membrane potential under normal physiological conditions, but only during OGD. The acute effects of RVT were also completely blocked by the BK channel blocker PAX. These results indicate that potentiation of BK channel by RVT is an important channel mechanism accounting for the early neuroprotection we find for RVT of these events.

In summary, by examining the effects of RVT on OGD-induced impairment of hippocampal pyramidal neuron excitability this study has demonstrated that RVT confers neuronal protection in terms of excitability and neuronal death, during the initial time period after ischemia onset. The mechanism of this neuroprotection seems to mostly derive from RVT's action in sustaining the neuronal membrane potential through activating BK channels and not through its antioxidant activity.

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Figure 1. OGD induced neuronal death in the hippocampal CA1 region was largely reduced by RVT

(A) Representative photomicrographs of hippocampal CA1 regions show TO-PRO-3-I fluorescence immediately after 20 min exposure to different treatments: OGD, OGD +100 μ M Resveratrol, OGD + 100 μ M PQQ and control bath solution. Quantification of TO-PRO-3-I staining from 20 min OGD treatment and with the presence of DMSO, RVT, PQQ are shown in B. Bars represent the mean $\pm$ SEM, * compare between the different OGD treatment groups with control * $P < 0.05$, ** $P < 0.01$; ‡ compare between the OGD with OGD + RVT group, ‡ $P < 0.05$, ‡†† $P < 0.01$.

Figure 2. RVT attenuates OGD-induced impairment of pyramidal neuron excitability

A. OGD-induced changes in pyramidal neuron excitability recorded in current clamp mode. During the pre-OGD control period, OGD period and recovery from OGD period, the Vm and excitability changes were periodically measured every 20 seconds by delivering a 200–300 pA/100 ms duration current pulse to the recorded cell. Representative traces from the control and after 15 min. OGD and 5 min. recovery phases, as indicated by the arrows, are displayed in the expanded scale in the lower panel in **A**, showing a marked Vm depolarization and loss of spike number over a 15 min. OGD insult. **B.** Shows that in another pyramidal neuron recording that when 100 μ M RVT was present during OGD Vm and spike number was maintained (**a–c** in the lower panel in **B**).

Figure 3. Quantifications of acute effect of RVT and PQQ on OGD induced excitability changes in pyramidal neuron

The neuronal membrane potential and spike number before, during and after OGD treatment were compared between OGD and OGD plus 100 μ M RVT groups (A–B) or OGD and OGD plus 100 μ M PQQ groups. (A) 15 min OGD causes a time-dependant membrane potential depolarization in the control OGD group which was significantly reduced in the present of RVT. Similarly, RVT also inhibited the OGD-induced the spikes decrease (B). In the same condition, however, PQQ failed to show similar effects (C, D). * and ** indicate that the differences in between OGD and OGD + RVT groups or OGD and OGD + PQQ group were statistically significant at the levels of $p < 0.05$ and $p < 0.01$, respectively.

Figure 4. RVT attenuates the OGD-induced increased spontaneous EPSCs in pyramidal neurons

A. Shows the OGD-induced increased sEPSCs (downward current deflections) for different periods of OGD. **B.** In the presence of 100 μ M RVT, both the OGD-induced increased frequency and amplitude increase of the sEPSCs were essentially inhibited. **C.** Comparison of cumulative probability of sEPSC amplitudes between the control OGD (dashed line) and OGD + RVT (solid line). The data used were taken from **A** and **B** during the 15 min. OGD treatment. **D.** Summary of all the experiments showing that the OGD-induced increase in sEPSC frequency is greatly inhibited by 100 μ M RVT. For the analyses presented in **C** and **D**, only downward current deflections greater than -4 pA from the baseline in all the recordings were considered as acceptable sEPSC events and were included for these analyses. * and ** indicate

that the differences in between OGD and OGD + RVT groups were statistically significant at the levels of $p < 0.05$ and $p < 0.01$, respectively.

Figure 5. The acute effect of RVT on pyramidal neuron during early OGD is mediated by potentiation of BK channels

Voltage-clamp recordings of CA1 pyramidal neurons exposed to a 15 min. OGD treatment at -60 mV holding potential under different conditions as indicated (A). The traces represent the changes in holding current (down indicates negative current reflecting the Vm depolarization shown in Fig. 2A) for OGD, OGD+RVT ($100 \mu\text{M}$) and OGD + RVT + PAX ($10 \mu\text{M}$), as noted on the figure. The relative speed and degree of Vm depolarization under these recording conditions were proportional to the slope and amplitude of the negative holding currents. The OGD induced currents were reduced in the presence of RVT; which was completely blocked by PAX). The negative holding current was greatly increased with PAX. **B**. The inhibition by RVT of the OGD induced sEPSCs frequency was reduced by $10 \mu\text{M}$ PAX to greater than control levels. At each time point, the sEPSC events in each minute during OGD were summed and presented as sEPSC frequency/per min as indicated on the y-axis. **C**. Summary of all the

experiments including additional controls of recording in the bath alone, PAX alone in the bath aCSF and PAX+OGD; the holding currents in of recordings in the bath control was -2.7 ± 3.0 pA, the values of holding currents shift at the end of OGD were -10.9 ± 6.1 pA for PAX alone, -71.6 ± 9.0 pA for OGD alone, -50.6 ± 4.0 pA for OGD+RVT and -128.6 ± 22.7 pA for OGD+RVT+10 μ M PAX.. All p values were determined by one-way ANOVA test. **. Indicates $p < 0.01$ of the difference between OGD and OGD+RVT. ‡‡. Indicates $p < 0.01$ of the difference between OGD+RVT and OGD+RVT+PAX. No difference was found between OGD+PAX and OGD+PAX+RVT groups.