Intracellular Ca\(^{2+}\) release through ryanodine receptors contributes to AMPA receptor-mediated mitochondrial dysfunction and ER stress in oligodendrocytes

A Ruiz\(^1\), C Matute\(^*\)\(^1,2\) and E Alberdi\(^1,2\)

Overactivation of ionotropic glutamate receptors in oligodendrocytes induces cytosolic Ca\(^{2+}\) overload and excitotoxic death, a process that contributes to demyelination and multiple sclerosis. Excitotoxic insults cause well-characterized mitochondrial alterations and endoplasmic reticulum (ER) dysfunction, which is not fully understood. In this study, we analyzed the contribution of ER-Ca\(^{2+}\) release through ryanodine receptors (RyRs) and inositol triphosphate receptors (IP\(_3\)Rs) to excitotoxicity in oligodendrocytes in vitro. First, we observed that oligodendrocytes express all previously characterized RyRs and IP\(_3\)Rs. Blockade of Ca\(^{2+}\)-induced Ca\(^{2+}\) release by TMB-8 following \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor-mediated insults attenuated both oligodendrocyte death and cytosolic Ca\(^{2+}\) overload. In turn, RyR inhibition by ryanodine reduced as well the Ca\(^{2+}\) overload whereas IP\(_3\)R inhibition was ineffective. Furthermore, AMPA-triggered mitochondrial membrane depolarization, oxidative stress and activation of caspase-3, which in all instances was diminished by RyR inhibition. In addition, we observed that AMPA induced an ER stress response as revealed by \(x\) subunit of the eukaryotic initiation factor 2x phosphorylation, overexpression of GRP chaperones and RyR-dependent cleavage of caspase-12. Finally, attenuating ER stress with salubrinal protected oligodendrocytes from AMPA excitotoxicity. Together, these results show that Ca\(^{2+}\) release through RyRs contributes to cytosolic Ca\(^{2+}\) overload, mitochondrial dysfunction, ER stress and cell death following AMPA receptor-mediated excitotoxicity in oligodendrocytes.

Cell Death and Disease (2010) 1, e54; doi:10.1038/cddis.2010.31; published online 15 July 2010

Subject Category: Neuroscience

Oligodendrocytes, the myelinating cells of the CNS, express functional ionotropic glutamate receptors,\(^1\) which can trigger cell death both in vivo and in vitro.\(^2,3\) Therefore, the relevance of glutamate excitotoxicity to neurons in acute injury to the CNS and chronic neurodegenerative disorders\(^4\) has been expanded to oligodendrocytes and demyelinating diseases such as, multiple sclerosis (MS).\(^5\)

Mitochondria are crucial to intracellular Ca\(^{2+}\) homeostasis and accumulation of Ca\(^{2+}\) induced by excitotoxic insults leads to mitochondrial membrane depolarization, increased production of oxygen free radicals and caspase-dependent or -independent oligodendrocyte death.\(^6\) In turn, the endoplasmic reticulum (ER) is also critical to Ca\(^{2+}\) homeostasis and its stress contributes to demyelinating disorders.\(^7\) ER serves as a rapidly exchanging Ca\(^{2+}\) store and contributes to the cytosolic Ca\(^{2+}\) signalling cascade by releasing Ca\(^{2+}\) mainly through ryanodine (RyR) and inositol triphosphate (IP\(_3\)R) receptors.\(^8\) The RyR family has three isoforms, all expressed in the brain, and has multiple allosteric Ca\(^{2+}\)-binding sites responsible for triggering Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) to the cytosol.\(^9\) IP\(_3\)R isoforms I, II and III are also expressed in the brain and activated by Ins(1,4,5)P\(_3\), a metabolic product of phospholipase C (PLC) activity, and are also regulated by IP\(_3\)-independent pathways.\(^10\) These RyR/IP\(_3\)R have a central role in cell survival as well as in apoptotic cell death.\(^11\)

CICR from ER can activate mitochondria-dependent apoptosis by Ca\(^{2+}\) overload of this organelle. Indeed, ER and mitochondria are physically and functionally coupled by microdomains, which include RyRs and IP\(_3\)Rs.\(^12,13\) Thus, Ca\(^{2+}\) signalling between these organelles can initiate apoptosis through mitochondria-specific toxicity events.\(^14\) In addition, ER can induce apoptosis by intrinsic pathways activated by impairment of ER function. In particular, accumulation of unfolded proteins or Ca\(^{2+}\) depletion from ER initiates the unfolded protein response (UPR)\(^15\) by activating the PERK/\(\alpha\) subunit of the eukaryotic initiation factor 2 (eIF2\(\alpha\)) pathway.\(^16\) UPR leads to a shutdown of translation and an overexpression of GRP chaperones to restore ER protein-folding capacity. However, under severe stress ER itself can induce apoptosis by activating caspase-12, which is activated under Ca\(^{2+}\) homeostasis disruption and accumulation of excess of proteins within the ER.\(^17\)

ER stress and excitotoxicity have been associated with demyelinating disorders, but whereas the link between these

---

\(^{1}\)Centro de Investigación Biomédica en Red en Enfermedades Neurodegenerativas (CIBERNED) and Departamento de Neurociencias, Universidad del País Vasco, E-48940 Leioa, Spain

\(^{2}\)Neurotek, Parque Tecnológico de Bizkaia, E-48170 Zamudio, Spain

\(^{*}\)Corresponding author: C Matute, Departamento de Neurociencias, Universidad del País Vasco, Barrio de Sarriena s/n, 48940-Leioa, Spain.

Abbreviations: ER, endoplasmic reticulum; RyR, ryanodine receptor; IP\(_3\)R, inositol triphosphate receptor; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; ROS, reactive oxygen species; eIF2\(\alpha\), \(\alpha\) subunit of the eukaryotic initiation factor 2; GRP78, glucose-regulated protein 78; MS, multiple sclerosis

Received 17.2.10; revised 09.6.10; accepted 10.6.10; Edited by D Bano
two phenomena have been described in neurons, it remains unknown in oligodendrocytes. In this study, we have investigated the contribution of ER-Ca²⁺ release to oligodendroglial excitotoxicity in vitro. In this study, we show that α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor activation induces ER stress and the subsequent activation of caspase-12, as well as cytosolic Ca²⁺ overload and mitochondrial dysfunction, which are all dependent on ER-Ca²⁺ release through RyRs.

Results

RyRs and IP₃Rs are expressed in cultured oligodendrocytes. Both RyRs and IP₃Rs have been widely described in the CNS although the specific distribution of these receptors along the brain remains unclear. To confirm the expression of the different Ry and IP₃ isoforms in our in vitro oligodendroglial model, we performed immunofluorescence labelling for each isoform and the myelin basic protein (MBP) as specific oligodendroglial marker. Oligodendrocytes showed co-expression of the three isoforms of IP₃ (I, II and III) and the MBP (Figure 1). The immunofluorescence and co-expression was also positive for the three isoforms of the RyR (Figure 1). Double immunofluorescence staining using MBP antibodies revealed that IP₃/RyR isoform and MBP co-expression occurred mainly along the body and proximal processes of myelin-producing mature oligodendrocytes. Together, these results indicate that the three isoforms of IP₃Rs and RyRs are present in our in vitro oligodendrocytes derived from rat optic nerve.

Blocking of ER Ca²⁺ release through RyRs reduces cytosolic Ca²⁺ overload and excitotoxicity in oligodendrocytes. AMPA receptor activation induces an increase in cytosolic Ca²⁺ levels that is sufficient to trigger excitotoxicity in cultured oligodendrocytes, with no requirement of Ca²⁺ entry through voltage-activated channels nor Na⁺-Ca²⁺ exchanger. To study the contribution of the ER-Ca²⁺ release to oligodendroglial excitotoxicity, cells were exposed to increasing concentrations of AMPA in the presence or absence of the CICR inhibitor TMB-8. AMPA (25–100 μM) induced a cell death of 34.1 ± 3.6 to 52.3 ± 3.7% of control (untreated cells, n = 4), which was significantly reduced in the presence of TMB-8 (Figure 2a). However, the selective RyR inhibitor ryanodine was not protective (Figure 2b).

To analyze the contribution of CICR, and particularly of RyRs and IP₃Rs, to the AMPA-induced cytosolic Ca²⁺ increase, we measured by microfluorimetry the intracellular Ca²⁺ concentration ([Ca²⁺]i) after application of AMPA (25 μM) to oligodendrocytes in the presence of TMB-8 (50 μM), ryanodine (50 μM) and the IP₃R inhibitor 2APB (10 μM). In AMPA-stimulated oligodendrocytes [Ca²⁺]i increased to 841 ± 65 nM (n = 78 cells), an effect that was greatly reduced in the presence of TMB-8 (492.3 ± 67.4, n = 33 cells) and ryanodine (264 ± 23 nM, n = 96 cells; Figure 2c). However, inhibition of IP₃Rs by 2APB (10 μM) did not attenuate [Ca²⁺]i significantly (Figure 2c). These results indicate that Ca²⁺ release through RyRs but not through IP₃Rs contributes to AMPA-induced cytosolic Ca²⁺ overload and excitotoxicity in oligodendrocytes.

Inhibition of RyRs attenuates AMPA-induced mitochondrial damage and activation of caspase-3 in oligodendrocytes. Mitochondrial Ca²⁺ uptake during excitotoxic insults causes membrane depolarization as well as generation of reactive oxygen species (ROS) in cultured...
First, we used DCFDA to measure ROS generation in AMPA-stimulated (25 μM, 5 min) oligodendrocytes after 30 min of the insult. In these experimental conditions, exposure to AMPA increased ROS levels up to 46.9 ± 11.2%, n = 7 comparing with control (untreated cells, 100%). These levels were significantly attenuated by ryanodine (10 μM) to 11.8 ± 6.9%, n = 7 (Figure 3a).

We next examined whether mitochondrial depolarization was affected by ryanodine after an excitotoxic insult. Oligodendrocytes were stimulated by AMPA (25 μM, 5 min) and mitochondrial membrane potential was measured 1 h later using the fluorescent dye JC-1. AMPA induced in oligodendrocytes a mitochondrial membrane depolarization loss up to 79.01 ± 4% of control (untreated cells, n = 6), which was significantly reduced by ryanodine (91.6 ± 3.8% of control, n = 6) (Figure 3b).

Previous studies have shown that submaximal activation of AMPA receptors lead to apoptosis in oligodendrocytes. As this process is mainly preceded by mitochondrial dysfunction and activation of caspases, we next analyzed whether ER-Ca\(^{2+}\) release through RyRs contributes to this apoptotic cascade. In AMPA-stimulated oligodendrocytes (25 μM, 5 min), pro-caspase-3 protein levels (inactive), 4 h after the stimulus, was reduced to a 57.3% as compared with non-treated cells (control, 100%), a feature that was inhibited in the presence of ryanodine (107.1% of control) (Figure 3c). To confirm caspase-3 activation, the cleaved-caspase-3 activity was quantified by luminescent methods. In AMPA-treated oligodendrocytes (25 μM, 5 min) cleaved caspase-3 activity increased to 120.7 ± 2.6% compared with control (untreated cells, 100%), whereas ryanodine treatment reduced the activity significantly to 104.3 ± 9.8%, n = 5 (Figure 3d). Together, these results indicate that ER Ca\(^{2+}\) release through RyRs contribute to mitochondrial dysfunction and ensuing apoptosis.

**AMPA-mediated excitotoxicity induces an ER stress response in oligodendrocytes.** Neurons undergo ER stress during excitotoxicity-induced Ca\(^{2+}\) dyshomeostasis, an effect that can be mediated by RyRs. To study whether AMPA induces ER stress in cultured oligodendrocytes, we analyzed the phosphorylation of eIF2α, a downstream target of the ER stress sensor PERK, as well as the upregulation of chaperones such as, glucose-regulated protein 78 (GRP78) and GRP94. AMPA stimulus (25 μM, 5 min) produced a strong phosphorylation of eIF2α, as did thapsigargin (5 μM, 30 min), which induces store depletion by blocking ER Ca\(^{2+}\) uptake and serves as positive control (Figure 4a). AMPA triggered a p-eIF2α increase to 474.2 ± 107.6%, n = 4, as compared with non-treated cells (control, 100%) (Figure 4b). We next analyzed GRP78 and GRP94 protein levels during the same excitotoxic insult and found that both were upregulated, up to 169.3 ± 38.9 and 144.5 ± 21.8% of control (non-treated cells, 100%), respectively (Figure 4c). These results indicate that AMPA receptor-mediated excitotoxic insults induce ER stress and a subsequent UPR in cultured oligodendrocytes.

**AMPA produces a RyR-dependent ER stress-induced apoptosis in oligodendrocytes.** Severe or prolonged ER

---

**Figure 2** Inhibition of ER Ca\(^{2+}\) release reduces AMPA-induced excitotoxicity and cytosolic Ca\(^{2+}\) overload in oligodendrocytes. (a, b) Toxicity induced by AMPA is significantly reduced by TMB-8 but not by ryanodine. Oligodendrocytes (DIV 1) were pretreated with TMB-8 (50 μM, 30 min) or ryanodine (50 μM, 45 min) and exposed to increasing concentrations of AMPA in the presence of CTZ (100 μM). Viability was quantified 24 h later by calcein-AM method. Data represent means ± S.E.M. of normalized calcein-AM fluorescence values of at least n = 4 cultures. Statistical significance was assessed with t-test (*P < 0.05) and two-way analysis of variance (ANOVA) analysis (P = 0.034). (c) AMPA (25 μM) together with CTZ (100 μM) induces a rapid increase in cytosolic Ca\(^{2+}\), which is significantly reduced in the presence of ryanodine (50 μM) and TMB-8 (50 μM) but not by 2-APB (10 μM). Neurons were loaded with Fura-2 AM for 30 min before drug application. Traces represent the time course of the [Ca\(^{2+}\)] (means ± S.E.M.) during exposition to agonist of three independent experiments.
stress compromise cell death viability, a mechanism that may be involved in the pathophysiology of brain diseases.\textsuperscript{27} To analyze whether ER-induced apoptosis contributes to excitotoxicity in oligodendrocytes, we analyzed the activation of caspase-12, which resides on the outside of ER membrane and is cleaved and activated during ER stress.\textsuperscript{17} Cells stimulated with AMPA (25 μM, 5 min) underwent caspase-12 cleavage as indicated by pro-caspase-12 levels, which were reduced to 80.7 ± 4%, n = 8 and 65.4 ± 15.9%, n = 5, as compared with non-treated cells (100%), respectively (Figure 5a). In the presence of ryanodine, AMPA-induced caspase-12 cleavage was significantly reduced to 101 ± 8.3% of control, n = 7 (Figure 5b).

Finally, we analyzed whether ER stress-dependent apoptosis is involved in AMPA excitotoxicity. To that end, we tested the protective potential of salubrinal, an inhibitor of p-eIF2α dephosphorylation, which has been shown to ease ER stress apoptosis.\textsuperscript{28} AMPA-induced cell death was attenuated from a control (non-treated cells, 0%) to 6.4 ± 1.3 and 7.5 ± 2.6% by salubrinal 10 and 50 μM treatments, respectively (Figure 5c). Together, these results suggest that AMPA induces an ER stress response in oligodendrocytes, which contributes to excitotoxicity by activation of caspase-12 and is dependent on RyRs.

**Discussion**

ER Ca\textsuperscript{2+} dyshomeostasis, ER stress and neurodegeneration are closely associated because depletion of ER stores causes upregulation of ER stress markers and neuronal death.\textsuperscript{15} In this study, we show that ER-Ca\textsuperscript{2+} release contributes to AMPA-induced cytosolic Ca\textsuperscript{2+} overload and mitochondrial dysfunction leading to apoptosis in oligodendrocytes.
In addition, we show that excitotoxic insults to oligodendrocytes induce ER stress and activation of caspase-12, which is attenuated by blocking RyRs. These data provide novel evidence regarding the association between excitotoxicity and ER stress in oligodendrocytes.

**RyR and IP₃R expression in oligodendrocytes.** IP₃Rs and RyRs are co-expressed in neurons and glia and Ca²⁺ mobilization through these receptors have a crucial role in Ca²⁺ signalling in these cells.²⁹ However, most of the previous studies regarding the expression of these receptors in the brain do not distinguish among different isoforms or cell types. To assess the expression of RyRs and IP₃Rs in oligodendrocytes in vitro, we carried out immunofluorescence experiments to examine the presence in these cells of all previously described isoforms of these receptors. Immunolabelling for the RyRs was positive for the three isoforms in cultured oligodendrocytes, a finding that confirms and extends those reported in a previous study regarding RyR expression in these cells that did not distinguish among different isoforms.²¹ In addition, we found that the three IP₃R isoforms are also present in oligodendrocytes, which is largely consistent with immunohistochemical studies in the rat brain.²⁰

**ER Ca²⁺ release and excitotoxicity.** We showed previously that ER-Ca²⁺ release contributes to neuronal excitotoxicity, but there is no strong evidence yet regarding the association between ER-Ca²⁺ release and excitotoxicity in oligodendrocytes. After assessing the presence of RyRs and IP₃Rs in oligodendrocytes in vitro, we analyzed the contribution of ER-Ca²⁺ release to the cytosolic Ca²⁺ overload and cell death induced by an excitotoxic insult. First, we observed that the prototypic intracellular Ca²⁺ antagonist TMB-8, which has been shown to inhibit RyR-mediated CICR,³³ attenuated excitotoxic cell death in oligodendrocytes. This result is in agreement with previous studies carried out in cultured cerebellar granule cells, which were protected against glutamate neurotoxicity by TMB-8,³⁰ and suggested that ER might be contributing to excitotoxicity.
by eliciting CICR in oligodendrocytes exposed to AMPA. In neurons, CICR is mainly mediated by RyRs and although ryanodine attenuates excitotoxicity in these cells, we did not observe a consistent protective effect of this antagonist in oligodendrocytes in the experimental paradigm used. However, inhibition of RyRs significantly reduced the AMPA-induced cytosolic Ca\(^{2+}\) overload in oligodendrocytes, consistent with previous experiments carried out in neurons and in oligodendrocytes in which the RyR antagonist dantrolene protected these cells against kainic acid-, amyloid \(\beta\)- and glutamate-induced toxicity. In contrast, it has been shown that IP\(_3\)Rs can also be regulated by cytosolic Ca\(^{2+}\) and therefore take part in a CICR. However, treating cells with 2APB, a commonly used IP\(_3\)R inhibitor did not influence AMPA-induced Ca\(^{2+}\) overload in oligodendrocytes. Thus, these data, together with the fact that TMB-8 reduced as well the AMPA-mediated [Ca\(^{2+}\)]\(_i\) overload, suggest that AMPA receptor activation provokes a CICR in a RyR-selective manner in oligodendroglial cells.

**ER-mitochondria crosstalk in excitotoxicity.** It has been shown that ER and mitochondria are physically and functionally coupled in terms of Ca\(^{2+}\) signalling. In particular, previous data suggest that interactions between subdomains involving RyRs in the ER and mitochondria permit Ca\(^{2+}\) signal transmission between these two organelles. Moreover, Ca\(^{2+}\) coupling between RyRs and mitochondria is involved in the activation of mitochondrial apoptosis pathways. Like in neurons, massive Ca\(^{2+}\) influx generated by overactivation of AMPA receptors in oligodendrocytes leads to mitochondrial depolarization, oxidative stress and cleavage of caspase-3. These characteristic events of mitochondrial damage were attenuated by inhibition of RyR-Ca\(^{2+}\) release in AMPA-stimulated oligodendrocytes, consistent with previous results, which showed that RyR- and IP\(_3\)R-mediated Ca\(^{2+}\) release activates the mitochondrial apoptotic pathway in neurons exposed to \(\beta\)-amyloid or to NMDA. These results indicate that although the protective effect of ryanodine has not been directly observed, it interferes with the molecular

![Figure 5](image-url)
mechanisms leading to oligodendrocyte death and therefore suggest that RyR-mediated CICR contributes to mitochondrial damage and apoptosis during oligodendroglial excitotoxicity.

Excitotoxicity and ER stress in oligodendrocytes. Severe ER stress and neurodegeneration are closely related, because UPR activation and ER Ca\(^{2+}\) homeostasis disruption can lead to neuronal apoptosis. In particular, excitotoxic insults induce ER stress and a subsequent UPR in neurons. Furthermore, ER Ca\(^{2+}\) release through RyRs regulates this stress response in neurons. Oligodendrocytes synthesize a large amount of proteins during myelination and as a consequence of that, they are highly sensitive to the disruption of the secretory pathway and ER homeostasis. Recently, activated ER stress pathways have been found in some inherited myelin disorders and MS, but little is known regarding how ER stress is triggered in these cells under pathological conditions. Our results show that AMPA receptor-mediated excitotoxic insults induce an activation of the UPR in oligodendrocytes, probably because of Ca\(^{2+}\) homeostasis disruption. As previously described in neurons exposed to kainate and to NMDA, we observed a fast activation of the eIF2\(\alpha\) pathway, which has been also shown to protect against experimental autoimmune encephalomyelitis (EAE)-induced oligodendrocyte death and demyelination. Upregulation of GRP78 and GRP94 was observed as well, a characteristic event of neuronal injury-related UPR, also found in MS demyelinated lesions and consistent with results obtained from neurons exposed to excitotoxic insults.

In addition to UPR, results reported here suggest that stimulation of AMPA receptors in cultured oligodendrocytes induce an ER stress-induced apoptotic cell death. AMPA-produced excitotoxic cell death was decreased in the presence of salubrinal, an inhibitor of p-eIF2\(\alpha\) dephosphorylation and of ER stress-induced apoptosis. Treatment with salubrinal was previously shown to ameliorate IFN-\(\gamma\)-induced oligodendrocyte loss and hypomyelination. Consistent as well with an ER stress-induced apoptosis, AMPA caused cleavage of caspase-12 in oligodendrocytes, an event that has been observed in ER stress-induced neurodegeneration models and specifically in neurons during excitotoxicity. Furthermore, caspase-12 activation during excitotoxicity was inhibited when RyRs were blocked by ryanodine, indicating that AMPA-induced ER-Ca\(^{2+}\) release might trigger apoptosis through mitochondria-independent pathways.

In summary, the results reported here indicate that ER-Ca\(^{2+}\) release through RyRs contributes to oligodendroglial excitotoxicity in vitro. In AMPA-stimulated oligodendrocytes, inhibition of ER-Ca\(^{2+}\) release results in an attenuation of cytosolic Ca\(^{2+}\) overload, mitochondrial damage and apoptosis. In addition, our data provide evidence that AMPA receptor-mediated excitotoxic insults induce an ER stress response in these cells, which contribute to excitotoxicity by a RyR-dependent activation of caspase-12 and thus ER-specific apoptosis pathways.

Glutamate excitotoxicity is relevant to demyelinating disorders of the CNS including MS. This idea is supported by data showing that AMPA and kainate receptor antagonists ameliorate neurological symptoms in several forms of EAE used to model various stages of MS. However, the contribution of ER-Ca\(^{2+}\) release to oligodendroglial excitotoxicity had not been assessed yet. Thus, our findings indicate that ER-Ca\(^{2+}\) release through RyRs and subsequent ER stress following glutamate insults contribute to oligodendrocyte excitotoxicity. In addition, the molecular intermediaries of ER stress unveiled in this study may represent candidate targets for neurodegenerative and demyelinating diseases undergoing oligodendroglial excitotoxic death.

Materials and Methods

Animals. All experiments were conducted under the supervision and with the approval of our internal animal ethics committee (University of the Basque Country, UPV/EHU). Animals were handled in accordance with the European Communities Council Directive. All possible efforts were made to minimize animal suffering and the number of animals used.

Reagents. AMPA, cyclothiazide (CTZ) and ryanodine were obtained from Ascent Scientific (Bristol, UK). Calcein-AM (calcium acetoxymethyl ester), CM-H\(_2\)DCFDA and JC-1 were purchased from Invitrogen (Barcelona, Spain). HBSS, poly-D-lysine and TMB-8 were obtained from Sigma (St Louis, MO, USA), and salubrinal and 2-APB from Calbiochem (Merck Chemicals, Nottingham, UK).

Optic nerve cultures. Primary cultures of oligodendrocytes derived from the optic nerves of 12-day-old Sprague–Dawley rats (typically 8–10 animals per culture) were obtained as described previously, with modifications. Cells were plated at the density stated below for each experimental procedure, into 24-well plates bearing poly-D-lysine (10\(\mu\)g/ml) coated 12 or 14-mm-diameter coverslips and maintained at 37°C and 5% CO\(_2\) in a chemically defined medium.

Toxicity assays. Cell toxicity assays were performed as described previously with modifications. Cells (1\(\times\)10\(^4\) per well at 1-day in vitro (DIV)) were exposed to AMPA plus CTZ (100\(\mu\)M) in previously described medium for 5 min at 37°C. Antagonists were present before and during the excitotoxic insult and cell viability was assessed 24 h later using calcein-AM fluorimetric assay. All experiments were performed in triplicate/quadruplicate and the values provided are the normalized mean ± S.E.M. of at least three independent experiments.

Intracellular ROS and mitochondrial membrane potential measurements. Oligodendrocytes (DIV 1–3) were exposed to AMPA 25\(\mu\)M plus CTZ 100\(\mu\)M for 5 min in the presence or absence of ryanodine (10\(\mu\)M, 45 min) and loaded with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H\(_2\)DCFDA) for 30 min for the measurement of generated ROS. Calcein-AM (1\(\mu\)M) was used to quantify the number of cells in the reading field and fluorescence was measured as described previously. For quantification of mitochondrial membrane potential, cells were exposed to AMPA plus CTZ (100\(\mu\)M) for 30 min after the excitotoxic stimulus with JC-1 dye for 15 min and red/green fluorescence ratio was measured. All experiments were performed in quadruplicate and the values provided are the normalized mean ± S.E.M. of at least three independent experiments.

Caspase-3 activity quantification. Oligodendrocytes (1\(\times\)10\(^4\) per well) were plated onto poly-D-lysine coated 96-well plates and, after 1-day in vitro, cells were exposed to AMPA 25\(\mu\)M plus CTZ 100\(\mu\)M for 5 min in the presence or absence of ryanodine. After 1 h, Caspase-Glo -3/7 substrate (Promega, Madison, WI, USA) was added, and after 2h caspase-3 activity was measured according to manufacturer’s instructions. All experiments were performed in triplicate and data provided are the mean ± S.E.M. of luminescence values normalized to cell viability values (calcein-AM, 1\(\mu\)M) for each condition.

Immunocytochemistry. For the iPSCs and RyRs expression analysis 1 DIV oligodendrocytes were fixed with 4% paraformaldehyde for 20 min and permeabilized in 1% BSA, 1% normal serum, 0.05% Triton X-100 in PBS for 30 min. Then cells were blocked in 10% BSA, 1% normal serum in PBS for 1 h and incubated first with the anti-MBP antibody (1:500, Stemberger Monoclonals Inc., Baltimore, MD, USA) for 1 h at room temperature in 1% BSA, 1% normal serum.
in PBS. After washing with PBS, cells were labelled with Alexa Fluor 594-conjugated IgG (1 : 200, Molecular Probes, Invitrogen, Barcelona, Spain) for 2 h at RT. Cells were washed in PBS and incubated overnight at 4 ºC with the primary antibody: anti-iP1-R-I (1 : 1000, Affinity Bioreagents, Rockford, IL, USA); anti-iP1-R-II (1 : 50, Santa Cruz Biotechnologies, Heidelberg, Germany); anti-iP1-R-III (1 : 1000, Chemicon, Millipore Ibérica, Madrid, Spain); anti-Ry-R-I (1 : 1000, Chemicon); anti-Ry-R-II (1 : 1000, Chemicon); anti-Ry-R-III (1 : 50, Santa Cruz Biotechnolog). After washing with PBS, Alexa Fluor 488-conjugated secondary antibody (1 : 200, Molecular Probes) was added for 1 h followed by Hoechst 33342 staining (5 μg/ml, 10 min). Finally, coverslips were washed in PBS, mounted using glycergel mounting medium (Dako, Glostrup, Denmark) and analyzed by laser-scanning confocal microscopy (Olympus Fluoview FV500, Barcelona, Spain). Controls without a primary antibody showed no staining.

For the pFL2± quantification, anti-pFL2± (1 : 100, Cell Signalling, Denver, MA, USA) was used as primary antibody and the staining was performed as above.

**Western blotting.** Cells (×10^6) were washed with phosphate buffered saline (PBS, 0.1 M) and harvested in 20 μl of ice-cold electrophoresis sample buffer. Lysates were boiled for 10 min and separated by 10–15% SDS-polyacrilamide gel electrophoresis, depending on the experiment. Samples were transferred overnight to nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Barcelona, Spain), blocked in 5% skimmed milk, 5% serum in TTBS overnight at 4 ºC, washed with TTBS, then incubated with specific primary antibodies in 5% BSA in TTBS overnight at 4 ºC, washed again with TTBS, and proteins detected by chemiluminescence (Super Signal West Dura, Pierce, Rockford, IL, USA). Signals were quantified using Image-J software (NIH, Bethesda, MA, USA) and values were normalized to β-actin signal and provided as the mean ± S.E.M. of at least three independent experiments.

**Measurement of [Ca2+].** The concentration of intracellular Ca2+ ([Ca2+]i) was determined as previously described in detail 4,5. Oligodendrocytes (10^4 per well) were pre-incubated with fura-2 AM (Invitrogen) at 5 μM in culture medium for 30–45 min at 37 ºC. Antagonists were continuously perfused for 5 min before and during the AMPA plus CTZ stimulus. The ([Ca2+]i) was estimated by the 340/380 ratio method and data were analyzed with Excel (Microsoft; Seattle, WA, USA) and Prism (Lake Forest, CA, USA) software.

**Data analysis.** All data are expressed as mean ± S.E.M. (n), where n refers to the number of cultures assayed, each obtained from a different group of animals. In [Ca2+]i measurement experiments, n refers to number of cells obtained from at least three different cultures obtained from different groups of animals. Statistical analysis was carried out with the two-way analysis of variance or paired Student’s t-test and significance was determined at P<0.05.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgements.** We thank O López, S Martín and H Gómez, for technical assistance. This study was supported by CIBERNED, European Leukodystrophy Association, and from Ministerio de Educación y Ciencia, Gobierno Vasco and Universidad del País Vasco. Technical and human support provided by SGiker (UPV/EHU, MECINN, GVEJ, ESF) is gratefully acknowledged. AR held a fellowship from Gobierno Vasco and Universidad del País Vasco.
34. Csordas G, Thomas AP, Hajnoczky G. Calcium signal transmission between ryanodine receptors and mitochondria in cardiac muscle. Trends Cardiovasc Med 2001; 11: 269–275.
35. Ferreiro E, Oliveira CR, Pereira CM. The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. Neurobiol Dis 2008; 30: 331–342.
36. Lin W, Bailey SL, Ho H, Harding HP, Ron D, Miller SD et al. The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage. J Clin Invest 2007; 117: 448–456.
37. Mhaille AN, McQuaid S, Windebank A, Cunnea P, McMahon J, Samali A et al. Increased expression of endoplasmic reticulum stress-related signaling pathway molecules in multiple sclerosis lesions. J Neuropathol Exp Neurol 2008; 67: 200–211.
38. Hetz C, Ruscellakis-Carneiro M, Maundrell K, Castilla J, Soto C. Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. EMBO J 2003; 22: 5435–5445.
39. Matute C, Alberdi E, Domeroc M, Perez-Cerda F, Perez-Samartin A, Sanchez-Gomez MV. The link between excitotoxic oligodendroglial death and demyelinating diseases. Trends Neurosci 2001; 24: 224–230.
40. Barres BA, Hart IK, Bums HS, Voyvodic JT, Richardson WD et al. Cell death and control of cell survival in the oligodendrocyte lineage. Cell 1992; 70: 31–46.
41. Sanchez-Gomez MV, Matute C. AMPA and kainate receptors each mediate excitotoxicity in oligodendroglial cultures. Neurobiol Dis 1999; 6: 475–485.
42. Campos-Esparza MR, Sanchez-Gomez MV, Matute C. Molecular mechanisms of neuroprotection by two natural antioxidant polyphenols. Cell Calcium 2009; 45: 358–368.
43. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260: 3440–3450.