

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

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Overactivation of ionotropic glutamate receptors in oligodendrocytes induces cytosolic  $\text{Ca}^{2+}$  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- $\text{Ca}^{2+}$  release through ryanodine receptors (RyRs) and inositol triphosphate receptors ( $\text{IP}_3\text{Rs}$ ) to excitotoxicity in oligodendrocytes *in vitro*. First, we observed that oligodendrocytes express all previously characterized RyRs and  $\text{IP}_3\text{Rs}$ . Blockade of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release by TMB-8 following  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor-mediated insults attenuated both oligodendrocyte death and cytosolic  $\text{Ca}^{2+}$  overload. In turn, RyR inhibition by ryanodine reduced as well the  $\text{Ca}^{2+}$  overload whereas  $\text{IP}_3\text{R}$  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  $\alpha$  subunit of the eukaryotic initiation factor 2 $\alpha$  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  $\text{Ca}^{2+}$  release through RyRs contributes to cytosolic  $\text{Ca}^{2+}$  overload, mitochondrial dysfunction, ER stress and cell death following AMPA receptor-mediated excitotoxicity in oligodendrocytes.

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

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

a metabolic product of phospholipase C (PLC) activity, and are also regulated by  $\text{IP}_3$ -independent pathways.<sup>10</sup> These RyR/ $\text{IP}_3\text{R}$  have a central role in cell survival as well as in apoptotic cell death.<sup>11</sup>

CICR from ER can activate mitochondria-dependent apoptosis by  $\text{Ca}^{2+}$  overload of this organelle. Indeed, ER and mitochondria are physically and functionally coupled by microdomains, which include RyRs and  $\text{IP}_3\text{Rs}$ .<sup>12,13</sup> Thus,  $\text{Ca}^{2+}$  signalling between these organelles can initiate apoptosis through mitochondria-specific toxicity events.<sup>14</sup> In addition, ER can induce apoptosis by intrinsic pathways activated by impairment of ER function. In particular, accumulation of unfolded proteins or  $\text{Ca}^{2+}$  depletion from ER initiates the unfolded protein response (UPR)<sup>15</sup> by activating the PERK/ $\alpha$  subunit of the eukaryotic initiation factor 2 (eIF2) $\alpha$  pathway.<sup>16</sup> 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  $\text{Ca}^{2+}$  homeostasis disruption and accumulation of excess of proteins within the ER.<sup>17</sup>

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

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

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two phenomena have been described in neurons,<sup>18,19</sup> it remains unknown in oligodendrocytes. In this study, we have investigated the contribution of ER-Ca<sup>2+</sup> release to oligodendroglial excitotoxicity *in vitro*. In this study, we show that  $\alpha$ -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 Ca<sup>2+</sup> overload and mitochondrial dysfunction, which are all dependent on ER-Ca<sup>2+</sup> release through RyRs.

## Results

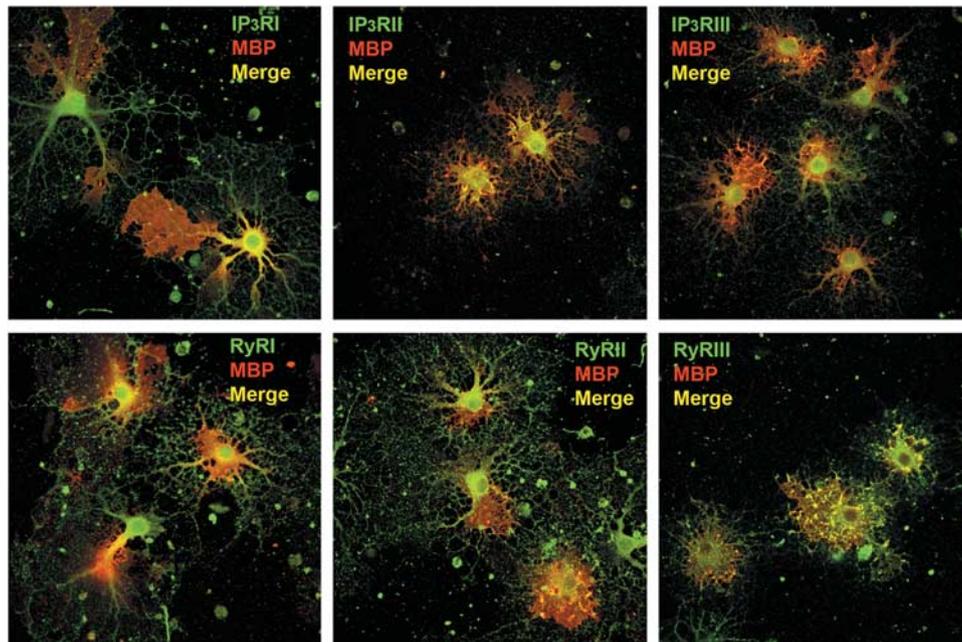
**RyRs and IP<sub>3</sub>Rs are expressed in cultured oligodendrocytes.** Both RyRs and IP<sub>3</sub>Rs have been widely described in the CNS<sup>20,21</sup> although the specific distribution of these receptors along the brain remains unclear. To confirm the expression of the different RyR and IP<sub>3</sub>R 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 IP<sub>3</sub>R (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 IP<sub>3</sub>R/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 IP<sub>3</sub>Rs and RyRs are present in our *in vitro* oligodendrocytes derived from rat optic nerve.

**Blocking of ER Ca<sup>2+</sup> release through RyRs reduces cytosolic Ca<sup>2+</sup> overload and excitotoxicity in oligodendrocytes.** AMPA receptor activation induces an

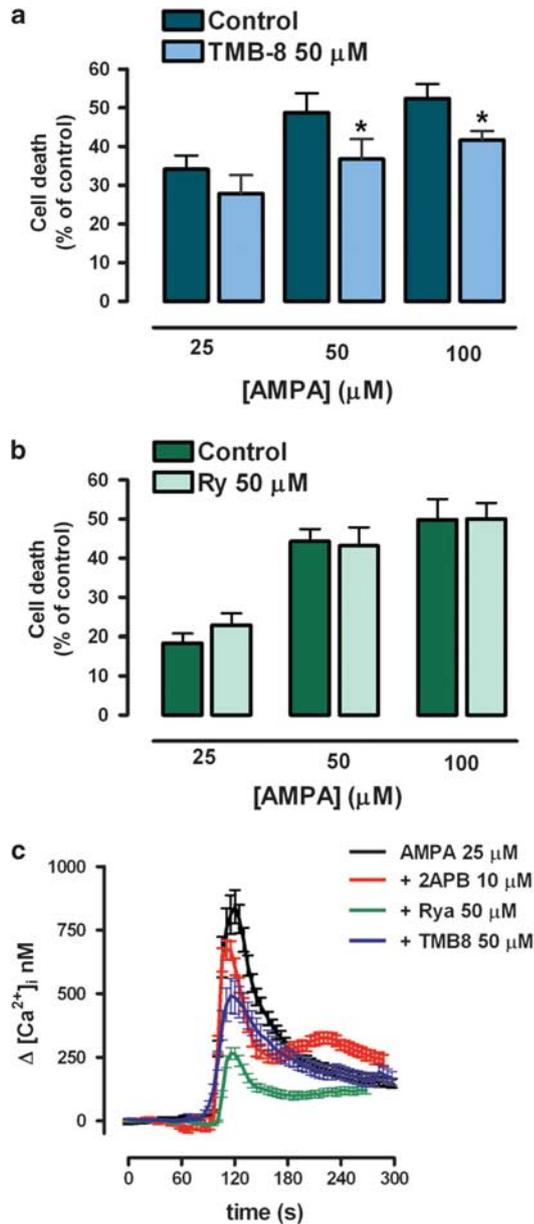
increase in cytosolic Ca<sup>2+</sup> levels that is sufficient to trigger excitotoxicity in cultured oligodendrocytes, with no requirement of Ca<sup>2+</sup> entry through voltage-activated channels nor Na<sup>+</sup>-Ca<sup>2+</sup> exchanger.<sup>22</sup> To study the contribution of the ER-Ca<sup>2+</sup> release to oligodendroglial excitotoxicity, cells were exposed to increasing concentrations of AMPA in the presence or absence of the CICR inhibitor TMB-8.<sup>23</sup> AMPA (25–100  $\mu$ M) induced a cell death of 34.1  $\pm$  3.6 to 52.3  $\pm$  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<sup>9</sup> was not protective (Figure 2b).

To analyze the contribution of CICR, and particularly of RyRs and IP<sub>3</sub>Rs, to the AMPA-induced cytosolic Ca<sup>2+</sup> increase, we measured by microfluorimetry the intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) after application of AMPA (25  $\mu$ M) to oligodendrocytes in the presence of TMB-8 (50  $\mu$ M), ryanodine (50  $\mu$ M) and the IP<sub>3</sub>R inhibitor 2APB (10  $\mu$ M).<sup>24</sup> In AMPA-stimulated oligodendrocytes [Ca<sup>2+</sup>]<sub>i</sub> increased to 841  $\pm$  65 nM ( $n=78$  cells), an effect that was greatly reduced in the presence of TMB-8 (492.3  $\pm$  67.4,  $n=33$  cells) and ryanodine (264  $\pm$  23 nM,  $n=96$  cells; Figure 2c). However, inhibition of IP<sub>3</sub>Rs by 2APB (10  $\mu$ M) did not attenuate [Ca<sup>2+</sup>]<sub>i</sub> significantly (Figure 2c). These results indicate that Ca<sup>2+</sup> release through RyRs but not through IP<sub>3</sub>Rs contributes to AMPA-induced cytosolic Ca<sup>2+</sup> overload and excitotoxicity in oligodendrocytes.

**Inhibition of RyRs attenuates AMPA-induced mitochondrial damage and activation of caspase-3 in oligodendrocytes.** Mitochondrial Ca<sup>2+</sup> uptake during excitotoxic insults causes membrane depolarization as well as generation of reactive oxygen species (ROS) in cultured



**Figure 1** IP<sub>3</sub>Rs and RyRs are expressed in cultured oligodendrocytes. Oligodendrocytes (oligodendrocyte-specific myelin basic protein (MBP), red) are stained with antibodies to IP<sub>3</sub>R-I, II, III and to RyR-I, II, III (both in green). Co-expression of receptors and MBP immunofluorescence is shown in merged images (yellow). All six isoforms are expressed in MBP<sup>+</sup> cells. Scale bar: 50  $\mu$ m



**Figure 2** Inhibition of ER Ca<sup>2+</sup> release reduces AMPA-induced excitotoxicity and cytosolic Ca<sup>2+</sup> 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<sup>2+</sup>, 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<sup>2+</sup>]<sub>i</sub> (means ± S.E.M.) during exposition to agonist of three independent experiments

rat oligodendrocytes.<sup>6</sup> As mitochondria and ER can interact through RyRs,<sup>25</sup> we analyzed whether inhibition of RyRs may attenuate mitochondrial dysfunction as a consequence of AMPA-induced excitotoxicity.

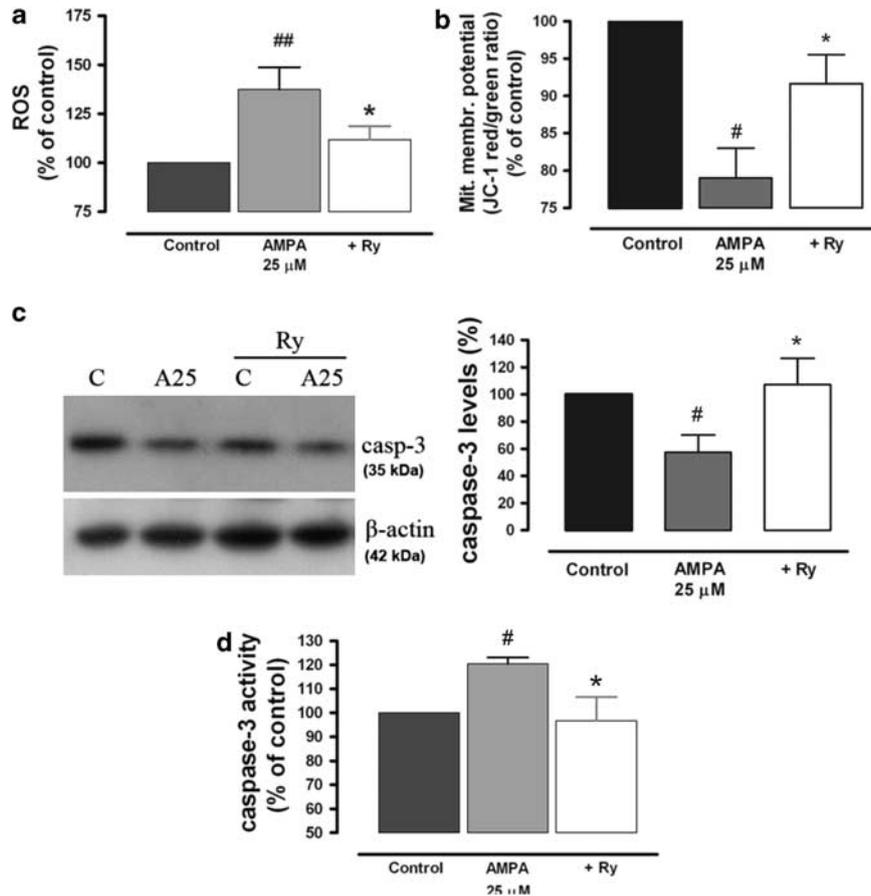
First, we used DCFDA to measure ROS generation in AMPA-stimulated (25 μM, 5 min) oligodendrocytes after 30 min of the insult. In these experimental conditions, exposure to AMPA increased ROS levels up to 46.9 ± 11.2%, *n* = 7 comparing with control (untreated cells, 100%). These levels were significantly 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.<sup>6</sup> As this process is mainly preceded by mitochondrial dysfunction and activation of caspases, we next analyzed whether ER-Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> dyshomeostasis,<sup>18,19</sup> an effect that can be mediated by RyRs.<sup>26</sup> 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<sup>2+</sup> 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 3** RyR blockade attenuates oxidative stress, mitochondrial depolarization and apoptosis caused by AMPA. (a) Ryanodine reduces AMPA-induced ROS generation in cultured oligodendrocytes. Cells were stimulated with AMPA (25 μM, 5 min) plus CTZ (100 μM) in the presence of ryanodine (10 μM). ROS levels were quantified at 30 min after the insult using the CM-H<sub>2</sub>DCFDA probe (30 μM). Data represent means ± S.E.M. of the % of CM-DCFDA/calcein-AM of *n* = 7 cultures. (b) Inhibition of ER Ca<sup>2+</sup> release through RyRs attenuates AMPA-induced mitochondrial membrane depolarization. Cells were stimulated with AMPA (25 μM, 5 min) and CTZ (100 μM) in the presence of ryanodine (10 μM) and mitochondrial membrane potential was measured using JC-1 fluorescent dye 1 h after AMPA application. Data represent normalized means ± S.E.M. of the JC-1 red/green fluorescence ratio of *n* = 6 cultures. (c) Caspase-3 cleavage is diminished in the presence of ryanodine in AMPA-stimulated oligodendrocytes. Cells were exposed to AMPA (25 μM, 5 min) plus CTZ (100 μM) after preincubation with ryanodine (10 μM) and harvested 4 h later for the detection of procaspase-3 by western blot. Data represent optical density of procaspase-3 levels normalized to β-actin values of *n* = 4 cultures. (d) AMPA-induced caspase-3 activity is reduced in the presence of ryanodine. Cells were exposed to AMPA (25 μM, 5 min) and CTZ (100 μM) and Caspase-Glo 3/7 Reagent was added 1 h later. After 2 h, luminescent signal was measured and data represent mean ± S.E.M. of luminescence values normalized to cell viability values (calcein-AM, 1 μM) for each condition of *n* = 5 cultures. #*P* < 0.05, ##*P* < 0.01 compared with control (untreated cells); \**P* < 0.05 compared with control (AMPA alone), paired Student's *t*-test

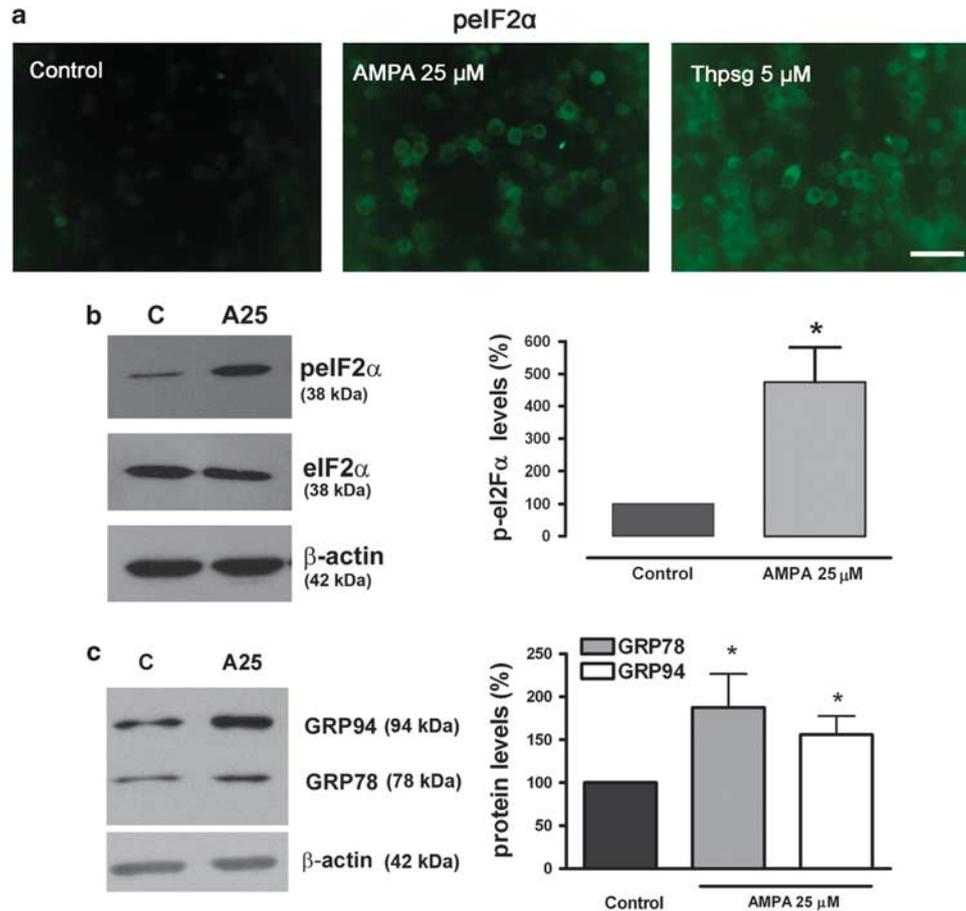
stress compromise cell death viability, a mechanism that may be involved in the pathophysiology of brain diseases.<sup>27</sup> To analyze whether ER-induced apoptosis contributes to excitotoxicity in oligodendrocytes, we analyzed the activation of caspase-12, which resides on the outside of ER membrane and is cleaved and activated during ER stress.<sup>17</sup> Cells stimulated with AMPA (25 and 100 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.<sup>28</sup> AMPA-induced cell death was attenuated from a 13% of 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 that is dependent on RyRs.

## Discussion

ER Ca<sup>2+</sup> dyshomeostasis, ER stress and neurodegeneration are closely associated because depletion of ER stores causes upregulation of ER stress markers and neuronal death.<sup>15</sup> In this study, we show that ER-Ca<sup>2+</sup> release contributes to AMPA-induced cytosolic Ca<sup>2+</sup> overload and mitochondrial dysfunction leading to apoptosis in oligodendrocytes.



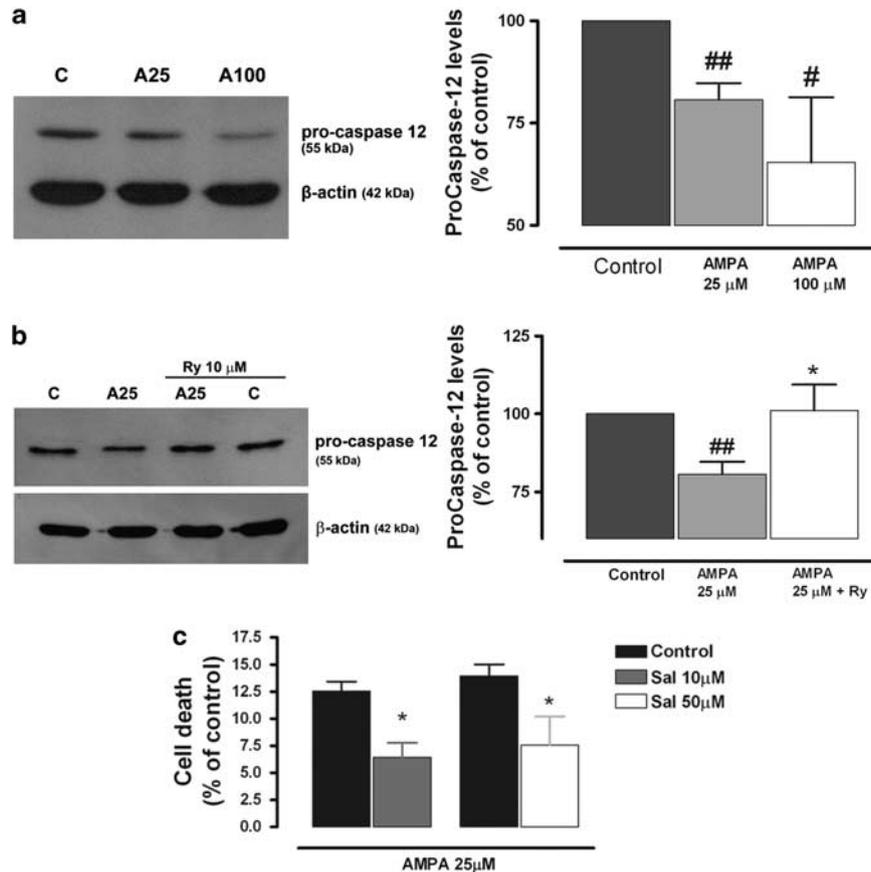
**Figure 4** AMPA induces an ER stress response in oligodendrocytes *in vitro*. (a) Excitotoxic insults induce an increase in pelf2 $\alpha$  levels in oligodendrocytes. Cells were stimulated with AMPA (25  $\mu$ M, 5 min) together with CTZ (100  $\mu$ M) and thapsigargin (5  $\mu$ M, 30 min), fixed 30 min later and labelled with an antibody against pelf2 $\alpha$ . Scale bar: 50  $\mu$ m. (b) Total protein samples were extracted after AMPA (25  $\mu$ M, 5 min, A25) incubation and eIF2 $\alpha$  and pelf2 $\alpha$  levels were quantified by western blot. Data represent the means  $\pm$  S.E.M. of optical density of pelf2 $\alpha$  levels normalized to  $\beta$ -actin values of  $n = 4$  cultures. (c) Excitotoxicity induces an upregulation of GRP chaperones in oligodendrocytes. Total protein samples were extracted 30 min after the AMPA (25  $\mu$ M, 5 min, A25) plus CTZ (100  $\mu$ M) stimulus and GRP78 and GRP94 were identified by western blot using an anti-KDEL sequence antibody. Data represent the means  $\pm$  S.E.M. of optical density values normalized to corresponding  $\beta$ -actin signal of  $n = 8$  cultures. \* $P < 0.01$  compared with non-treated cells; paired Student's *t*-test

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 provides novel evidence regarding the association between excitotoxicity and ER stress in oligodendrocytes.

**RyR and IP<sub>3</sub>R expression in oligodendrocytes.** IP<sub>3</sub>R and RyRs are co-expressed in neurons and glia and Ca<sup>2+</sup> mobilization through these receptors have a crucial role in Ca<sup>2+</sup> signalling in these cells.<sup>29</sup> 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 IP<sub>3</sub>R 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.<sup>21</sup> In addition, we found that the three IP<sub>3</sub>R isoforms are also present in oligodendrocytes, which is largely consistent with immunohistochemical studies in the rat brain.<sup>20</sup>

**ER-Ca<sup>2+</sup> release and excitotoxicity.** We showed previously<sup>18</sup> that ER-Ca<sup>2+</sup> release contributes to neuronal excitotoxicity, but there is no strong evidence yet regarding the association between ER-Ca<sup>2+</sup> release and excitotoxicity in oligodendrocytes. After assessing the presence of RyRs and IP<sub>3</sub>R in oligodendrocytes *in vitro*, we analyzed the contribution of ER-Ca<sup>2+</sup> release to the cytosolic Ca<sup>2+</sup> overload and cell death induced by an excitotoxic insult. First, we observed that the prototypic intracellular Ca<sup>2+</sup> antagonist TMB-8, which has been shown to inhibit RyR-mediated CICR,<sup>23</sup> 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,<sup>30</sup> and suggested that ER might be contributing to excitotoxicity



**Figure 5** RyR-dependent ER stress-induced apoptosis contributes to excitotoxicity. (a) AMPA (25 and 100  $\mu$ M) plus CTZ (100  $\mu$ M) induces a dose-dependent cleavage of caspase-12. Cells were stimulated for 5 min, total protein extracts were prepared 1 h later and pro-caspase-12 was detected by western blot using a specific antibody. Data represent the means  $\pm$  S.E.M. of optical density caspase-12 values normalized to  $\beta$ -actin values of  $n=8$  and  $n=5$  cultures respectively. (b) Cleavage of pro-caspase-12 was reduced in the presence of ryanodine (10  $\mu$ M, 45 min) in AMPA-stimulated oligodendrocytes. Data represent the means  $\pm$  S.E.M. of optical density caspase-12 values normalized to  $\beta$ -actin values of  $n=8$  cultures. (c) AMPA-induced oligodendroglial death is reduced by salubrinal (Sal), an ER-stress apoptosis inhibitor. Cultured cells were exposed to AMPA (25  $\mu$ M, 5 min) and CTZ (100  $\mu$ M) after treatment with Sal 10 and 50  $\mu$ M for 30 min. To determine cell death, calcein-AM vital dye was used 24 h later ( $n=4$  cultures). Data represent normalized means  $\pm$  S.E.M. # $P<0.05$ ; ## $P<0.01$  compared with non-treated cells. \* $P<0.05$  compared with AMPA alone; paired Student's *t*-test

by eliciting CICR in oligodendrocytes exposed to AMPA. In neurons, CICR is mainly mediated by RyRs and although ryanodine attenuates excitotoxicity in these cells,<sup>18</sup> 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<sup>2+</sup> 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.<sup>31–33</sup> In contrast, it has been shown that IP<sub>3</sub>Rs can also be regulated by cytosolic Ca<sup>2+</sup><sup>10</sup> and therefore take part in a CICR. However, treating cells with 2APB, a commonly used IP<sub>3</sub>R inhibitor<sup>24</sup> did not influence AMPA-induced Ca<sup>2+</sup> overload in oligodendrocytes. Thus, these data, together with the fact that TMB-8 reduced as well the AMPA-mediated [Ca<sup>2+</sup>]<sub>i</sub> 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<sup>2+</sup> signalling.<sup>13</sup> In particular, previous data suggest that interactions between subdomains involving RyRs in the ER and mitochondria permit Ca<sup>2+</sup> signal transmission between these two organelles.<sup>25,34</sup> Moreover, Ca<sup>2+</sup> coupling between RyRs and mitochondria is involved in the activation of mitochondrial apoptosis pathways.<sup>11</sup> Like in neurons, massive Ca<sup>2+</sup> influx generated by overactivation of AMPA receptors in oligodendrocytes leads to mitochondrial depolarization, oxidative stress and cleavage of caspase-3.<sup>6</sup> These characteristic events of mitochondrial damage were attenuated by inhibition of RyR-Ca<sup>2+</sup> release in AMPA-stimulated oligodendrocytes, consistent with previous results, which showed that RyR- and IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release activates the mitochondrial apoptotic pathway in neurons exposed to  $\beta$ -amyloid<sup>35</sup> or to NMDA.<sup>18</sup> These results indicate that although the protective effect of ryanodine has not been directly observed, it interferes with the molecular

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<sup>2+</sup> homeostasis disruption can lead to neuronal apoptosis.<sup>15</sup> In particular, excitotoxic insults induce ER stress and a subsequent UPR in neurons.<sup>18,19</sup> Furthermore, ER Ca<sup>2+</sup> release through RyRs regulates this stress response in neurons.<sup>18</sup>

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<sup>7</sup> 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<sup>2+</sup> homeostasis disruption. As previously described in neurons exposed to kainate<sup>19</sup> and to NMDA,<sup>18</sup> 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.<sup>36</sup> Upregulation of GRP78 and GRP94 was observed as well, a characteristic event of neuronal injury-related UPR,<sup>27</sup> also found in MS demyelinated lesions<sup>37</sup> and consistent with results obtained from neurons exposed to excitotoxic insults.<sup>18,19</sup>

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.<sup>28</sup> Treatment with salubrinal was previously shown to ameliorate IFN- $\gamma$ -induced oligodendrocyte loss and hypomyelination.<sup>36</sup> 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<sup>17,38</sup> and specifically in neurons during excitotoxicity.<sup>19</sup> Furthermore, caspase-12 activation during excitotoxicity was inhibited when RyRs were blocked by ryanodine, indicating that AMPA-induced ER-Ca<sup>2+</sup> release might trigger apoptosis through mitochondria-independent pathways.

In summary, the results reported here indicate that ER-Ca<sup>2+</sup> release through RyRs contributes to oligodendroglial excitotoxicity *in vitro*. In AMPA-stimulated oligodendrocytes, inhibition of ER-Ca<sup>2+</sup> release results in an attenuation of cytosolic Ca<sup>2+</sup> 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<sup>39</sup> used to model various stages of MS. However, the contribution of ER-Ca<sup>2+</sup> release to oligodendroglial excitotoxicity had not been assessed yet. Thus, our findings indicate that ER-Ca<sup>2+</sup> 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 (calcein acetoxymethyl ester), CM-H<sub>2</sub>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,<sup>40</sup> with modifications.<sup>22</sup> 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<sub>2</sub> in a chemically defined medium.<sup>40</sup>

**Toxicity assays.** Cell toxicity assays were performed as described previously<sup>41</sup> with modifications. Cells ( $1 \times 10^4$  per well at 1-day *in vitro* (DIV1)) were exposed to AMPA plus CTZ (100  $\mu$ M) in previously described medium<sup>40</sup> 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  $\pm$  S.E.M. of at least three independent experiments.

**Intracellular ROS and mitochondrial membrane potential measurements.** Oligodendrocytes (DIV 1;  $1 \times 10^4$  per well) were stimulated with AMPA 25  $\mu$ M plus CTZ 100  $\mu$ M for 5 min in the absence or presence of ryanodine (10  $\mu$ M, 45 min) and loaded with 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub> 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.<sup>42</sup> For quantification of mitochondrial membrane potential, cells were loaded 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  $\pm$  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 2 h caspase-3 activity was measured according to manufacturer's instructions. All experiments were performed in triplicate and data provided are the mean  $\pm$  S.E.M. of luminescence values normalized to cell viability values (calcein-AM, 1  $\mu$ M) for each condition.

**Immunocytochemistry.** For the IP<sub>3</sub>R and RyR 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, Stenberger 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 °C with the primary antibody: anti-IP<sub>3</sub>R-I (1:1000, Affinity Bioreagents, Rockford, IL, USA); anti-IP<sub>3</sub>R-II (1:50, Santa Cruz Biotechnologies, Heidelberg, Germany); anti-IP<sub>3</sub>R-III (1:1000, Chemicon, Millipore Iberica, Madrid, Spain); anti-RyR-I (1:1000, Chemicon); anti-RyR-II (1:1000, Chemicon); anti-RyR-III (1:50, Santa Cruz Biotechnology). After washing with PBS, Alexa Fluor 488-conjugated secondary antibody (1:200, Molecular Probes) was added for 1 h followed by Hoechst 33258 staining (5 µg/ml, 10 min). Finally, coverslips were washed in PBS, mounted using glycerol 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 pelf2F $\alpha$  quantification, anti-pelf2F $\alpha$  (1:100, Cell Signalling, Denver, MA, USA) was used as primary antibody and the staining was performed as above.

**Western blotting.** Cells ( $1 \times 10^5$ ) 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-polyacrylamide 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 and proteins detected by specific primary antibodies in 5% BSA in TTBS overnight at 4 °C: anti-caspase-3 (1:1000, Santa Cruz Biotechnologies); anti-pelf2F $\alpha$  and anti-elf2F $\alpha$  (1:1000, Cell Signalling); anti-KDEL (Grp78, Grp94) (1:1000, Stressgen Bioreagents, Ann Harbor, MI, USA); anti-caspase-12 (1:1000, Calbiochem); anti- $\beta$ -actin (1:2000, Sigma). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Sigma) in 5% skimmed milk, 1% normal serum in TTBS for 2-h RT and developed using enhanced chemiluminescence according to the manufacturer's instructions (SuperSignal West Dura, Pierce, Rockford, IL, USA). Signals were quantified using Image-J software (NIH, Bethesda, MA, USA) and values were normalized to  $\beta$ -actin signal and provided as the mean  $\pm$  S.E.M. of at least three independent experiments.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub>.** The concentration of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) was determined as previously described in detail.<sup>6,43</sup> 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 °C. Antagonists were continuously perfused for 5 min before and during the AMPA plus CTZ stimulus. The [Ca<sup>2+</sup>]<sub>i</sub> 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  $\pm$  S.E.M. (*n*), where *n* refers to the number of cultures assayed, each obtained from a different group of animals. In [Ca<sup>2+</sup>]<sub>i</sub> 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.

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