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A role for intracellular zinc in glioma alteration of neuronal chloride equilibrium

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Glioma patients commonly suffer from epileptic seizures. However, the mechanisms of glioma-associated epilepsy are far from being completely understood. Using glioma-neurons co-cultures, we found that tumor cells are able to deeply influence neuronal chloride homeostasis, by depolarizing the reversal potential of γ-aminobutyric acid (GABA)-evoked currents (E_{GABA}). E_{GABA} depolarizing shift is due to zinc-dependent reduction of neuronal KCC2 activity and requires glutamate release from glioma cells. Consistently, intracellular zinc loading rapidly depolarizes E_{GABA} in mouse hippocampal neurons, through the Src/Trk pathway and this effect is promptly reverted upon zinc chelation. This study provides a possible molecular mechanism linking glioma invasion to excitation/inhibition imbalance and epileptic seizures, through the zinc-mediated disruption of neuronal chloride homeostasis.

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Results

Glioma co-culture increases neuronal [Cl−], by a glutamatergic mechanism. To address the effect of glioma cells on neuronal Cl− equilibrium, we determined the reversal potential of the currents evoked by GABA application in mouse hippocampal cultured neurons. As shown in Figures 1a and b, co-culturing neurons with patient-derived glioma cells (MZC) caused a rightward shift in the current–voltage relationship of GABA-mediated responses, giving a positive shift of E_{GABA} from −73.9 ± 1.2 mV (control; n = 124) to −52.1 ± 1.6 mV (co-culture; n = 101, P < 0.001).

In control neurons, E_{GABA} was significantly below resting membrane potential (RMP); conversely, in co-cultured neurons, despite a small depolarization of resting potential (Figure 1b), E_{GABA} value was consistently more positive than RMP, reverting the driving force for GABA-mediated currents (Figure 1c). Similar results were observed also after a shorter (4h) co-culture duration (E_{GABA} = −52.2 ± 4.4 mV; n = 18). E_{GABA} shift resulted from the increase in [Cl−], as directly demonstrated using a genetically encoded Cl-Sensor, which gave values similar to those calculated by Nernst equation (Figure 1d).

To investigate the possible role of glutamate released by glioma cells in E_{GABA} depolarization, glutamate receptors (GlurRs) antagonists (D−)-2-Amino-5-phosphonopentanoic acid (APV), 20 μM; 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydronbenzo [f]quinoloxaline-7-sulfonamide disodium salt (NBQX), 10 μM) were added to the co-culture medium. In this condition,
glioma-induced $E_{\text{GABA}}$ depolarizing shift was prevented (Figure 2a). Similar effects were observed when co-culture experiments were performed in the sole presence of APV ($E_{\text{GABA}} = -73.9 \pm 1.2$ mV; $n = 124/101$, $P < 0.001$ ANOVA) or NBQX ($E_{\text{GABA}} = -70.5 \pm 7.7$; $n = 6$, $P < 0.01$), indicating that activation of both AMPA and NMDA receptors is necessary to cause $E_{\text{GABA}}$ shift.

In addition, when the system Xc blocker sulfasalazine (250 μM) was added to the co-culture medium, $E_{\text{GABA}}$ depolarization was prevented (Figure 2a), showing that this effect requires Xc-mediated glutamate release.

Moreover, acutely applied glioma-conditioned medium (GCM) activated APV/NBQX-sensitive inward currents (Figure 2b) in hippocampal neurons, confirming the presence of GluRs agonists in GCM.17

Altogether, these data show that in the co-culture conditions, glioma cells cause the depolarizing shift of $E_{\text{GABA}}$ equilibrium potential in neurons, through glutamate release and GluRs activation. Consistently, $E_{\text{GABA}}$ depolarization was observed in neurons co-cultured with different human and murine glioma cells, but not with astrocytes (Figure 2c, and Supplementary Table S1), indicating a tumor-specific effect.

**Glioma-induced $E_{\text{GABA}}$ depolarization is due to Cl⁻ transporters unbalance.** In cultured hippocampal neurons, KCC2 and NKCC1 transporters participate in the regulation of $E_{\text{GABA}}$. Indeed, the acute application of furosemide (100 μM; 5–15 min), the blocker of both transporters, caused a minor shift in neuronal $E_{\text{GABA}}$. Conversely, the specific blockers of NKCC1 (bumetanide; 10 μM; acutely applied 5–15 min) and KCC2 (R- (+)-butylindazone dihydroindenyl-oxy alkanoic acid, DIOA; 10 μM) in opposite directions (Figure 3a), indicating that, in our culture conditions, both transporters actively regulate $[\text{Cl}^-]_i$.

The alteration of Cl⁻ transporter activity is involved in glioma-induced $E_{\text{GABA}}$ shift in co-culture. In fact, in co-cultured neurons, furosemide, bumetanide or DIOA treatment abolished $E_{\text{GABA}}$ depolarization (Figure 3b). However, the acute application of bumetanide reverted the glioma-induced effect.
Glioma co-culture causes KCC2 impairment by increasing intracellular Zn$^{2+}$ in neurons. To investigate a possible role of intracellular Zn$^{2+}$ on glioma-induced $E_{\text{GABA}}$ shift, we used the membrane permeant Zn$^{2+}$ chelator N.N.N.N’-tetraakis(2-pyridylmethylen)ethylenediamine (TPEN). TPEN application (20 μM; 5–20 min) caused the rapid recovery of neuronal $E_{\text{GABA}}$ from glioma-induced depolarization (Figure 4a). This result suggests that glioma-induced $E_{\text{GABA}}$ shift can be ascribed to KCC2 inhibition, caused by neuronal Zn$^{2+}$ rise. Accordingly, when similar $E_{\text{GABA}}$ depolarization was obtained blocking KCC2 with the selective antagonist DIOA (20 μM; 1 h pre-treatment plus continuous superfusion), acute TPEN application had no effect (Figure 4a).

Using FluoZin-based fluorescence determinations, we also observed that basal intracellular Zn$^{2+}$ was significantly higher in co-cultured neurons in respect to control (Figure 4b). Neuronal Zn$^{2+}$ accumulation was prevented by APV/NBQX application in the co-culture medium (Figure 4c), indicating the requirement for GluR activation. Consistently, the application of GCM or glutamate (20 μM) onto FluoZin-loaded neurons, elicited an APV/NBQX-sensitive fluorescence increase, rapidly reverting to basal level upon TPEN application (Supplementary Figure S1 and S2).

To identify the source of Zn$^{2+}$, we performed experiments in the presence of tricine, a chelator of extracellular Zn$^{2+}$. Tricine treatment (1 mM, 24 h) did not abolish the effect of co-culture on $E_{\text{GABA}}$ (Supplementary Table S2), indicating that extracellular Zn$^{2+}$ is not required for $E_{\text{GABA}}$ depolarization. However, co-culture induced [Zn$^{2+}$] rise. Accordingly, when similar $E_{\text{GABA}}$ depolarization was not observed (not shown). Noteworthy, tricine treatment caused a reduction in basal Zn$^{2+}$ both in control and co-cultured neurons (n = 41/59, control/co-culture; not shown). These results suggest that extracellular Zn$^{2+}$ chelation modifies neuronal Zn$^{2+}$ homeostasis, altering basal cytosolic Zn$^{2+}$ level. To avoid possible effects on intracellular Zn$^{2+}$ homeostasis, experiments with Zn$^{2+}$ chelators were repeated reducing incubation time (4 h), following the observation that 4 h tricine treatment did not impair neuronal ability to release intracellular Zn$^{2+}$ in response to a glutamatergic stimulus (Supplementary Figure S2). Consistently, tricine did not prevent the depolarizing effect of 4 h co-culture on $E_{\text{GABA}}$ (Figure 4d). Conversely, when intracellular Zn$^{2+}$ was chelated (with FluoZin 5 μM, 4 h), co-culture-induced $E_{\text{GABA}}$ shift was abolished (Figure 4d).

All together, these data indicate that Cl$^{-}$ disequilibrium in co-cultured neurons is due to intracellular Zn$^{2+}$-dependent KCC2 impairment.

Zn$^{2+}$-mediated $E_{\text{GABA}}$ shift requires Src/TrkB activation.

To disclose the mechanisms underlying Zn$^{2+}$-mediated $E_{\text{GABA}}$ shift, neuronal [Zn$^{2+}$], was artificially increased through perforated (by gramicidin) patch pipette loading. We preliminarily verified the efficacy of intracellular Zn$^{2+}$ loading through perforated patch by fluorescence recordings; the presence of ZnCl$_2$ in the pipette solution caused a time- and concentration-dependent fluorescence increase in FluoZin-loaded neurons, indicating that, in our experimental conditions, Zn$^{2+}$ permeates through gramicidin pores (Supplementary Table S3; Supplementary Figure S3). When 0.1 μM ZnCl$_2$ was added to a BAPTA (5 mM) containing intracellular solution, giving a controlled free [Zn$^{2+}$]$_i$ of...
~ 10 nM (Supplementary Table S3) $E_{\text{GABA}}$ promptly shifted to more depolarized values (Figures 5a and b). Conversely, $E_{\text{GABA}}$ remained stable with control intracellular solution (calculated as ~ 0.1 pM free [Zn$^{2+}$], Figure 5a), or when free [Zn$^{2+}$], was below 2 nM ($n = 7$; Supplementary Table S3). This time-dependent $E_{\text{GABA}}$ depolarization was not observed when cultures were treated with the KCC2 blocker DIOA (20 $\mu$M; 1 h pre-treatment plus continuous superfusion; Figure 5a and Supplementary Table S4). In these conditions, neurons displayed a depolarized $E_{\text{GABA}}$, likely occluding the effects of intracellular Zn$^{2+}$ loading. Consistently, acute TPEN application (15 min; Figure 5b) rapidly reverted Zn$^{2+}$-induced $E_{\text{GABA}}$ depolarization on control neurons. These data indicate that intracellular Zn$^{2+}$ level rapidly and reversibly interferes with Cl$^-$/ equilibrium, through KCC2 activity modulation, highlighting intracellular Zn$^{2+}$ rise as the key step in glioma-induced $E_{\text{GABA}}$ depolarization.

Several mechanisms have been proposed to explain KCC2 downregulation in hyperexcitability models, including the phosphorylation of KCC2 residues by a number of different kinases. To explore the mechanism of Zn$^{2+}$-mediated $E_{\text{GABA}}$ shift, we investigated the involvement of Src/TrkB-dependent KCC2 tyrosine phosphorylation, as intracellular Zn$^{2+}$ has been reported to transactivate TrkB in a Src-dependent manner. When hippocampal cultures were treated with TrkB inhibitor K252A (200 nM; 1 h pre-application and perfused...
during the experiment), intracellular Zn$^{2+}$ failed to depolarize neuronal \(E_{GABA}\) (Figure 5c and Supplementary Table S4). Similarly, in the presence of Src kinase inhibitor PP2 (5 \(\mu\)M, 1 h pre-application and perfused during the experiment), \(E_{GABA}\) shift due to intracellular Zn$^{2+}$ loading was absent (Figure 5c and Supplementary Table S4). Thus, Zn$^{2+}$-induced \(E_{GABA}\) shift requires the integrity of Src/TrkB pathway.

Consistently, by western blots analysis, we demonstrated that GCM treatment (15 min) significantly increased neuronal Src phosphorylation (Figure 5d). This effect was Zn$^{2+}$-dependent, as it was prevented by TPEN application (20 \(\mu\)M, 15 min pre-treatment and during GCM application, \(n=6\); \(P=0.92\) with respect to TPEN, Figure 5e). Moreover, GCM treatment significantly increased neuronal TrkB phosphorylation (Figure 5f).

Altogether, these data indicate that glioma-released factors might alter neuronal Cl$^{-}$ homeostasis through Zn$^{2+}$-induced Src/TrkB-mediated KCC2 modulation, as illustrated in Figure 6.

**Discussion**

We used co-cultures of hippocampal neurons and glioma cells to unveil the mechanisms of glioma-induced hyperexcitability, reporting that glioma cells depolarize neuronal \(E_{GABA}\), increasing [Cl$^{-}$], and reverting the driving force for GABA-mediated currents. Our results show that \(E_{GABA}\) depolarization relies on Zn$^{2+}$-mediated KCC2 functional impairment, disclosing the underlying mechanism: glioma-released glutamate activates neuronal GluRs, causing neuronal intracellular Zn$^{2+}$ rise which, through Src/TrkB activation, reduces KCC2 activity, leading to intracellular [Cl$^{-}$] increase and \(E_{GABA}\) depolarization. We conclude that glioma might reduce neuronal inhibition through Zn$^{2+}$-mediated downregulation of KCC2 activity, causing hyperexcitability.

In glioma-co-cultured hippocampal neurons, the current-voltage relationship of GABA-mediated responses is shifted to more depolarized potentials, compared with control, giving a more depolarized \(E_{GABA}\). This indicates a higher basal neuronal [Cl$^{-}$], in co-cultures, confirmed by an independent estimation in neurons transfected with a YFP-based Cl$^{-}$ sensor. Although glioma co-culture induces a small neuronal depolarization, the shift of \(E_{GABA}\) is more relevant, resulting in the inversion of the driving force for GABA-mediated currents.

According to previous studies, Cl$^{-}$ homeostasis in cultured hippocampal neurons is determined by the activity of both NKCC1 and KCC2. Indeed, we show here that both transporters are expressed in control neurons and their activity is required to maintain Cl$^{-}$ equilibrium as blocking either NKCC1 or KCC2 leads to a shift in basal \(E_{GABA}\). Conversely, in co-cultured neurons, NKCC1 activity is
Figure 5  Intracellular Zn\(^{2+}\) modulates \(E_{\text{GABA}}\), through Src and TrkB activation. (a) Bottom, \(E_{\text{GABA}}\) is rapidly depolarized in cultured hippocampal neurons loaded with -10 nM free Zn\(^{2+}\) through the patch pipette (dark red circles); t=0, -79.6 ± 4.7 mV; t=5, -58.8 ± 4.8 mV; n=17; **P<0.01, paired (t) test, but not with standard EGTA-containing intracellular solution (empty circles; free Zn\(^{2+}\) = 0.1 μM; P=0.54; n=8). Top, in cultures treated with the KCC2 blocker DIOA (20 μM, 1 h pre-application and during the experiment), \(E_{\text{GABA}}\) was significantly depolarized at t=0 with both intracellular solutions (dark red squares, free Zn\(^{2+}\) = 10 nM, −47.5 ± 1.2 mV; n=5; empty squares, free Zn\(^{2+}\) = 0.1 μM, −52.8 ± 6.1 mV, n=6; **P<0.01; respect to control, ANOVA), and remained stable despite the intracellular Zn\(^{2+}\) loading. (b) TPEN application reverts Zn\(^{2+}\)-induced \(E_{\text{GABA}}\) shift. Top, sample current traces from 10 nM free Zn\(^{2+}\)-loaded neuron, at start of recording (t=0), 5′ after and following TPEN application (20 μM, 15′). Bottom, Zn\(^{2+}\)-induced \(E_{\text{GABA}}\) depolarization at t=0, 5′ and 20′ (n=7, **P<0.01, ANOVA); dashed column represents \(E_{\text{GABA}}\) after 15′ TPEN application (following 5′ Zn\(^{2+}\) loading; n=8, **P<0.01 versus 20′, ANOVA-Tukey test). (c) Impairment of Zn\(^{2+}\)-induced \(E_{\text{GABA}}\) depolarization (\(\Delta E_{\text{GABA}}\) at t=5′, 22.8 ± 3.8 mV; n=9 in the presence of Src kinases inhibitor (PP2, 5 μM, n=9, P<0.01) or TrkB inhibitor (K252A, 200 nM, n=7, P<0.01) (versus control, ANOVA). \(E_{\text{GABA}}\) and RMP were depolarized in control following, K252A or PP2 treatment as reported in Supplementary Table S4. (d) GCM increases Src phosphorylation. Top, typical immunoblot experiment; bottom, GCM treatment (15′) increases Src (n=10, P<0.01; ANOVA) phosphorylation (platelet-derived growth factor represent positive control). (e) TPEN application prevents GCM-induced Src phosphorylation; Top, typical immunoblot experiment; bottom, GCM treatment (15′) failed to increase Src (n=6, P=0.92; ANOVA) phosphorylation in cultures pre-treated with TPEN (20 μM, 15′; y-axis as in (d); (f) GCM increases TrkB phosphorylation. Top, typical immunoblot experiment; bottom, GCM treatment (15′) increases TrkB (n=3, P<0.05; ANOVA) phosphorylation (BDNF represents positive control).

apparently not balanced by KCC2-mediated Cl\(^-\) extrusion and transporter disequilibrium leads to an increase in neuronal [Cl\(^-\)]. This picture is demonstrated pharmacologically by the use of selective antagonists of Cl\(^-\) co-transporters: (i) furosemide, the blocker of both transporters, abolishes \(E_{\text{GABA}}\) shift, demonstrating the involvement of Cl\(^-\) transport; (ii) the specific NKCC1 antagonist bumetanide reverses co-culture-induced \(E_{\text{GABA}}\) depolarization, demonstrating that Cl\(^-\) accumulation requires the activity of NKCC1; (iii) neurons treated with the specific KCC2 blocker DIOA display depolarized \(E_{\text{GABA}}\), likely occluding the co-culture effect. Altogether, these data support the proposal that in neurons, a new Cl\(^-\) equilibrium is established during glioma co-culture, characterized by higher [Cl\(^-\)], and caused by reduced KCC2 activity.

We provide evidence that glioma-induced KCC2 impairment, observed in co-cultured neurons, is due to intracellular Zn\(^{2+}\) rise.\(^{16}\) Indeed, intracellular Zn\(^{2+}\) chelation by TPEN rapidly reverts co-culture-induced \(E_{\text{GABA}}\) shift and basal [Zn\(^{2+}\)], is significantly higher in neurons after glioma co-culture.

The effect of neuronal Zn\(^{2+}\) rise on \(E_{\text{GABA}}\) was directly evaluated in experiments where intracellular [Zn\(^{2+}\)] was artificially increased from pico to nanomolar, as in pathological conditions.\(^{23,24}\) The resulting Zn\(^{2+}\)-dependent \(E_{\text{GABA}}\) shift is ascribable to KCC2 impairment, because it was absent when
KCC2 was pharmacologically blocked by DIOA. Indeed, the effect of Zn$^{2+}$ on $E_{\text{GABA}}$ is likely occluded by pharmacological KCC2 block, as DIOA treated neurons already show a depolarized $E_{\text{GABA}}$, which is, consistently, not rescued by TPEP application.

Glioma-induced alteration of neuronal Cl$^{-}$ homeostasis likely depends on functional KCC2 block, rather than on reduction of protein expression (see Lee et al.$^7$). This view is based on the unaltered expression of neuronal KCC2 after glioma co-culture and on the rapid rescue exerted by Zn$^{2+}$ chelation, demonstrating a dynamic modulation of Cl$^{-}$ transport mechanisms. The functional modulation of KCC2 activity has been observed both in physiological and pathological models, such as prolonged post-synaptic spiking,$^{25}$ brain-derived neurotrophic factor (BDNF) stimulation$^{26}$ and oxygen glucose deprivation.$^{18}$ Our data suggest that the mechanism responsible for glioma-induced KCC2 inhibition relies on Zn$^{2+}$-mediated Src/TrkB activation. Indeed, Zn$^{2+}$-induced $E_{\text{GABA}}$ shift was prevented by the application of Src or TrkB kinase inhibitors (PP2 or K252A). Consistently, GCM increased the level of TrkB and Src phosphorylation in hippocampal cultures, the latter effect being prevented by the Zn$^{2+}$ chelator TPEN. These results are in line with the notion that intracellular Zn$^{2+}$ may transactivate TrkB by a neurotrophin-independent and Src-dependent mechanism, as reported in models of intense neuronal activity.$^{22}$

In neurons, Zn$^{2+}$-induced TrkB transactivation may mimic BDNF-TrkB signaling, leading to KCC2 phosphorylation on tyrosine residues$^{27,28}$ and driving neuronal disinhibition.$^{29}$ We speculate that co-culture induced $E_{\text{GABA}}$ depolarization needs KCC2 phosphorylation on tyrosine residues because of Zn$^{2+}$-induced TrkB transactivation.

We report that glioma-induced $E_{\text{GABA}}$ shift requires the release of glutamate in the extracellular space by glioma cells and the consequent activation of neuronal ionotropic GluRs. Indeed, the application of APV and NBQX during co-culture prevents $E_{\text{GABA}}$ depolarization.

It is known that glutamate can induce intracellular Zn$^{2+}$ increase through different mechanisms, including AMPARs and Ca$^{2+}$-channel-mediated influx or Ca$^{2+}$-dependent intracellular release.$^{25,30}$ We observed that both APV and NBQX abolished co-culture-induced $E_{\text{GABA}}$ depolarization highlighting the role for both ionotropic GluRs in this effect. The simplest explanation for this evidence is that AMPAR-mediated neuronal depolarization drives NMDAR activation thus allowing Ca$^{2+}$-dependent intracellular Zn$^{2+}$ release.$^{30}$ Our data indicate that the source of Zn$^{2+}$ is intracellular, as the extracellular Zn$^{2+}$ chelator tricine was ineffective, whereas FluoZin prevented co-culture-induced $E_{\text{GABA}}$ depolarization. It has to be considered that tricine treatment, although did not prevent co-culture-induced $E_{\text{GABA}}$ depolarization, inhibited co-culture-induced basal [Zn$^{2+}$]$^\text{extracellular}$, preventing cytosolic Zn$^{2+}$ accumulation. However, tricine-treated neurons retained the ability to release intracellular Zn$^{2+}$ in response to a glutamatergic stimulus, and this event is likely sufficient to trigger the intracellular signaling leading to KCC2 impairment.

It is well established that intracellular Zn$^{2+}$ rise induces cell death, and Zn$^{2+}$ exposure is toxic to neurons both in vitro and in vivo. It is now evident that increased cytosolic Zn$^{2+}$ resulting from liberation from intracellular stores, rather than cytoplasmic influx of synaptically released Zn$^{2+}$, can be highly toxic during oxidative and other types of neuronal injury.$^{31}$ and Zn$^{2+}$ dyshomeostasis appears to be a common feature of numerous neuropathological conditions.$^{23,32}$ We speculate that the reported mechanism, leading to reduced GABAergic transmission, could underlie the etiology of glioma-related epilepsy, pointing to Zn$^{2+}$ accumulation as a possible therapeutic target to restore KCC2 function and the excitatory/inhibitory balance. In this view, it is possible to speculate that Zn$^{2+}$ homeostatic drugs may be helpful in the treatment of Zn$^{2+}$-related neurological disorders such as neuronal hyperexcitability or Alzheimer’s disease.$^{33}$

The use of co-cultures$^{34}$ allowed to disclose the molecular mechanisms involved in glutamate-mediated overexcitation induced by glioma. Our results are in line with recent works showing that Xc-mediated glutamate release is responsible for the generation of tumor-associated epileptic events in glioma-bearing mice.$^{6,35}$ Indeed, increased concentration of extracellular glutamate has been found in peritumoral tissue in both humans and mice$^{6,36}$ supporting its role in tumor growth, survival and peritumoral seizure activity.$^6$ Additional sources of glutamate in peritumoral tissue may be microglial Xc system or reverse activity of Na$^{+}$-dependent glutamate transporters in neurons or astrocytes.$^{37,38}$ Thus, the co-culture system likely retains the feature of excessive glutamate release typical of glioma. Consistently, neuronal $E_{\text{GABA}}$ Shift was observed in co-culturing neurons with different glioblastoma cell lines, but not with astrocytes, supporting the view of a tumor-specific effect.$^6$

Glutamate-induced alteration of neuronal Cl$^{-}$ homeostasis may act concomitantly with other mechanisms, including the direct depolarizing effect of glutamate on neurons, displacing the excitation/inhibition balance toward an increased network excitability, thus promoting seizure onset.$^{35}$

In conclusion, our study provides a possible explanation of the mechanisms by which glioma cells affect neuronal Cl$^{-}$ equilibrium, highlighting the role of Zn$^{2+}$, recently emerged in a variety of excitotoxic conditions, such as epilepsy, ischemia and brain trauma.$^{32}$
Materials and Methods

Animals. Procedures using laboratory animals were in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used in accordance with the European Communities Council Directive of September 20, 2010 (2010/63/EU).

Primary hippocampal neuronal cultures. Hippocampal neuronal cultures were prepared from newborn (P0-P1) C57BL/6 mice of either sex (Charles River - Research Models and Services, Lecco, Italy). In brief, after careful dissection from diencephalic structures, the meninges were removed and the hippocampi were chopped and digested in 1.25 mg/ml trypsin for 20 min at 37 °C. Cells were mechanically dissociated and plated at a density of 10^5 in poly-L-lysine-coated glass coverslip (12 mm diameter) in serum-free Neurobasal Medium ( Gibco Life Science, Life Technologies Italia, Monza, Italy), supplemented with B27 2 mM L-glutamine and 100 μg/ml gentamycin (neuronal culture medium). Then, cells were kept at 37 °C in 5% CO2 for 10–13 days with medium replacement (1:1 ratio) three times per week. With this method, we obtained cultures composed by 70–80% neurons, 30–35% astrocytes and 4–5% microglia, as determined with β-tubulin III, glial fibrillary acidic protein and isolectin IB4 staining.38 The same procedure was followed to prepare rat hippocampal culture used for some immunoblot experiments.

Glia primary cultures. Primary cortical glial cells were prepared from P0–P2 mice. Cerebral cortices were chopped and digested in 30 U/ml papain for 40 min at 37 °C and gently triturated. The dissociated cells were washed, suspended in Dulbecco's Modified Eagle Medium ( Gibco Life Science, Italy) with Glutamax and supplemented with B27 2 mM L-glutamine and 100 μg/ml gentamycin (neuronal culture medium). Then, cells were kept at 37 °C in 5% CO2 and 100 μM gentamicin (neuronal culture medium). After 6 days, cultures were mechanically dissociated and plated at a density of 10^5 in poly-L-lysine-coated glass coverslip (12 mm diameter) in serum-free Neurobasal Medium ( Gibco Life Science, Life Technologies Italia, Monza, Italy), supplemented with B27 2 mM L-glutamine and 100 μg/ml gentamycin (neuronal culture medium). Then, cells were kept at 37 °C in 5% CO2 and 100 μM gentamicin (neuronal culture medium). After 6 days, cultures were mechanically dissociated and plated at a density of 10^5 in poly-L-lysine-coated glass coverslip (12 mm diameter) in serum-free Neurobasal Medium ( Gibco Life Science, Life Technologies Italia, Monza, Italy), supplemented with B27 2 mM L-glutamine and 100 μg/ml gentamycin (neuronal culture medium). Then, cells were kept at 37 °C in 5% CO2 for 10–13 days with medium replacement (1:1 ratio) three times per week. With this method, we obtained cultures composed by 70–80% neurons, 30–35% astrocytes and 4–5% microglia, as determined with β-tubulin III, glial fibrillary acidic protein and isolectin IB4 staining.

Neuronal culture transfection. Hippocampal neuronal cultures were transfected at 9–10 DIV. One day before transfection, 50% of the culture medium was replaced with fresh medium. For transfection, 100 μl of Neurobasal media were mixed with 2 μl of Neuroferm (OZ Biosciences, Marseille, France) and 1 μg of CI-Sensor DNA.15,16 The mixture was incubated for 15–20 min at room temperature and thereafter distributed dropwise over the neuronal culture. Neuronal cultures were placed on a magnetic board (OZ Biosciences) and incubated for 15 min at 37 °C, 5% CO2. One hour later, 50% of neuronal culture medium was substituted with fresh neuronal culture medium. Cells were used for experiments 24–76 h after transfection.

Glioma cell culture. Patient-derived glioma cells (MZC; kindly provided by Dr. Antonietta Arcella, Neuromed, Italy) within passage 40,14,39 glioblastoma cell lines: 6% of microglia contamination), as verified by staining with glial fibrillary acidic protein and isolectin IB4.34

For non-invasive monitoring of [Cl−], cells were transfected with CPF-YFP-based CI-Sensor15 and the optic system equipped with an emission filter D535/540 nm and a dichroic mirror 505DCLP. Zn2+ fluorescence changes were determined using an HEPES-buffered extracellular solution (nominally CO2- and bicarbonate-free43,44), so that EGABA was an estimation of ECl. Membrane currents, recorded with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Foster city, CA, USA), were filtered at 2 kHz, digitized (10 kHz) and acquired with Clampex 10 software (Molecular Devices). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiment, and recordings were discarded when any of these parameters changed by > 10%.

Zn2+ loading through gramicidin pores. When indicated (Figure 5 and Supplementary Figure S3), intracellular [Zn2+]i was artificially increased in neurons through the perforated patch, using a BAPTA-based intracellular solution (in mM: 140 KCl, 2 MgCl2, 10 HEPES, 2 MgATP, 5 BAPTA) supplemented with ZnCl2 (0.01–100 μM). The corresponding free Zn2+ concentrations were calculated using the MaxChelator software: http://www.stanford.edu/~cpporton/maxc.html, and are reported in Supplementary Table S3.

Zn2+ imaging. Fluorescence images were acquired at room temperature (24–25°C) using a customized digital imaging microscope. Excitation of fluorophores at various wavelengths was achieved using a 1-nm-bandwidth polychromatic light source (Till Polychrome V, equipped with a 150 W xenon lamp (Till Photonics, Hillsboro, OR, USA). Fluorescence was visualized using an upright microscope (Axioskope) equipped with a 40× oil-water immersion objective (Achroplan CarlZeiss, Oberkochen, Germany) and a digital 12 bit CCD camera system (SenseiCam, PCO AG, Kelheim, Germany). All the peripheral hardware control, image acquisition and image processing were achieved using customized software Till Vision v. 4.0 (Till Photonics). Changes in the intracellular Zn2+ level were monitored using the high-affinity Zn2+–sensitive indicator FluoZin-3AM (Invitrogen). Neurons were loaded by incubating coverslips for 45 min at 37 °C in 1 ml of HEPES-buffered salt solution containing: 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 20 mM HEPES-NaOH and 15 mM glucose (pH 7.3), plus 5 mg/ml of bovine serum albumin and 5 μM of FluoZin-3AM. For time-lapse recordings, FluoZin-3AM was excited at 480 nm (emission filter D535/540 nm; dichroic mirror 505DCLP). Zn2+ fluorescence changes were determined using an HEPES-buffered extracellular solution (nominally CO2- and bicarbonate-free43,44), so that EGABA was an estimation of ECl. Membrane currents, recorded with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Foster city, CA, USA), were filtered at 2 kHz, digitized (10 kHz) and acquired with Clampex 10 software (Molecular Devices). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiment, and recordings were discarded when any of these parameters changed by > 10%.

CI− imaging. For non-invasive monitoring of [Cl−], cells were transfected with CPF–YFP-based CI-Sensor15 and the optic system equipped with an emission filter D535/540 nm and a dichroic mirror 505DCLP (505 nm) was used. Cells expressing CI-Sensor were excited at 445 and 485-nm wavelengths alternatively (50 ms, 0.1 Hz). [Cl−] changes are expressed as a ratio (R) of background-subtracted F445/F485 (ΔF = F445/F485). For [Cl−] imaging, CI− fluorescence intensity after agonist application and FTPen represents fluorescence intensity after 5 min of TPEN application.

Glioma-conditioned medium (GCM). GCM was prepared as follows: when cultures of glioma cells became confluent (−3 × 10^5 cells) were harvested. The culture medium was substituted with 10 ml of filtered normal extracellular solution. The conditioned medium (GCM) was collected after incubating for 4 h, centrifuged, pH adjusted to 7.35 and then used for electrophysiological and imaging
12-well cultures plates. Fabrizio Eusebi, who initiated and inspired this direction of research. The work was supported by PhD programme in Neurophysiology, Sapienza University, Rome. Authors wish to thank Dr. Stefano Senisi for critical reading of the manuscript and precious suggestions.

Author contributions

SDA designed, carried out and analyzed all perforated patch clamp experiments; SC, FS and MGM performed whole-cell recordings; CL and CL designed, carried out and analyzed western blot experiments. Imaging experiments were designed, carried out and analyzed by EM, CB, PB and DR, DR and SDA conceived the project and wrote the manuscript with the help of PB and original input from Fabrizio Eusebi.

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