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RESEARCH ARTICLE

Paracrine effect of carbon monoxide – astrocytes promote neuroprotection through purinergic signaling in mice

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ABSTRACT

The neuroprotective role of carbon monoxide (CO) has been studied in a cell-autonomous mode. Herein, a new concept is disclosed – CO affects astrocyte–neuron communication in a paracrine manner to promote neuroprotection. Neuronal survival was assessed when co-cultured with astrocytes that had been pre-treated or not with CO. The CO-pre-treated astrocytes reduced neuronal cell death, and the cellular mechanisms were investigated, focusing on purinergic signaling. CO modulates astrocytic metabolism and extracellular ATP content in the co-culture medium. Moreover, several antagonists of P1 adenosine and P2 ATP receptors partially reverted CO-induced neuroprotection through astrocytes. Likewise, knocking down expression of the neuronal P1 adenosine receptor A_{2A}-R (encoded by *Adora2a*) reverted the neuroprotective effects of CO-exposed astrocytes. The neuroprotection of CO-treated astrocytes also decreased following prevention of ATP or adenosine release from astrocytic cells and inhibition of extracellular ATP metabolism into adenosine. Finally, the neuronal downstream event involves TrkB (also known as NTRK2) receptors and BDNF. Pharmacological and genetic inhibition of TrkB receptors reverts neuroprotection triggered by CO-treated astrocytes. Furthermore, the neuronal ratio of BDNF to pro-BDNF increased in the presence of CO-treated astrocytes and decreased whenever A_{2A}-R expression was silenced. In summary, CO prevents neuronal cell death in a paracrine manner by targeting astrocytic metabolism through purinergic signaling.

KEY WORDS: Apoptosis, Brain, Carbon monoxide, Co-cultures, Purinergic, Preconditioning

INTRODUCTION

The astrocyte–neuron network is crucial for cerebral homeostasis and is very complex (a single astrocyte enwraps multiple neurons, and one neuron interacts with 4–8 astrocytes) (Theodosis et al., 2008). Astrocytic function modulates extracellular ionic homeostasis, neurotransmission, glutathione metabolism and amino acid recycling (Theodosis et al., 2008; Verkhratsky et al., 2016). Astrocytes are also important for neurogenesis, normal dendritic maturation, spine formation and functional integration of adult-born neurons (Sultan et al., 2015). Astroglial cells play an important biological role in neuroinflammation and neuroprotection, and scientists have been identifying the increasing importance of astrocytes in pathologies such as Alzheimer disease, amyotrophic lateral sclerosis and cerebral ischemia (Allaman et al., 2011). Communication between neurons and astrocytes is bidirectional and tightly regulated, and any dysfunction can affect both cell populations. Understanding this cell-to-cell communication is crucial, in particular under pathological conditions.

Carbon monoxide (CO) is an endogenous product, resulting from heme degradation by heme-oxygenase activity, along with biliverdin and free iron (Queiroga et al., 2014). There are two isoforms of heme-oxygenase described: HO-1 (inducible; encoded by *Hmox1*) and HO-2 (constitutive; encoded by *Hmox2*), whose expression or activation is promoted by several cell-damaging stimuli (Ryter et al., 2006), resulting in HO being classified as a stress-response enzyme. Beneficial roles of heme-oxygenase activity have also been studied in the central nervous system (Dore, 2002; Queiroga et al., 2014). Likewise, low amounts of CO prevent neuroinflammation (Chora et al., 2007; Fagone et al., 2011), vasoconstriction (Zimmermann et al., 2007) and cerebral damage following ischemia (Zeynalov and Dore, 2009; Queiroga et al., 2012, 2014; Yabluchanskiy et al., 2012). Moreover, in neurons (primary cultures and cell lines), CO prevents apoptosis through activation of soluble guanylate cyclase and through reactive oxygen species (ROS) signaling (Vieira et al., 2008; Schallner et al., 2013). Likewise, in astrocytes, CO limits mitochondrial membrane permeabilization (MMP) and the subsequent release of pro-apoptotic factors into the cytosol, which in turn inhibits apoptosis (Queiroga et al., 2010). Furthermore, CO increases ATP production and improves mitochondrial metabolism, which promotes cytoprotection in astrocytes. This metabolic improvement is due to the strengthening of mitochondrial oxidative phosphorylation, inducing mitochondrial biogenesis and increasing cytochrome *c* oxidase (COX) activity through the COX–Bcl-2 interaction (Almeida et al., 2012).

ATP is a classic energy source molecule but is also a signaling molecule. This molecule is crucial for astrocyte–astrocyte, astrocyte–microglia and astrocyte–neuron communication (Shinozaki et al., 2005; Abbracchio and Ceruti, 2006; Fields and Burnstock, 2006; Sebastiao and Ribeiro, 2009), and it is released by neurons and glial cells in response to neurotransmitter stimulation, hypoxia, inflammation or mechanical stress (Rodrigues et al., 2015; Fields and Burnstock, 2006). Extracellular ATP acts as a chemoattractant factor (Fields and Burnstock, 2006), which can induce the expression of important genes for cell survival (McKee et al., 2006), can protect astrocytes from oxidative stress (Schock et al., 2007; Fields and Burnstock, 2006) and can prevent neuronal excitotoxicity (Schock et al., 2007). In addition, the protective mechanisms related to ischemic preconditioning involve the increasing availability of energy substrates (Kavianipour et al., 2003). Adenosine is an important signaling nucleoside and a neuroprotective factor in the

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brain. There are two sources of extracellular adenosine – metabolism of ATP by ectonucleotidases and its direct release from the cytosol through equilibrative nucleoside transporters (ENTs) (Fields and Burnstock, 2006; Sebastiao and Ribeiro, 2009). Mild hypoxia increases adenosine release from cerebral cells (McKee et al., 2006; Abbracchio and Ceruti, 2006) in a defensive way, and adenosine is a well-described neuroprotector factor (Sebastiao and Ribeiro, 2009). Biological signaling activities of ATP and adenosine occur through purinergic receptors, which are divided into two main classes of purinergic receptors: P1, which respond to adenosine and are associated with G-proteins, and P2, which are activated by ATP and can be ion channels (P2X or ionotropic) or G-protein coupled [P2Y or metabotropic (Fields and Burnstock, 2006)]. ATP and adenosine often have antagonistic actions as a way to refine the regulatory mechanism (Fields and Burnstock, 2006). In summary, ATP and adenosine act as intercellular signaling molecules (Schock et al., 2007; Rodrigues et al., 2015), and adenosine is neuroprotective (Sebastiao and Ribeiro, 2009).

To date, the cytoprotective role of CO has been exclusively studied as being a cell-autonomous effect. Herein, a cell-dependent effect of CO in controlling neuronal activity through cell–cell chemical communication is proposed. Therefore, the role of CO in the modulation of purinergic signaling in astrocyte–neuron communication is explored. Indeed, CO promotes neuroprotection in a paracrine manner by acting on astrocytic metabolism. CO-pre-treated astrocytes prevent neuronal cell death that is induced by oxidative stress. Purinergic signaling is involved in this cell-to-cell communication through activation of P1 and P2 receptors, as well as through TrkB (also known as NTRK2) receptors and brain-derived neurotrophic factor (BDNF), and these are associated with neuroprotective signaling.

RESULTS

Paracrine effect of CO – neuroprotection promoted by astrocytes

In order to closer mimic the *in vivo* environment, we used a co-culture system as an *in vitro* model in which primary cultures of mouse neurons and astrocytes are in the same environment – sharing metabolites – but do not physically interact. A saturated solution containing 50 µM of CO (see Materials and Methods) was added to primary cultures of astrocytes. After 3 h, the co-culture was established, and neurons were challenged with oxidative stress [*tert*-butylhydroperoxide (*t*-BHP) addition] for 18 h (Fig. 1A). It is worthy of note that upon opening the sealed vial of CO-saturated solution, CO gas diffuses out quickly from the cell culture system. Indeed, after 30 min, more than 50% of CO content is already lost to the atmosphere (personal communication João Seixas). Furthermore, co-culture is established using neuronal medium as culture medium. Thus, there is no CO gas present in the co-culture system, and any neuroprotective effect found is due to the presence of astrocytes. Neuronal survival levels were higher in the case of CO-treated astrocyte co-cultures, in particular at the highest concentrations of *t*-BHP (Fig. 2). Of note, t-BHP at these concentrations does not kill astrocytes (Fig. S1), being toxic only to neurons. Neuronal cell death was assessed by taking three different approaches: (i) apoptosis-related chromatin condensation followed by Hoechst staining (Fig. 2A and B), (ii) cell viability assessed by measuring plasma membrane integrity through the entry of propidium iodide (Fig. 2A and C) and (iii) activation of caspase-3 (Fig. 2D and E). Representative micrographs of fluorescent microscopy of live cells and immunocytochemistry are presented in Fig. 2A and E, respectively. The neuronal marker microtubule associated protein 2 (MAP-2) was evaluated with immunocytochemistry to assess the neuronal cytoskeleton. In accordance with neuroprotection-indicative data, CO-treated astrocytes partially prevented neuronal cytoskeleton rupture due to oxidative stress (Fig. 2E). Of note, in the case of monocultures of neurons challenged with *t*-BHP, higher levels of cell death were found than in the presence of non-treated-CO astrocytes, which is in agreement with the fact that astrocytes promote cytoprotection and metabolic support for neurons (Allaman et al., 2011). In summary, CO improved astrocyte neuroprotective function in order to prevent neuronal apoptosis.

There are two possible hypotheses for how CO promotes neuroprotection by acting on astrocytes: by stimulating astrocytes to release pro-survival molecules or by promoting astrocytes to take up toxic factors. Both events would improve the neuronal environment and, therefore, promote neuronal survival. In order to clarify this question, a conditioned medium experiment was performed. Half of the neuronal medium was incubated with monocultures of astrocytes, with or without CO pre-treatment for 3 h. Then, the conditioned neuronal medium was returned to the neuronal monoculture and neurons were exposed to *t*-BHP. Conditioned medium derived from CO-treated astrocytes increased neuronal survival (Fig. 2F and G). However, conditioned medium that had not received CO treatment decreased neuronal viability below the levels found in monocultures of neurons. This apparent toxicity can be explained by the fact that astrocytes are metabolically active cells, consuming nutrients and excreting toxic metabolic products at a higher rate (Fig. 2F and G). Thus, the presence of astrocytes is important for maintaining neuron viability because co-culture conditions led to higher neuronal survival than astrocyte-conditioned medium. Nevertheless, these data clearly indicate that CO promotes the release of neuroprotective factors from astrocytes into the intercellular space. In summary, CO has an important modulator effect on astrocytes, which is beneficial for neurons by increasing the release of neuroprotective factors.

Carbon monoxide influences ATP extracellular content

Several factors have been described as being implicated in astrocyte–neuron communication (Theodosis et al., 2008; Allaman et al., 2011). Among them, it is known that (i) extracellular ATP is a signaling molecule, (ii) adenosine is a neuroprotector molecule and (iii) purinergic receptors have been described as being implicated in protective mechanisms. Furthermore, we have previously demonstrated that CO reinforces oxidative phosphorylation and ATP intracellular content in primary cultures of astrocytes (Almeida et al., 2012). Therefore, ATP and adenosine are strong candidate molecules for playing a role in the communication between astrocytes and neurons, in particular for the CO-triggered neuroprotection.

Indeed, intracellular ATP concentration in monocultures of astrocytes was higher following 3 h of treatment with CO (Fig. 3A). Moreover, in monocultures of astrocytes, rates of serine and cysteine production and consumption were assessed in the presence of CO (Fig. 3B). In both cases, up to 6 h following CO treatment, the velocity profiles of these metabolites changed drastically, from production to consumption. Both amino acids might be converted to pyruvate in order to feed the citric acid cycle, reinforcing oxidative phosphorylation and, therefore, ATP production. Thus, along with our previous published results (Almeida et al., 2012), these data confirmed that CO improves astrocytic mitochondrial metabolism and enhances mitochondrial ATP production. For assessing the role of ATP as a signaling molecule in astrocyte–neuron communication, ATP extracellular content in the co-cultures at 1, 4 and 24 h was measured (Fig. 3C). Extracellular ATP in co-cultures at time zero was

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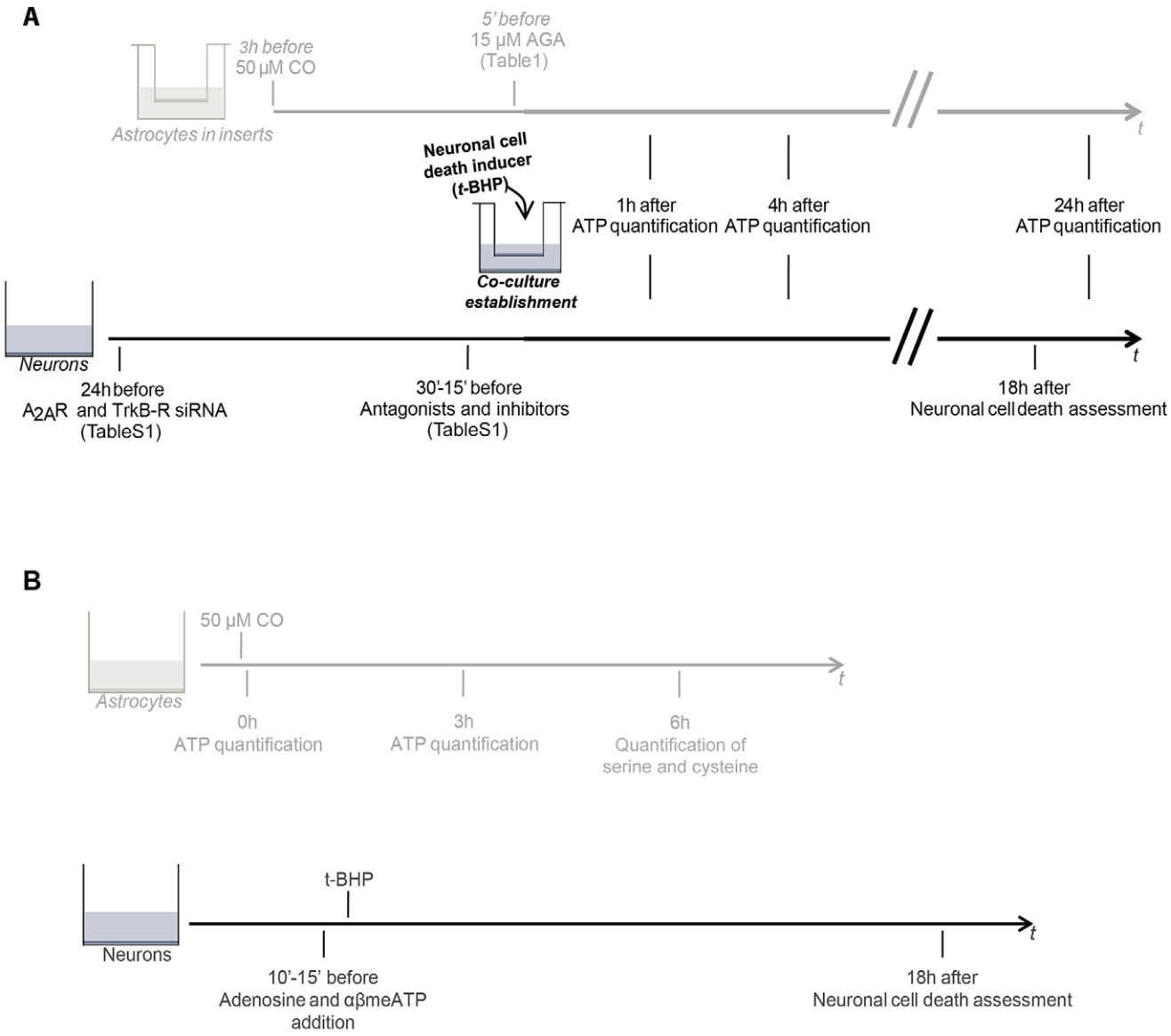

Fig. 1. Representation of experimental time line. The chemical compounds were added at the indicated timings and concentrations. In panel A, timings of the co-culture experiments are shown (Figs 2, 4–6). Panel B represents time lines in monoculture experiments (Fig. 3).

not quantified because the used medium is neuronal. After 1 h of co-culture establishment, the presence of CO decreased the amount of ATP in the supernatant, whereas at 4 h, the equilibrium had already been re-established and was maintained after 24 h. These results indicate that CO might stimulate ATP metabolism, in particular in the first hour following co-culture establishment, when neuronal stimulation might occur.

Adenosine and αβmeATP prevent neuronal cell death in monocultures

For studying the potential neuroprotective role of adenosine and ATP, cell death was induced by oxidative stress (treatment with *t*-BHP) in primary monocultures of neurons. In accordance with previous reports (Haschemi et al., 2007; Sebastiao and Ribeiro, 2009), the addition of 5 µM of adenosine and 10 µM of α,β-methyleneadenosine 5′-triphosphate lithium salt (αβmeATP; an ATP analog, resistant to degradation) increased neuronal survival in monocultures of neurons (Fig. 3D and E).

Effect of neuronal purinergic receptors on CO protection

In order to study ATP and adenosine signaling, the role of purinergic receptors in neurons was assessed. From the P1 family, the high-affinity receptors for adenosine (A_1 and A_{2A}, encoded by *Adora1* and *Adora2a*, respectively) were chosen. For the P2 family (specific for ATP), because the number of receptors is higher, the subfamily of receptors P2X was chosen, instead of a particular receptor.

The effect of P2X-receptor and A_{2A}-receptor antagonists was examined in the CO protective mechanism (Fig. 4A and B). Both in the presence of suramin (P2X-receptor antagonist) and SCH58261 (SCH, A_{2A}-receptor antagonist), the neuroprotection conferred by the CO pre-treatment in astrocytes was reverted. This result confirms that these receptors play a role in astrocyte–neuron communication and are implicated in the neuronal protection that CO pre-treatment in astrocytes confers. 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an A_1-receptor antagonist, was also tested (Fig. 4A and B). However, DPCPX had no effect on

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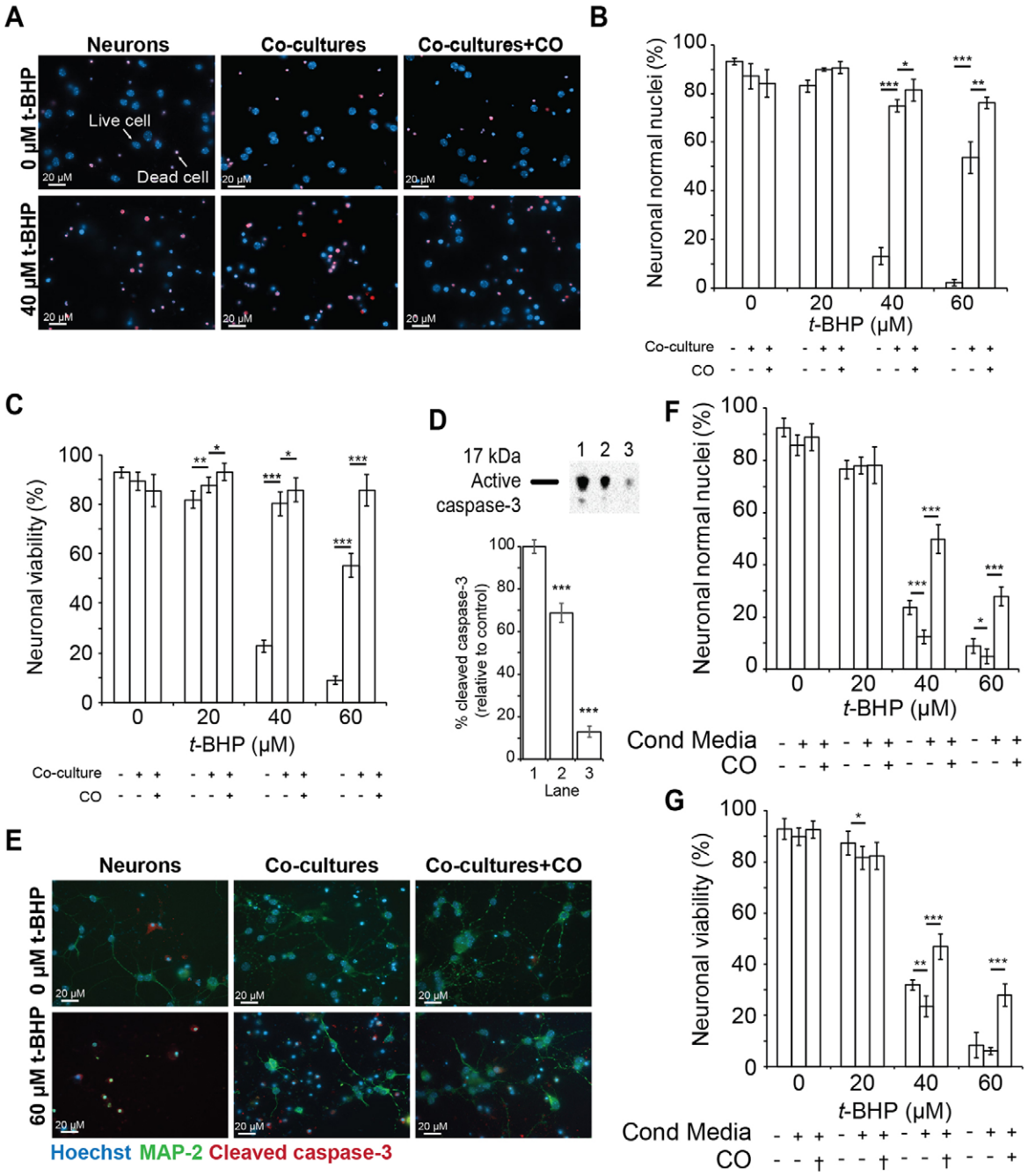

Fig. 2. Effect of astroglial pre-treatment with CO on neuronal apoptosis. Primary cultures of astrocytes were pre-treated for 3 h with 50 µM of CO, followed by establishment of co-culture and 18 h of *t*-BHP (20 to 60 µM) treatment, as outlined in Fig. 1A. Neuronal cell death hallmarks were analyzed by performing fluorescent microscopy. (A) Merged representative micrographs of neurons in co-cultures or not under *t*-BHP treatment. Blue fluorescence (Hoechst 33342) was used to assess chromatin condensation, and red fluorescence reflects the loss of viability (propidium iodide). The images were obtained with a Zeiss Z2 microscope (magnification 630×). (B,C) Quantification of the hallmarks shown in A, regarding neuronal survival in co-cultures under *t*-BHP treatment. Quantification was based on counting the viable nuclei, nuclei presenting chromatin condensation (B) and non-viable cells (C), and for each coverslip, at least 600 cells were counted. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=5 biological replicates. In D, caspase-3 activation was assessed by western blotting. Representative immunoblot is shown in the upper panel and the respective quantification in the lower panel; lane 1, neurons with 60 µM *t*-BHP; lane 2, neurons in co-culture with 60 µM *t*-BHP treatment; lane 3, neurons in co-culture+CO with 60 µM *t*-BHP treatment. All values are mean±s.d. (error bars), ****P*<0.001, *n*=3 biological replicates. (E) Detection of cleaved caspase-3 with immunofluorescence in neurons in co-cultures with *t*-BHP. Blue, nuclei; green, MAP-2, as a marker for neuronal cytoskeleton; red, cleaved caspase-3. Images were taken with a Zeiss Z2 microscope, magnification 630×. (F,G) Results of neuronal cell death hallmark analysis when neurons were exposed to conditioned medium. Quantification was performed as described in B and C. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=5 biological replicates.

CO-induced protection, excluding the involvement of the A_1 receptor in this pathway. Additional controls for chemical inhibitors are presented in Fig. S2. Taken together, ATP and adenosine through neuronal P2X and A_{2A} receptors, respectively, are implicated in the signaling of the neuroprotection induced by CO-treated astrocytes.

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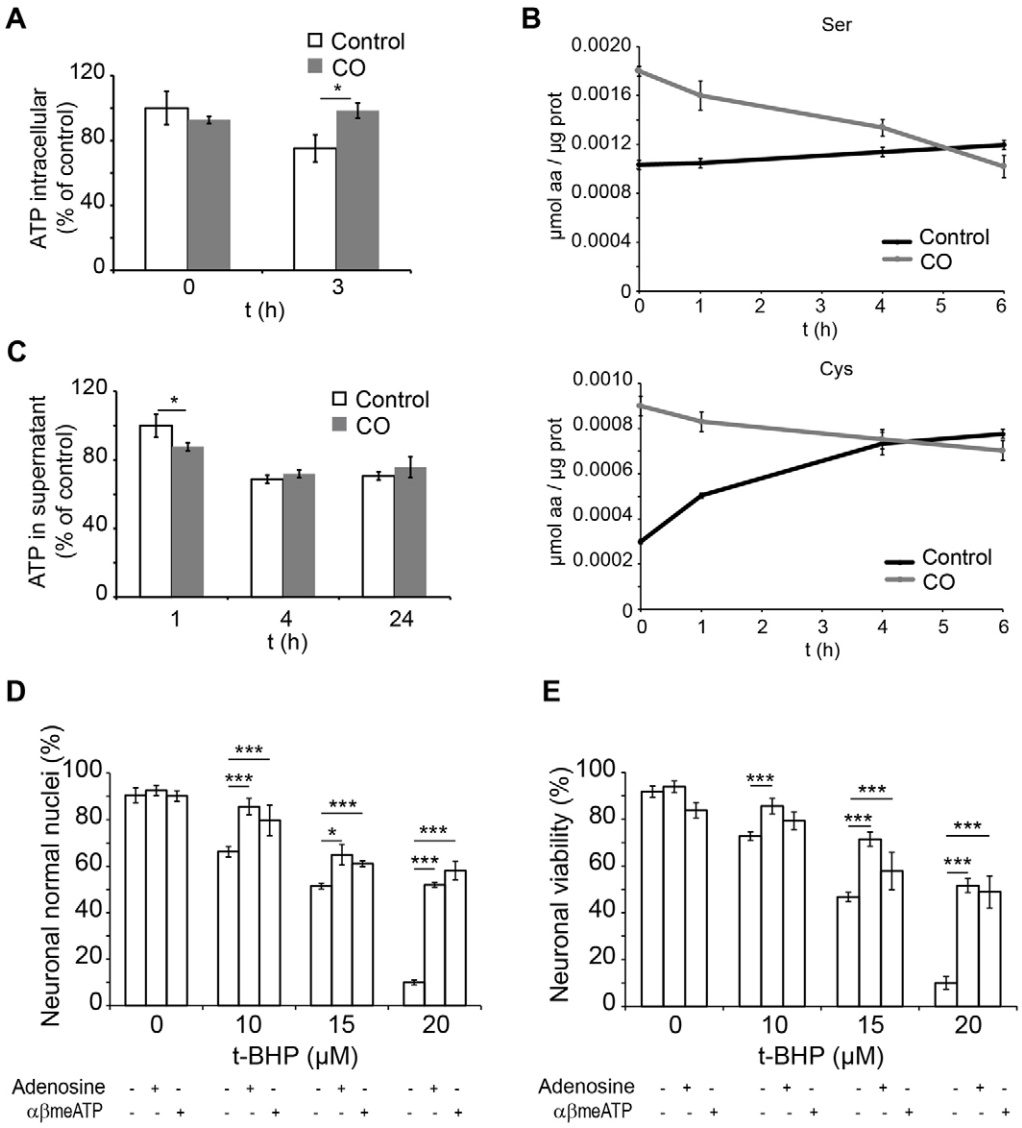

Fig. 3. CO influences intracellular ATP, serine and cysteine content in monocultures of astrocytes and ATP levels in co-culture supernatant. (A) Astrocytes were treated with 50 µM of CO, and intracellular ATP was quantified after 0 and 3 h, as described in the Materials and Methods and in Fig. 1B. All values are mean±s.d., **P*<0.05 (one-way ANOVA), *n*=3 biological replicates. t, time. (B) CO treatment changes the serine (Ser, top panel) and cysteine (Cys, bottom panel) profiles in monocultures of astrocytes. Monocultures of astrocytes were treated with 50 µM of CO. The indicated amino acid (aa) was quantified at 0 h, 1 h, 4 h and 6 h after CO treatment (Fig. 1B). Quantities were calculated and normalized to protein (prot) amount. The profile of these amino acids was inverted, from production to consumption, possibly to feed the TCA cycle and oxidative phosphorylation, in accordance with previous work. All values are mean±s.d., *n*=3 biological replicates. (C) 50 µM of CO was added to astrocytes for 3 h, followed by co-culture establishment. Co-culture supernatant was collected at 1, 4 and 24 h after co-culture establishment. ATP was quantified as described in Materials and Methods. All values are mean±s.d., **P*<0.05 (one-way ANOVA), *n*=3 biological replicates. (D,E) Adenosine and αβmeATP protect neurons against cell death. Neuronal cultures were treated with 5 µM of adenosine and 10 µM αβmeATP for 10 and 15 min, respectively. Then cell death was induced with *t*-BHP (Fig. 1A). Neuronal apoptotic hallmarks were analyzed by performing fluorescent microscopy, such as chromatin condensation (D) and cell viability (E). Quantification was based on counting the viable nuclei, nuclei presenting chromatin condensation and non-viable cells (presenting red-stained nuclei), and for each coverslip, at least 600 cells were counted. All values are mean±s.d., **P*<0.05 and ****P*<0.001 (one-way ANOVA), *n*=6 biological replicates.

Effect of adenosine, ATP release and ATP metabolism on CO protection

CO promotes a conditioning state in astrocytes and a metabolic change that signals to neurons to promote an increase in cell survival. ATP, adenosine, P2X and A_{2A} receptors are involved in this beneficial communication. Nevertheless, it is not clear whether ATP, adenosine or both are released from astrocytes. ATP can be released through connexin 43, and adenosine is released through an ENT. Whenever connexin 43 and ENTs (all isoforms) were inhibited with 18α-glycyrrhetinic acid (AGA) and with S-(ρ-nitrobenzyl)-6-thioinosine (NBTI), respectively (Fig. 4C and D), there was a reversion of the neuroprotective effect caused by CO-treated astrocytes. It is worthy of note that AGA and NBTI are added to astrocytic cultures before co-culture establishment. Thus, their effects are exclusively on astrocytes and not neurons because co-culture medium comprises only neuronal medium. Furthermore, ENT inhibition (adenosine release) caused a reversion of neuroprotection to a great extent, resulting in a much lower survival percentage than in the co-cultures that had not been subject to CO treatment. These data indicate that ENT inhibition might also affect other neuronal events.

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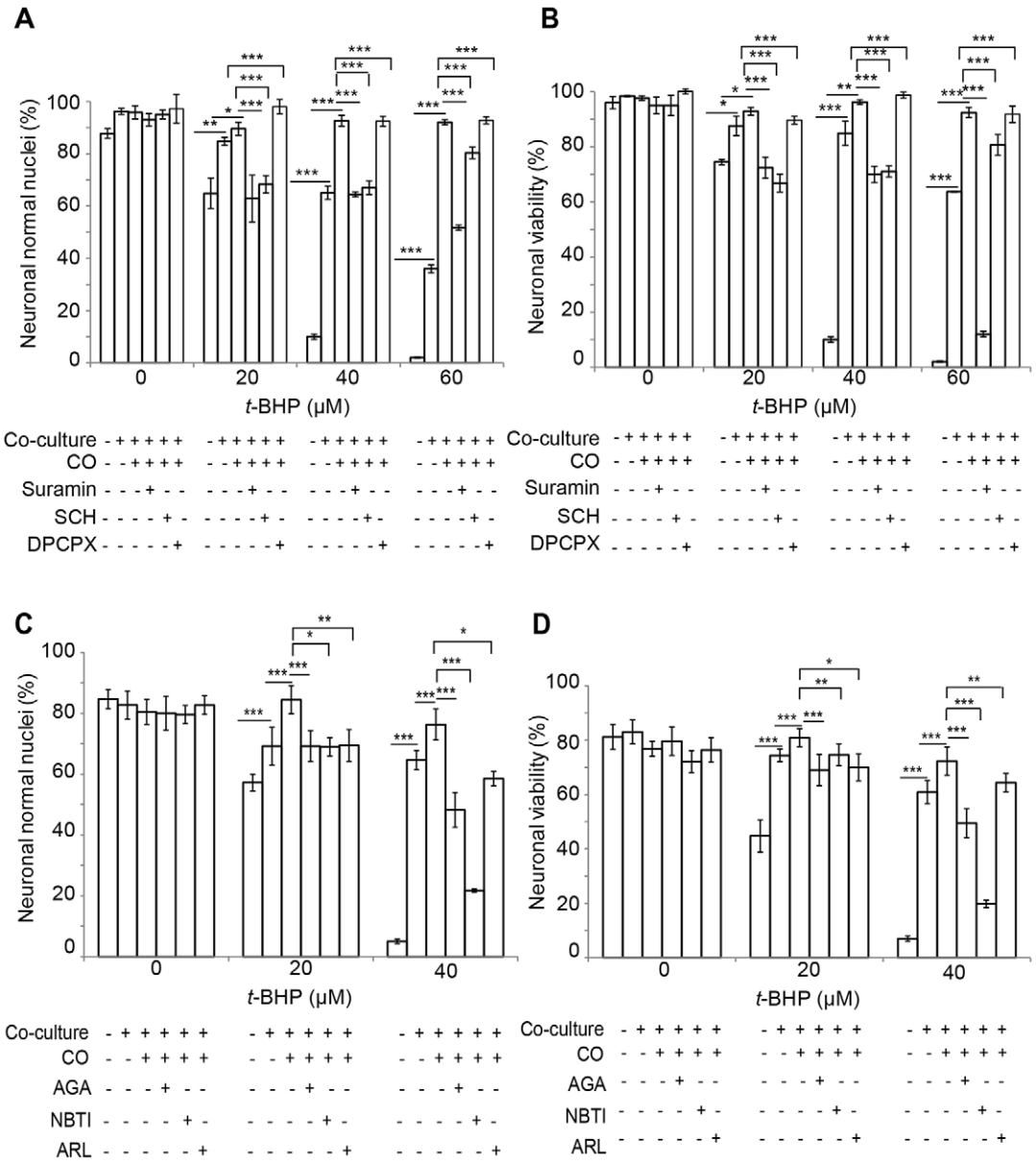

Fig. 4. Effects of suramin, SCH, DPCPX, AGA, NBTI and ARL in the CO mechanism. Primary cultures of astrocytes were pre-treated for 3 h with 50 µM of CO. 30 µM of suramin, 1 µM of SCH and 25 µM of DPCPX were added to primary cultures of neurons 15, 15 and 20 min, respectively, before co-culture establishment (A,B). 15 µM of AGA was added to primary cultures of astrocytes 5 min before CO treatment. 5 µM of NBTI or 50 µM of ARL was added to primary cultures of neurons 30 min before co-culture establishment (C,D). In every case, it was followed by 18 h of *t*-BHP (20 to 60 µM) treatment (Fig. 1A). Neuronal apoptotic hallmarks were analyzed with fluorescence microscopy, such as chromatin condensation (A,C) and cell viability (B,D). Quantification was based on counting the viable nuclei, nuclei presenting chromatin condensation and non-viable cells (presenting red-stained nucleus), and for each coverslip, at least 600 cells were counted. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=8 biological replicates. Additional chemical controls are presented in Fig. S2.

Moreover, adenosine can also be generated by ATP metabolism through ectonucleotidase action. In fact, the ectonucleotidase inhibitor ARL67156 (ARL) did result in reversion of CO protection (Fig. 4C and D). Thus, ATP metabolism has a great importance for CO-mediated astroglial protection in neurons, which is in accordance with previous results (Fig. 3). Indeed, ATP levels in the extracellular space decreased 1 h after establishment of co-cultures, which suggests ATP is metabolized in the presence of neurons. Additional controls for chemical inhibitors are presented in Fig. S2.

Altogether, one can speculate that one possible CO-induced pathway is the stimulation of ATP release from astrocytes that is then metabolized into adenosine, which activates its neuronal receptors – A_{2A} – in order to initiate downstream events to promote neuronal protection. Nevertheless, a role for P2X receptors cannot be excluded.

Silencing of A_{2A} receptor affects CO-mediated protection

We selected the A_{2A} receptor for genetic validation of the pharmacological inhibition data (Fig. 4). A_{2A} receptors were transiently silenced by transfecting neurons with small interfering (si)RNAs against the A_{2A} receptor. Controls for A_{2A} receptor silencing are demonstrated in Fig. S3A, which was unaffected by *t*-BHP treatment, as shown in Fig. S3B. In fact, knocking down the expression of the A_{2A} receptor completely reverted astrocyte-mediated CO protection, which was assessed by examining plasma

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membrane permeabilization (Fig. 5A). The internal control, lacking siRNA, did not change CO-induced neuroprotection, as expected (Fig. 5A). Furthermore, inhibition of caspase-3 activation in neurons in the presence of CO-pre-treated astrocytes was also reverted whenever A_{2A} receptor was downregulated (Fig. 5B).

TrkB receptors are involved in the CO-mediate protection

BDNF has been implicated in activity-dependent synaptic plasticity (Caldeira et al., 2007) and has been associated with neuroprotective outcomes through TrkB receptor activation (Gomes et al., 2012). In fact, pharmacological inhibition of TrkB receptors (Fig. 6A and B) reverts the neuroprotective effect that is promoted by CO-treated astrocytes, suggesting that it is involved in a neuronal downstream event in this pathway to protect against cell death. Additional controls for chemical inhibitors are presented in Fig. S4A and B. Furthermore, genetic downregulation of TrkB expression using siRNA was also performed to assess in a more specific manner the role of this receptor (control of silencing TrkB protein expression is demonstrated in Fig. S4C). In fact, knocking down the expression of TrkB receptor reverted the neuroprotection induced by CO-treated astrocytes (Fig. 6C). In order to shed light on whether TrkB receptors are activated directly by the A_{2A} receptor (which is involved in this pathway; Fig. 5) or through BDNF signaling, the amounts of BDNF and pro-BDNF were measured, and the ratio between the two was calculated (Fig. 6D). In fact, the BDNF-to-pro-BDNF ratio increased in the presence of CO, implicating BDNF in the neuroprotection signaling induced by CO-treated astrocytes (Fig. 6D), confirming the previous results. Moreover, whenever A_{2A} receptors were silenced, the BDNF-to-pro-BDNF ratio returned to the levels in cells that had not been treated with CO. In summary, one can conclude that BDNF is associated with neuroprotection in a paracrine manner through CO-treated astrocytes and purinergic signaling.

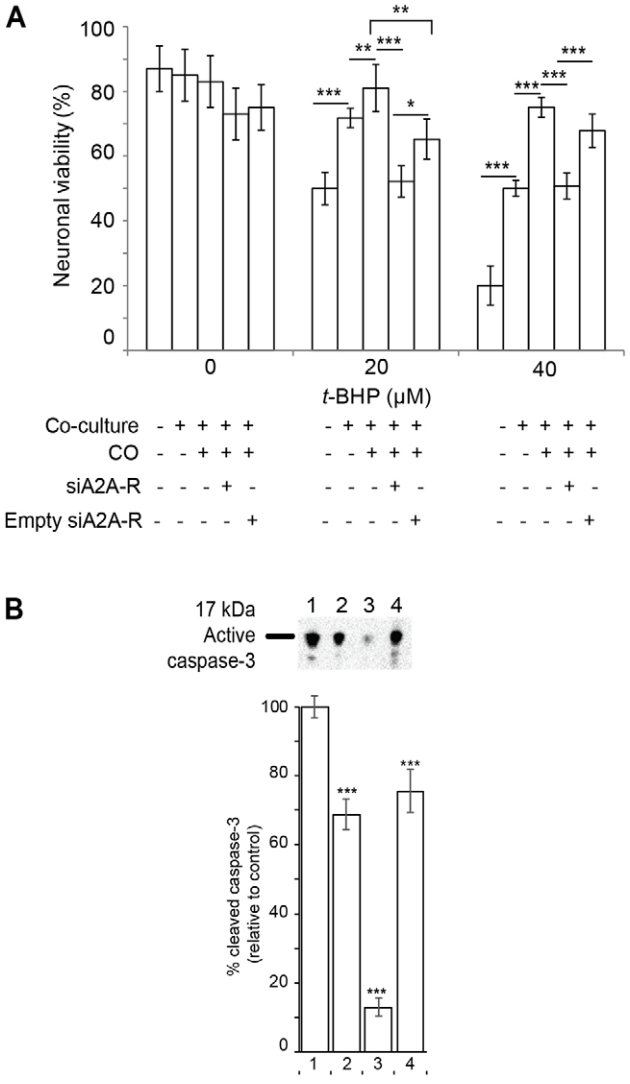

Fig. 5. The effects of silencing A2A receptor expression on CO protection. Primary cultures of astrocytes were pre-treated for 3 h with 50 µM of CO. 4 pmol of siRNA against A_{2A} receptor (siA2A-R) was added to primary cultures of neurons 24 h before co-culture establishment, followed with 18 h of *t*-BHP (20 and 40 µM) treatment (Fig. 1A). Cell viability (A) was analyzed with fluorescence microscopy. Quantification was based on counting non-viable cells (presenting red-stained nuclei), and for each coverslip, at least 600 cells were counted. Empty siA2A-R, mock, lacking siRNA. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=5 biological replicates. Assessment of caspase-3 activation by western blotting is shown in B. A representative immunoblot is shown in the upper panel, and the respective quantification is in the lower panel; lane 1, neurons with 15.6 µM *t*-BHP; lane 2, neurons in co-culture with 15.6 µM *t*-BHP treatment; lane 3, neurons in co-culture+CO with 15.6 µM *t*-BHP treatment; lane 4, neurons in co-culture+CO+ siA2A-R with 15.6 µM *t*-BHP treatment. All values are mean±s.d., ****P*<0.001 (one-way ANOVA), *n*=3 biological replicates.

DISCUSSION

The communication between neurons and astrocytes has been extensively studied (Shinozaki et al., 2005). Astrocytes are well known for the important functions of providing nutritional, structural and signaling support for neurons. They are responsible for providing nutrients like glutamine (glutamine–glutamate cycle) and lactate (astrocyte–neuron lactate shuttle), but also for scavenging the waste and toxic compounds resulting from neuronal metabolism (Belanger et al., 2011). In many pathological contexts, astrocytes are able to rescue neurons from death (Bezzi and Volterra, 2001; Allaman et al., 2011). For the last 15 years, the scientific community studying CO has been taking the approach of investigating the beneficial roles in a cell-autonomous manner, where this gas directly acts on cells and improves their function. For the first time, we demonstrate that CO has a paracrine effect, acting on astrocyte–neuron communication. Herein, it was shown that CO targets astrocytes and modulates their metabolism, releasing astrocytic factor(s), which in turn prevent neuronal death. Moreover, the main molecular events regulating cell-to-cell communication were disclosed (Fig. 7): (i) ATP and adenosine are released from astrocytes (Figs 3 and 4), (ii) ATP is metabolized into adenosine (Figs 3 and 4), (iii) A_{2A} and P2X (Figs 4 and 5) and (iv) TrKB neuronal receptors are activated and BDNF is involved (Fig. 6). Although further studies are necessary to better understand the role of P2X receptors.

The contribution of metabolized adenosine to this model is in agreement with previous reports about upregulation of ectonucleotidases following brain ischemia in order to provide cerebral protection (Shinozaki et al., 2005). A_{2A} receptor activation is also in accordance with the literature, as Boeck and colleagues (Boeck et al., 2005) have described previously that adenosine resulting from ectonucleotidase activity has a preference for A_{2A} receptors, whereas released adenosine prefers A_1 receptors. Moreover, genetic silencing of A_{2A} receptors in neurons confirmed their key roles in the CO paracrine effect (Fig. 5).

Adenosine binding to A_{2A} receptor activates HO-1 (Haschemi et al., 2007), which through CO generation, alters A_{2A} receptor

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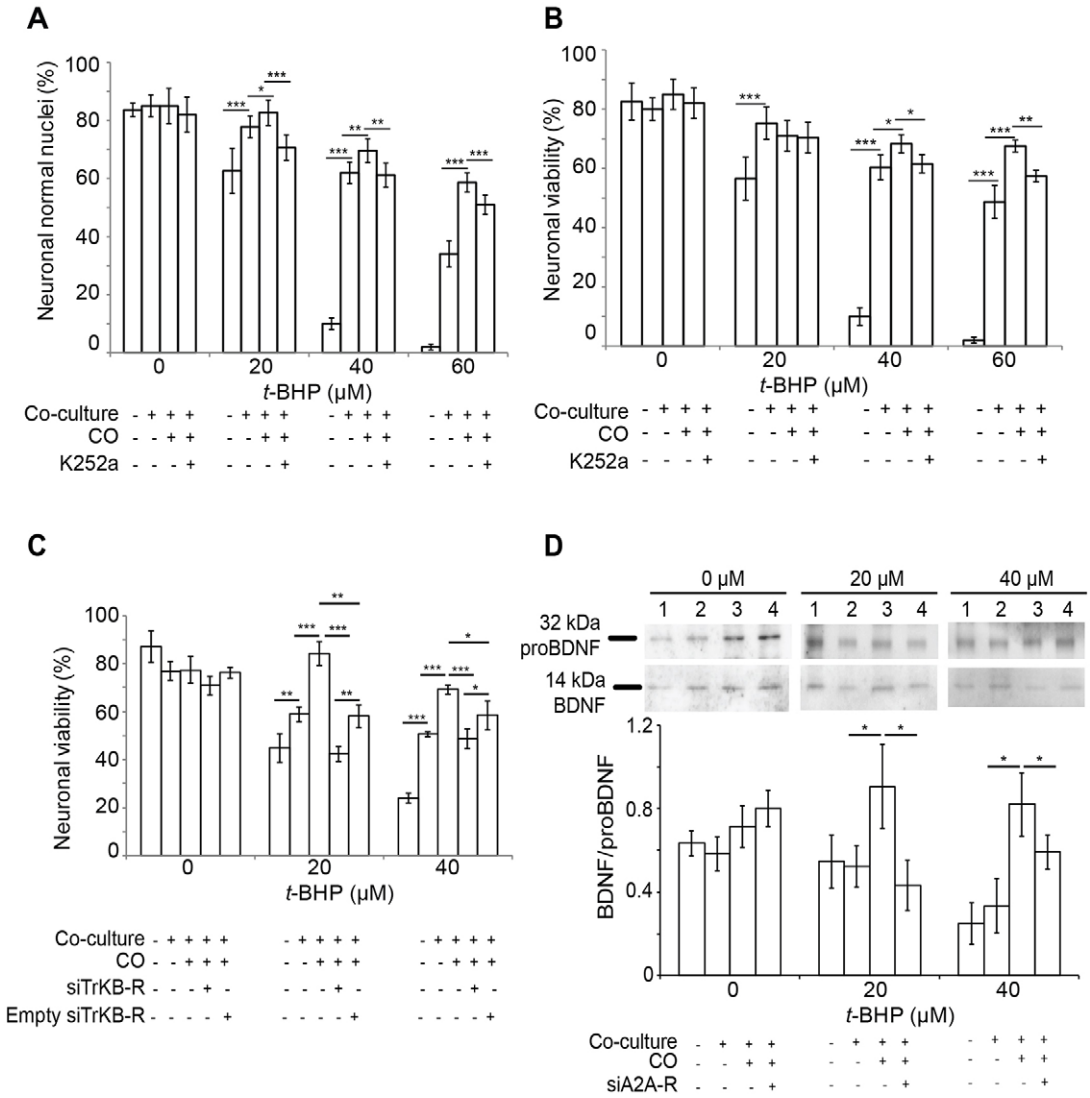

Fig. 6. Effect of TrkB receptor in the CO mechanism. Primary cultures of astrocytes were pre-treated for 3 h with 50 µM of CO. 200 nM of K252a was added to primary cultures of neurons 30 min before co-culture establishment, followed by 18 h of *t*-BHP (20 to 60 µM) treatment (Fig. 1A). Neuronal apoptotic hallmarks were analyzed by performing fluorescent microscopy, such as chromatin condensation (A) and cell viability (B). Quantification was based on counting the viable nuclei, nuclei presenting chromatin condensation and non-viable cells (presenting red-stained nuclei), and for each coverslip, at least 600 cells were counted. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=6 biological replicates. Additional chemical controls are presented in Fig. S4. Primary cultures of astrocytes were pre-treated for 3 h with 50 µM of CO. 2 pmol of siRNA against TrkB receptor (siTrKB-R) was added to primary cultures of neurons 24 h before co-culture establishment, followed by 18 h of *t*-BHP (20 and 40 µM) treatment (Fig. 1A). Cell viability (C) was analyzed by performing fluorescent microscopy. Empty siTrKB-R, mock, lacking siRNA. Quantification was based on counting non-viable cells (presenting red-stained nuclei), and for each coverslip, at least 600 cells were counted. All values are mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001 (one-way ANOVA), *n*=3 biological replicates. In D, the BDNF-to-pro-BDNF ratio was analyzed by western blotting. A representative immunoblot is shown in the upper panel, and the respective quantification is in the lower panel; lane 1, neurons; lane 2, neurons in co-culture; lane 3, neurons in co-culture+CO; lane 4, neurons in co-culture+CO+siA2A-R. All values are mean±s.d., **P*<0.05 (one-way ANOVA), *n*=3 biological replicates.

expression. Also, a positive-feedback loop among adenosine, HO-1, CO and A_{2A} receptors has been described during the chronological resolution in the inflammatory response in macrophages (Haschemi et al., 2007). Based on these data, one could hypothesize that the adenosine neuroprotective role could also be due to the contribution of HO-1 and CO. In the present study, a very interesting and new effect is proposed. CO induces preconditioning in astrocytes to exert a paracrine effect on neurons, through A_{2A} receptor activation, which augments CO content in the target neurons. Therefore, it can be hypothesized that neuronal protection can also occur owing to the action of endogenous CO.

A_{2A} receptors can directly induce translocation and activation of TrkB receptors, or this can occur in a BDNF-dependent manner (Assaife-Lopes et al., 2010). Our data demonstrate that TrkB is activated and that BDNF expression is dependent on A_{2A} receptor expression (Fig. 6), nevertheless, more experiments are needed to demonstrate whether TrkB receptor activation in the present model is dependent or independent of BDNF actions, or both.

CO improves mitochondrial metabolism, reinforcing oxidative phosphorylation and increasing ATP production in several distinct cell types: astrocytes (Almeida et al., 2012), neurons (Almeida et al., 2016) and cancer cells (Wegiel et al., 2013). Herein, it was shown that in monocultures of astrocytes, CO treatment modifies the serine and cysteine kinetic profiles. Accordingly, both amino acids might feed the citric acid cycle and lead to an increase in ATP production in an oxidative-phosphorylation-dependent manner (Almeida et al.,

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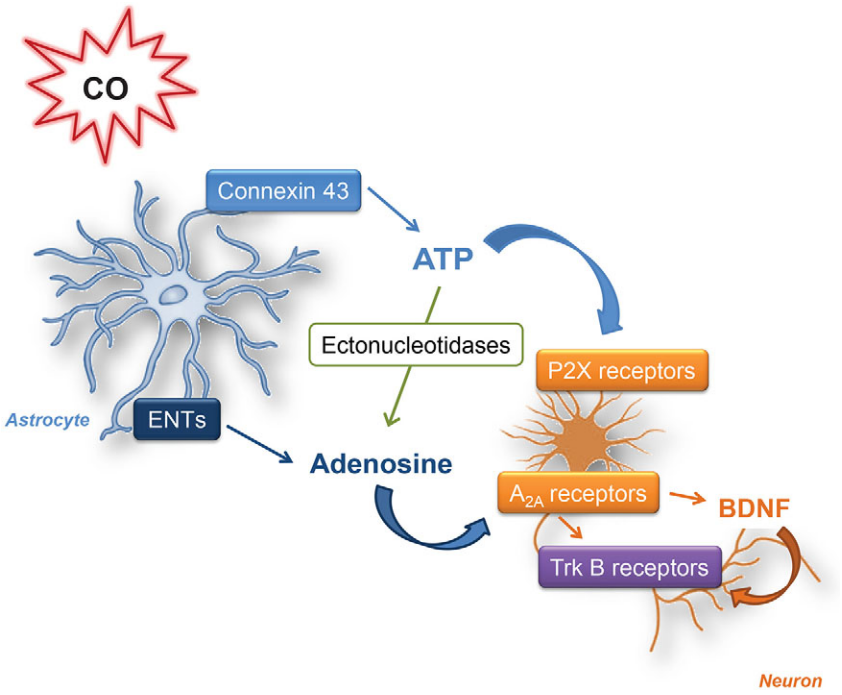

Fig. 7. Schematic representation of the hypothesis and main conclusions. We propose that the paracrine effect of CO in astrocyte–neuron communication is based on purinergic molecules and receptors.

2012). Another metabolic fate for cysteine is glutathione synthesis, in which cysteine is the limiting amino acid. The fact that cysteine consumption was stimulated can also be related to protein glutathionylation, which is a post-translational modification that has already been shown to occur in response to CO (Queiroga et al., 2010).

In conclusion, the brain is a dynamic and interconnected network of cells and signaling molecules. Most of the current therapies under investigation do not take this integrative nature into account and target one cell type, a single cell process or pathway. Herein, the main proteins in purinergic signaling modulating the paracrine protective communication between astrocytes and neurons induced by CO have been identified. Furthermore, the capability of CO in modulating astrocytic paracrine effects is demonstrated, taking advantage of the complex but efficient cell-to-cell communication processes, which opens a new field of CO studies and possible therapeutic applications.

MATERIALS AND METHODS

Materials

All the chemicals were of analytical grade and were obtained from Sigma (Germany) unless stated otherwise. Plastic tissue culture dishes were from Nunc; cell culture inserts were from BD Falcon; fetal bovine serum (FBS), glutamine, penicillin-streptomycin solution and Dulbecco's minimum essential medium (DMEM) were obtained from Gibco. C57/B6 mice were purchased from Instituto Gulbenkian de Ciência (Oeiras, Portugal). For primary culture preparation, mice were rapidly killed. The procedure was approved by the National Institutional Animal Care and Use Committee (Direção Geral de Alimentação e Veterinária with reference number 0421/000/000/2013) and in accordance with relevant national and international guidelines.

Primary cultures of brain cells

Astroglial cells

Primary cultures of astrocytes were prepared from 2-day-old mice brains. Cerebral hemispheres were carefully freed of the meninges, washed in ice-cold PBS and mechanically disrupted. Single-cell suspensions were plated in T-flasks (3 hemispheres/175 cm^2) in DMEM supplemented with 10 mM of glucose, 1% (v/v) penicillin-streptomycin solution and 10% (v/v) heat-inactivated fetal bovine serum. Cells were maintained in a humidified atmosphere of 7% CO_2 at 37°C. After 8 days, the microglia and other non-astrocyte contaminant cells growing on the astrocytic cell layer were separated by vigorous shaking and removed as described previously (McCarthy and de Vellis, 1980). The remaining astrocytes were detached gently with trypsinization using trypsin-EDTA (0.25% w/v) and subcultured in T-flasks for another 3 weeks. Growth medium was changed twice a week.

Neuronal cells

Cerebellar granule cells were isolated from cerebellum of 7-day-old mice. The brain tissue was trypsinized followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans. Cells were suspended (1.25×10^6 cells/ml) and cultured in Basal Medium Eagle (BME) containing 12 mM of glucose, 7.3 µM p-aminobenzoic acid, 4 µg/l insulin, 2 mM glutamine, 1% (v/v) penicillin-streptomycin solution and 10% (v/v) heat-inactivated fetal bovine serum. Cells were maintained in a humidified atmosphere of 7% CO_2 at 37°C. Cytosine arabinoside (20 µM) was added after 24–48 h to prevent glial cell proliferation. Neurons are considered mature between 7 to 11 days *in vitro*.

Co-cultures of neurons and astrocytes

The co-cultures were initiated by transferring cell culture inserts (0.4 µm) containing astroglial cells into a well with neuronal primary cultures. Small molecules are allowed to pass through the membrane but the astrocyte-to-neuron contact is prevented.

Preparation of CO solutions

Fresh stock solutions of CO gas were prepared each day and carefully sealed. PBS was saturated by bubbling 100% of CO gas for 30 min to produce 10^{-3} M stock solution. The concentration of CO in solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin, as in an assay described previously (Motterlini et al., 2002). 100% CO was purchased as compressed gas (Linde, Germany).

Cell death induction and prevention, and detection

Neuronal cells

5 µM of adenosine and 10 µM of αβmeATP were added to neuronal monocultures 10 min and 15 min, respectively, before induction of cell

death with *t*-BHP at 10 and 20 µM. Apoptosis-related parameters were analyzed 18 h later.

Co-cultures

Astrocytes were pre-treated with 50 µM of CO. After 3 h, astroglial cells (without astrocytic medium) were transferred to the neuron compartment. Then neuronal apoptosis was induced with *t*-BHP at 20 to 80 µM for 18 h. Afterwards, cell death-associated parameters were analyzed.

Cell-death-associated hallmarks

In both *in vitro* models, cell-death-associated parameters were analyzed by performing fluorescence microscopy using Hoechst 33342 (2 mM) for chromatin condensation assessment and propidium iodide (PI, 1 µg/ml, Invitrogen) to determine cell viability, based on the plasma membrane integrity. The results are expressed as the percentage relative to the control (100%). Several compounds were used to modulate CO effects and are listed in Table S1. The cells were observed on a Leica DMRB microscope (Leica, Wetzlar, Germany) using a filter cube giving a UV excitation range with a wavelength bandpass of 340–380 nm. For each coverslip, 8–10 fields containing around 200 cells were counted (a total of at least 1500 counted cells).

Immunoblotting

Neuronal samples were collected with lysis buffer, which consisted of 50 mM Tris-HCl, pH 6.8, 10% glycerol (v/v) and 2% SDS (w/v). Protein concentration was determined using the Pierce BCA Protein Assay Kit and was measured at 540 nm. Total protein extract (30 µg) was mixed with 10 mM DTT, 10% (v/v) and 0.005% (w/v) bromophenol blue, loaded into 12% polyacrylamide gels and electrically transferred to a nitrocellulose membrane (Hybond™-C extra, Amersham Biosciences). The membranes were blocked with 5% BSA in TBS with 0.1% Tween-20 (TBS-T) and subsequently incubated with primary antibodies in 5% BSA in TBS-T. Antibodies were against cleaved caspase-3 (sc-9664, Cell Signaling Technology), A_{2A} receptor (sc-70321, Santa Cruz Biotechnology), TrkB (ab33655, Abcam) and BDNF (sc-546 Santa Cruz Biotechnology) and used at 1/200 dilution for 2 h at room temperature. Blots were developed using the ECL (BioRad) detection system after incubation with horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG antibody (Amersham Bioscience), 1/5000, 1 h of incubation at room temperature. These experiments have been repeated three times with similar results.

Immunofluorescence

Neurons were fixed with 4% PFA for 15 min and then permeabilized with 0.1% SDS in PBS for 30 min, at room temperature. The cells were incubated for 2 h, at room temperature, with primary antibody diluted in 10% FBS in PBS. Antibodies were against cleaved caspase-3 (sc-9664, Cell Signaling Technology) and MAP-2 (Sigma-Aldrich, M1406) and used at 1/500. After 1 h of incubation with Alexa-Fluor-594-conjugated anti-rabbit IgG (A11012, 1/500) and Alexa-Fluor-488-conjugated anti-mouse IgG (A11001, 1/500), respectively, and Hoechst (33342, 1/2000), cells were mounted in mounting medium, and images were captured with a Zeiss Z2 microscope.

ATP quantification

The quantity of ATP was assessed with a PerkinElmer kit. It is a luminescent assay, based on a luciferase reaction. ATP reacts with luciferin, emitting luminescent light that can be detected and it is proportional to the intracellular ATP level in monocultures of astrocytes or ATP content present in the co-culture supernatant. The results are expressed in percentages relative to the control (100%).

Serine and cysteine quantification by HPLC

Amino acids in monocultures of astrocytes were quantified by high-performance liquid chromatography (HPLC) using a reverse phase 3.9×150 mm column (AccQ.Tag, Waters, USA). A pre-column derivatization method (Waters AccQ.Tag Amino Acid Analysis) was used, as described previously (Carinhas et al., 2010). An internal standard (α-aminobutyric acid) was added to ensure consistency between runs. Mobile phases were prepared following the manufacturer's instructions, filtered and degassed before usage.

siRNA transfection

A_{2A} receptor and TrkB expression were silenced by using siRNAs against A_{2A} receptor and TrkB according to manufacturer's instructions (Invitrogen, UK). Neurons were transfected using Lipofectamine™ RNAiMAX and Opti-MEM® medium (Invitrogen, UK); for 1.9 cm^2 of culture area, 1.25×10^6 cells/ml, 4 pmol of siRNA for A_{2A} receptor and 2 pmol of siRNA for TrkB were used. At room temperature, siRNA and culture medium were gently mixed with Lipofectamine to form liposomes, and then neurons were transfected in the absence of antibiotics. At 24 and 48 h after transfection, A_{2A} receptor and TrkB expression was assessed by western blotting, silencing was more efficient at 24 h so A_{2A}-receptor- and TrkB-silenced neurons were used within this time frame.

Statistical analysis of data

At least three biological replicates were performed for all experiments, using different cultures that had been isolated from different animal pools; values are mean±s.d., $n\geq3$. Error bars correspond to s.d. Statistical comparisons were performed in Microsoft Excel using ANOVA: single factor with replication, with $*P$-value<0.05, $**P$-value<0.01, $***P$-value<0.001, asterisks refer to all figures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.S.F.Q. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. R.M.A.A. and S.V.C. performed experiments. P.M.A. supervised experiments. H.L.A.V. conceived the study, supervised experiments and wrote the manuscript. All authors edited the manuscript.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.187260.supplemental

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Paracrine effect of CO: astrocytes promote neuroprotection *via* purinergic signaling

Supplementary material

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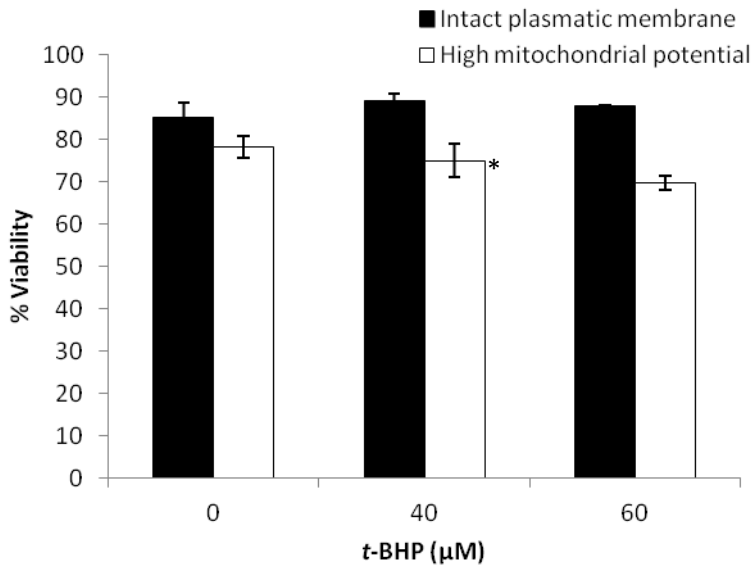
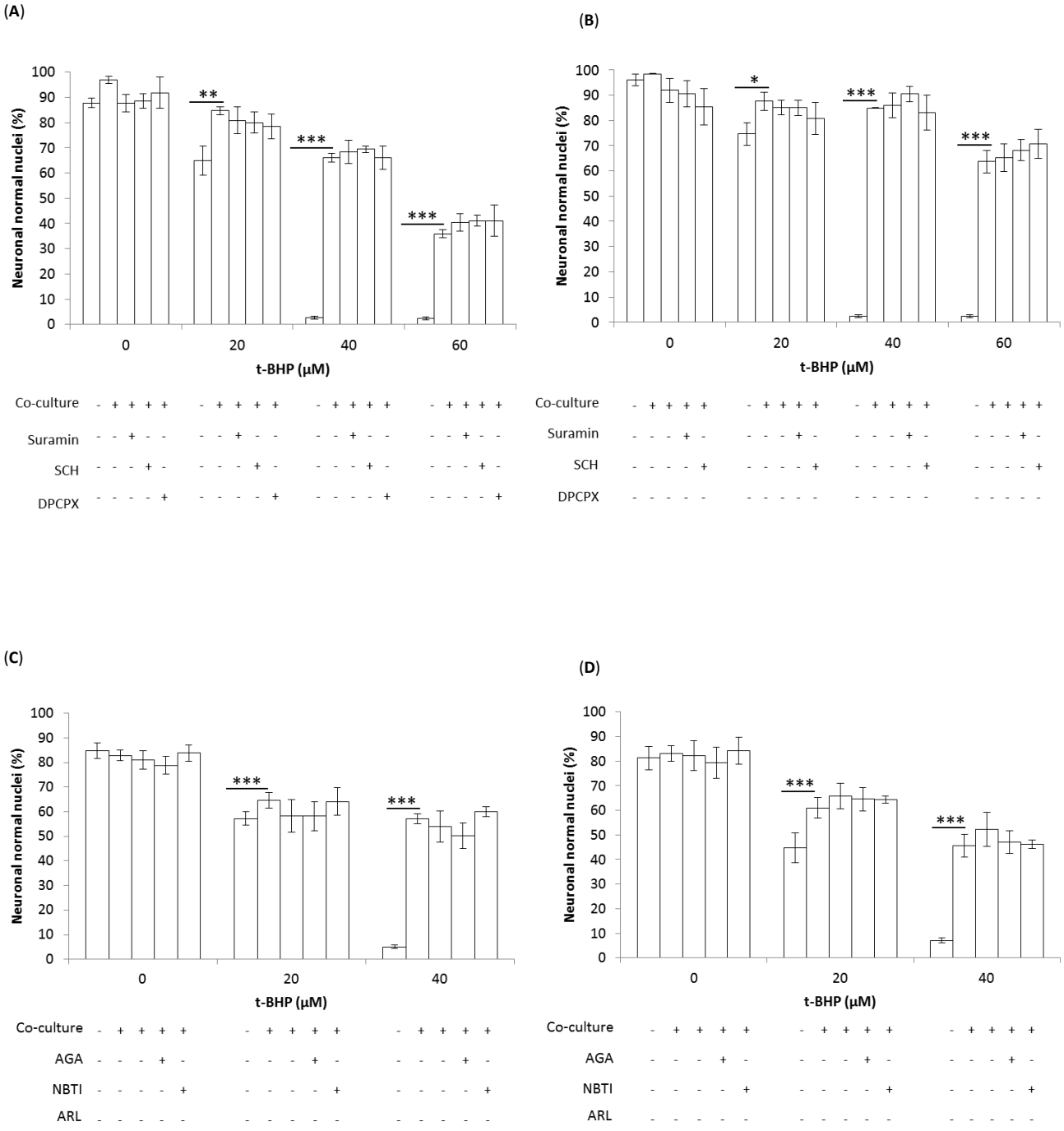
Herein are displayed the supplementary data on *tert*-butylhydroperoxide effect on astrocytes, the chemicals controls for Fig. 4 and 6 and experimental control of protein expression following siRNA silencing procedure for Fig. 5 and 6 of the main manuscript.

Journal of Cell Science • Supplementary information



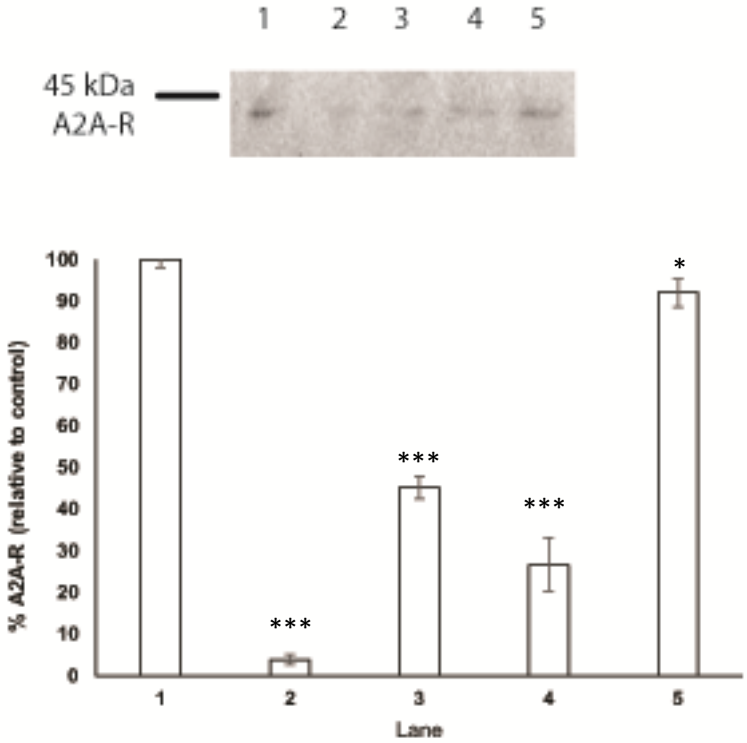
Supplementary Figure S1. Effect of *tert*-butylhydroperoxide (*t*-BHP) at 40 and 60 µM on monocultures of astrocytes. Primary cultures of astrocytes cultured in 24 well-plates were pre-treated with 50 µM of CO for 3h, following apoptosis induction by the 18h exposure to the oxidative stress inducer, *t*-BHP, at the same concentrations used in the co-culture approach. Cell death hallmarks were assessed by flow cytometry. It is expressed by the percentage of cells with high mitochondrial potential, detected with DiOC6(3) fluorochrome and by the percentage of viable cells (intact plasma membrane), assessed with propidium iodide (PI). All values are mean±SD, *p<0.5, comparing to 0 µM, n=4.



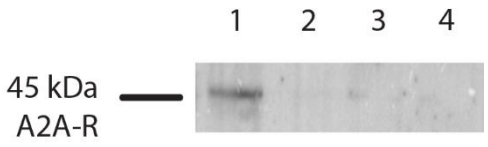
Supplementary Figure S2. Effect of suramin, SCH58261 (SCH), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) 18α-glycyrrhetinic acid (AGA), S-(p-nitrobenzyl)-6-thioinosine (NBTI) and ARL67156 (ARL) in neuronal survival under co-culture conditions. Chemical controls for Fig. 4 of the main manuscript. 30 µM of suramin, 1 µM of SCH or 25 µM of DPCPX were added to primary cultures of neurons 15, 15 or 20 min, respectively before co-culture

establishment (**A**) and (**B**). 15 µM of AGA was added to primary cultures of astrocytes, 5 min before CO treatment. 5µM of NBTI or 50µM of ARL were added to primary cultures of neurons 30 min before co-culture establishment (**C**) and (**D**). In every case, it was followed by 18h of *t*-BHP (20 to 60 µM) treatment (**Figure 1A**). Neuronal apoptotic hallmarks were analyzed by fluorescent microscopy, such as chromatin condensation (**A**) and (**C**) and cell viability (**B**) and (**D**). Quantification was based on counting the viable nucleus, nucleus presenting chromatin condensation and unviable cells (presenting red stained nucleus), and for each cover slip, at least 600 cells were counted. All values are mean±SD, *p<0.05, **p<0.01 and ***p<0.001, n=8.

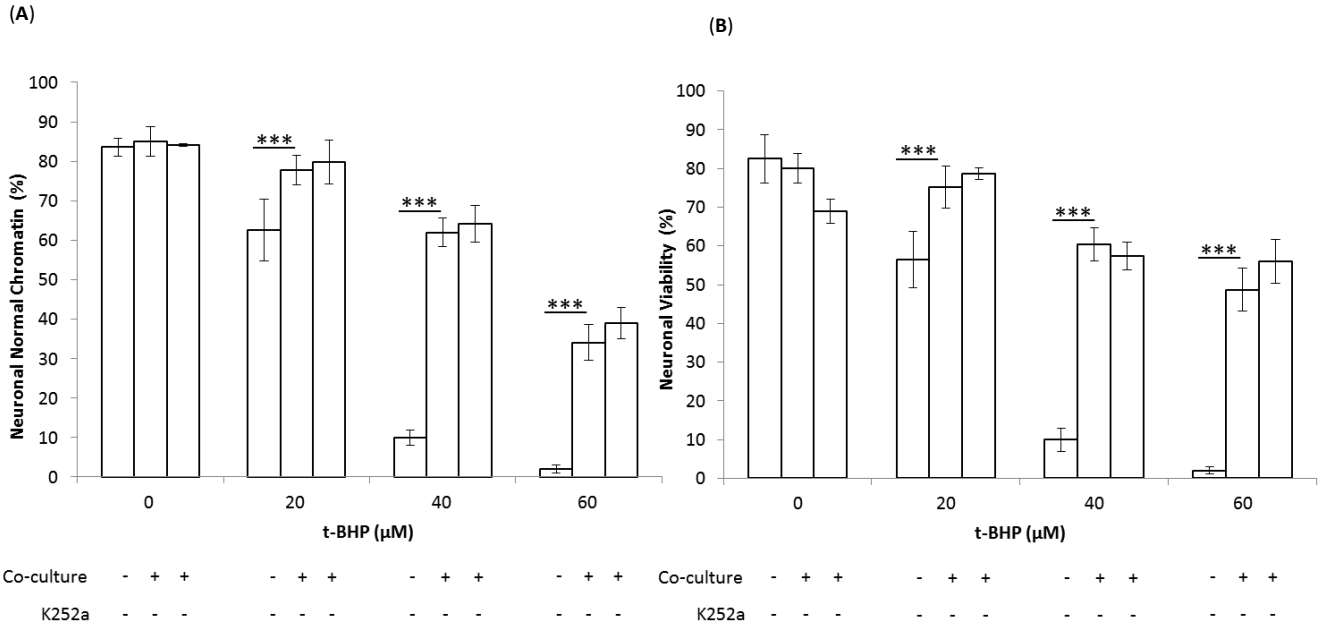
(A)



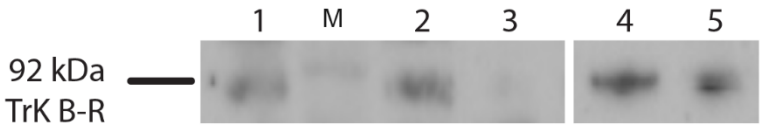
B)

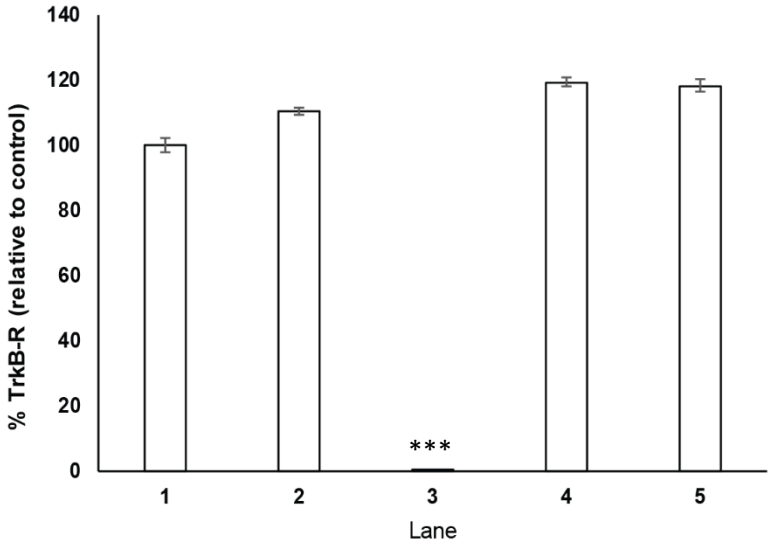


Supplementary Figure S3. Effect of A_{2A}-R in CO mechanism. (**A**) A_{2A}-R siRNA experiment optimization. Several conditions were tested and then the cellular extract was analysed by western blot. Representative immunoblot in the upper panel and the respective quantification in the lower panel; lane 1, neurons under normal conditions, lane 2, neurons with siA_{2A}-R after 24h and with an incubation of 10 minutes with Opti-MEM, lane 3, neurons with siA_{2A}-R after 24h and with an incubation of 20 minutes with Opti-MEM, lane 4, neurons with siA_{2A}-R after 48h and with an incubation of 10 minutes with Opti-MEM, lane 5, neurons with siA_{2A}-R after 48h and with an incubation of 20 minutes with Opti-MEM. This experiment was repeated three times with similar results. All values are mean±SD (error bars), ***p<0.001, n=3. (**B**) A_{2A}-R down regulation under *t*-BHP treatment. 4 pmol of siRNA A_{2A}-R were added to primary cultures of neurons and 24h later *t*-BHP was added to cells, during 18h; lane 1, neurons under normal conditions, lane 2, neurons+siA_{2A}-R under 0 µM *t*-BHP, lane 3, neurons+siA_{2A}-R under 20 µM *t*-BHP, lane 4, neurons+siA_{2A}-R under 40 µM *t*-BHP. This experiment was repeated three times with similar results.



(C)





Supplementary Figure S4. Effect of TrkB-R in neuronal survival under co-culture conditions. Chemical controls for Fig. 6 of the main manuscript. 200 nM of K252a was added to primary cultures of neurons 30 min before co-culture establishment, followed by 18h of *t*-BHP (20 to 60 µM) treatment (**Figure 1A**). Neuronal apoptotic hallmarks were analysed by fluorescent microscopy, such as chromatin condensation (**A**) and cell viability (**B**). Quantification was based on counting the viable nucleus, nucleus presenting chromatin condensation and unviable cells (presenting red stained nucleus), and for each cover slip, at least 600 cells were counted. All values are mean±SD, ***p<0.001, n=6. (**C**) TrkB-R siRNA experiment optimization. Several conditions were tested and then the cellular extract was analysed by western blot. Representative immunoblot in the upper panel and the respective quantification in the lower panel; lane 1, neurons under normal conditions, M, molecular marker, lane 2, neurons with siTrkB-R after 24h and with an incubation of 10 minutes with Opti-MEM, lane 3, neurons with siTrkB-R after 24h and with an incubation of 20 minutes with Opti-MEM, lane 4, neurons with siTrkB-R after 48h and with an incubation of 10 minutes with Opti-MEM, lane 5, neurons with siTrkB-R after 48h and with an incubation of 20 minutes with Opti-MEM. This experiment was repeated three times with similar results. All values are mean±SD (error bars), ***p<0.001, n=3.

Table S1. List of compounds used to modulate CO effect

Compound	Action	Final concentration	Target cell population	Time of addition (before co-cultures establishment)
Suramin	P2X antagonist	30 µM	Neuronal medium	15 min
SCH58261 (SCH)	A_{2A} antagonist	1 µM	Neuronal medium	15 min
8-cyclopentyl-1,3-dipropylxanthine (DPCPX)	A_1 antagonist	25 µM	Neuronal medium	20 min
18α-glycyrrhetinic acid (AGA)	Connexin 43 inhibitor	15 µM	Astrocytic medium	5 min
S-(ρ-nitrobenzyl)-6-thioinosine (NBTI)	Adenosine Equilibrative Nucleotide Transporter inhibitor	5 µM	Astrocytic medium	30 min
ARL67156 (ARL)	Ectonucleotidase inhibitor	50 µM	Neuronal medium	30 min
K252a	TrK B receptor antagonist	200 nM	Neuronal medium	30 min
siRNA ADORA2A	A_{2A}R silencing	4 pmol	Neurons	24 h
siRNA Ntrk2	TrK B silencing	2 pmol	Neurons	24 h