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Contribution of adenosine and ATP to the carotid body chemosensory activity in ageing

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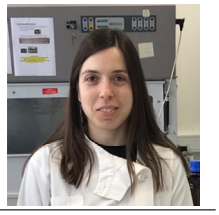
Key points

- Adenosine and ATP are excitatory neurotransmitters involved in the carotid body (CB) response to hypoxia.
- During ageing the CB exhibits a decline in its functionality, demonstrated by decreased hypoxic responses.
- In aged rats (20–24 months old) there is a decrease in: basal and hypoxic release of adenosine and ATP from the CB; expression of adenosine and ATP receptors in the petrosal ganglion; carotid sinus nerve (CSN) activity in response to hypoxia; and ventilatory responses to ischaemic hypoxia. There is also an increase in SNAP25, ENT1 and CD73 expression.
- It is concluded that, although CSN activity and ventilatory responses to hypoxia decrease with age, adjustments in purinergic metabolism in the CB in aged animals are present aiming to maintain the contribution of adenosine and ATP.
- The possible significance of the findings in the context of ageing and in CB-associated pathologies is considered.

Abstract During ageing the carotid body (CB) exhibits a decline in its functionality. Here we investigated the effect of ageing on functional CB characteristics as well as the contribution of adenosine and ATP to CB chemosensory activity. Experiments were performed in 3-month-old and 20- to 24-month-old male Wistar rats. Ageing decreased: the number of tyrosine hydroxylase immune-positive cells, but not type II cells or nestin-positive cells in the CB; the expression of $P2X_2$ and A_{2A} receptors in the petrosal ganglion; and the basal and hypoxic release of adenosine and ATP from the CB. Ageing increased ecto-nucleotidase (CD73) immune-positive cells and the expression of synaptosome associated protein 25 (SNAP25) and equilibrative nucleoside transporter 1 (ENT1) in the CB. Additionally, ageing did not modify basal carotid sinus nerve (CSN) activity or the activity in response to hypercapnia, but decreased CSN activity in hypoxia.

Joana F. Sacramento has been part of the Neuronal Control of Metabolic Disturbances research group of CEDOC, NOVA Medical School in Lisbon since 2011. She graduated with a BSc in Molecular and Cellular Biology in 2010 and obtained her masters degree in Biotechnology in 2012, both from Faculdade de Ciências e Tecnologia of NOVA University, Portugal. This year she obtained her PhD degree at NOVA Medical School under the supervision of Sílvia V. Conde. Her project focused on the modulation of carotid body activity as a therapeutic target in metabolic disturbances. In 2017 she was awarded the Fernando De Castro Award from the International Society for Arterial Chemoreception.



Constancio Gonzalez passed away during the development of the work.

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The contribution of adenosine and ATP to stimuli-evoked CSN chemosensory activity in aged animals followed the same pattern of 3-month-old animals. Bilateral common carotid occlusions during 5, 10 and 15 s increased ventilation proportionally to the duration of ischaemia, an effect decreased by ageing. ATP contributed around 50% to ischaemic-ventilatory responses in young and aged rats; the contribution of adenosine was dependent on the intensity of ischaemia, being maximal in ischaemias of 5 s (50%) and much smaller in 15 s ischaemias. Our results demonstrate that both ATP and adenosine contribute to CB chemosensory activity in ageing. Though CB responses to hypoxia, but not to hypercapnia, decrease with age, the relative contribution of both ATP and adenosine for CB activity is maintained.

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Introduction

The carotid bodies (CBs) are small paired organs located in the bifurcation of the common carotid artery that classically sense changes in the levels of O_2, CO_2 and pH in arterial blood, specifically through the chemoreceptor cells (glomus cells or type I cells). These stimuli activate the CBs, inducing an increase in the frequency of discharges in the carotid sinus nerve (CSN), which once integrated in the brainstem lead to a set of cardiorespiratory and metabolic reflexes aimed to restore levels of blood gases, blood pressure and cardiac performance. Among the neurotransmitters released from the chemoreceptor cells are ATP and adenosine. In the last few years a large body of evidence has demonstrated the significance of adenosine and ATP in the hypoxic chemotransduction in the CB (Buttigieg & Nurse, 2004; Conde *et al.* 2009, 2017; Murali & Nurse, 2016). The CB releases adenosine and ATP simultaneously in response to hypoxia, the release of ATP and its extracellular catabolism to adenosine being the main origin of extracellular adenosine at high-intensity hypoxia (Conde & Monteiro, 2004; Conde *et al.* 2012*a*). During mild hypoxia, adenosine is also released via the equilibrative nucleoside transporter (ENT) (Conde & Monteiro, 2004; Conde *et al.* 2012*a*). Also, it has been shown that both adenosine and ATP are the main players in hypoxic chemotransmission in the CB sensory synapse (Rong *et al.* 2003; Conde *et al.* 2006*a*), with ATP contributing more than adenosine to generate CSN activity in high-intensity hypoxias and adenosine showing a more pronounced role during mild/moderate hypoxia (Conde *et al.* 2012*a*).

Ageing is associated with a gradual decline in all body functions including a marked reduction in the maximum breathing capacity (Knapowski *et al.* 2002), a reduction in O_2 supply to the tissues (Chan & Welsh, 1998) and a decrease in arterial blood P_{O_2} (Cerveri *et al.* 1995; Chan & Welsh, 1998). To some extent the deficit in ventilation has been attributed to a decline in the chemoreceptor functionality of the CB. Morphological studies found degenerative ultrastructural changes developing with age in the CB (Di Giulio *et al.* 2003; Pokorsky *et al.* 2004; for a review see Di Giulio, 2018). It was described as an increase in extracellular matrix, a reduction in the number of chemoreceptor cells and a decrease in the mitochondrial volume similar somehow to that observed in chronic hypoxia (Wang & Bisgard, 2002). These morphological alterations are in agreement with our previous observations that the CB enlarges with age but with a concomitant decrease in the proportion of chemoreceptor cells (Conde *et al.* 2006*b*). We have also found that old animals exhibit a smaller catecholamine release in response to a hypoxic challenge than young animals, while it was similar in response to an unspecific depolarizing stimulus (Conde *et al.* 2006*b*). Recording the activity in the CSN, whose integration leads to a ventilatory response, we found accordingly that hypoxia was unable to elicit similar responses in old animals, while the response to hypercapnia/acidosis was similar (Conde *et al.* 2006*b*; Quintero *et al.* 2016), demonstrating that the CBs of aged animals are less functional. Consistent with these findings it was found in humans that hypoxic chemosensitivity decreased with age (Peterson *et al.* 1981; García-Río *et al.* 2007) but remained stable from the age of 75 onward, an effect that the authors suggest to be mainly due to loss of peripheral chemosensitivity (García-Río *et al.* 2007).

In the last decade a lot of diseases that are associated with age such as hypertension, type 2 diabetes, obstructive sleep apnoea and chronic heart failure have been associated with an increased CB and sympathetic nervous system activity (Peng *et al.* 2003; Abdala *et al.* 2012; Del Rio *et al.* 2013; Ribeiro *et al.* 2013) suggesting that modulation of CB function might be used to treat these pathologies (Pijacka *et al.* 2016; Sacramento *et al.* 2018). Knowing from our previous studies the importance of adenosine and ATP as key players in the chemotransduction at the CB, here we investigated the effect of ageing in key proteins involved in the purinergic metabolism in the CB and also the contribution of adenosine and ATP to the CB chemosensory activity. We found that both ATP and adenosine contribute to CB chemosensory activity in ageing. Though

CB function in response to hypoxia but not to hypercapnia decreases with age, the relative contribution of both ATP and adenosine to CB activity is maintained via adjustments in CB purinergic metabolism.

Methods

Ethical approval

All animal experimental and care procedures were approved by the Institutional Committee of the University of Valladolid and Nova Medical School. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Our work complies with the animal ethics guidelines as outlined in the editorial by Grundy (2015).

Animals and surgical procedures

Experiments were performed in adult Wistar rats of both sexes aged 3 months and 20–24 months obtained from the vivarium of the NOVA Medical School of Lisbon and from the vivarium of the Faculty of Medicine of the University of Valladolid. The rats were kept at a constant temperature (21°C) and a regular light (08.00–20.00 h) and dark (20.00–08.00 h) cycle, with food and water *ad libitum*. The animals were anaesthetized with sodium pentobarbital (60 mg kg^{-1} I.P.), tracheostomized and the carotid bifurcation was removed and dissected *in vitro* in a Lucite Chamber. Adenosine CB content, and adenosine and ATP release experiments, were performed as previously described (Conde & Monteiro 2006; Conde *et al.* 2012*a*). Briefly, the carotid bifurcation was collected from the animal and the CBs were cleared free of CSN and nearby connective tissue. For CB content evaluation, the organs were placed in 3 M perchloric acid (PCA) and processed as previously described (Conde *et al.* 2006*a*). For adenosine and ATP release the CBs were placed in 500 µl of ice-cold 95% O_2-equilibrated Tyrode medium until the onset of the experiments (see protocol in Results section). For the recording of CSN activity, the CB–CSN preparation was dissected in ice-cold 100% O_2-equilibrated Tyrode medium. The CB–CSN preparation was digested in collagenase type I (1 mg ml^{-1}) solution, to loosen the perineurium, and maintained in ice-cold 100% O_2-equilibrated Tyrode medium until it was transferred to the recording chamber (Conde *et al.* 2006*b*, 2012*a*,*b*). To investigate A_{2A} and $P2X_2$ receptor expression in the petrosal ganglion and the expression of equilibrative nucleoside transporter type 1 (ENT1) and synaptosomal-associated protein of 25 kDa (SNAP25) in the carotid body, petrosal ganglions and CBs were dissected as previously described (Conde *et al.* 2012*b*) and placed in cryovials in liquid nitrogen until further homogenization. For immunohistochemistry studies, the carotid bifurcation was collected from the animal, and the CB, with a small piece of the carotid artery, was bilaterally dissected and immersion-fixed in 4% paraformaldehyde (PFA). Samples were embedded into OCT (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and frozen, then serial sections of 8 µm thick were obtained with a Leica CM3050 S cryostat (Leica Biosystems, Nussloch, Germany).

At the end of the experiments, the rats were killed by an intracardiac overdose of sodium pentobarbital (60 mg kg^{-1}), except when heart puncture was performed to collect blood. Death was confirmed by cervical dislocation.

Effect of ageing on ATP and adenosine release by the carotid body

In all ATP and adenosine release experiments, before the incubation periods, the CBs were submitted to a 30 min period of pre-incubation in hyperoxia (95% O_2 + 5% CO_2) at 37°C to allow recovery from the surgery. Due to their small size (CB wet weight ∼50 µg), four CBs were used in each experiment. After pre-incubation the CBs were incubated for 10 min at 37°C in 500 µl of Tyrode solution in the presence of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; an inhibitor of adenosine deaminase, 2.5 µM) equilibrated with the following percentages of O_2 and CO_2: normoxia, 20% O_2 + 5% CO_2; hyperoxia, 95% O_2 + 5% CO_2; and hypoxia, 2% and 10% O_2 + 5% CO_2, depending on the hypoxic intensity to be studied; in all cases the balance gas was N_2. After the incubation period the CBs were removed and adenosine and ATP were extracted from the incubation medium (Conde *et al.* 2012*a*).

Western blot analysis of A_{2A} adenosine and $P2X_2$ ATP receptors in the petrosal ganglion, and 5′-ectonucleotidase (CD73), ENT1 and SNAP25 in the carotid body

Petrosal ganglions and CBs were homogenized in Zurich medium containing a cocktail of protease inhibitors (Conde *et al.* 2012*b*). Samples containing subcellular suspension were subjected to SDS–PAGE (10% or 12%) analysis and transferred onto nitrocellulose membrane (0.2 mmol l^{-1}; BioRad, Madrid, Spain) as described (Conde *et al.* 2012*b*). The membranes were blocked for 1 h in I-Block (Applied Biosystems, Foster City, CA, USA) in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) at room temperature, followed by incubation with primary antibodies against A_{2A} (mouse, 1:100), $P2X_2$ (rabbit, 1:100), SNAP25 (mouse, 1:200), ENT1 (mouse, 1:100) (Santa Cruz Biotechnology, Madrid, Spain) and tyrosine hydroxylase (mouse, 1:4000; Sigma,

Madrid, Spain) at room temperature with gentle agitation. Membranes where then washed 4× for 15 min with 0.02% TBST, and incubated in 0.1% TBST containing biotin-conjugated goat anti-mouse (1:10,000; Millipore, Madrid, Spain) and mouse anti-rabbit (1:10,000; Pierce, Madrid, Spain) for 90 min with gentle agitation. After washes membranes were incubated for 30 min in 0.1% TBST containing horseradish peroxidase-conjugated streptavidin (1:10,000; Pierce), as described (Ribeiro *et al.* 2013). Immunoreactive bands developed with enhanced chemiluminescence reagents (Immobilon Western; Millipore) and signals were detected in a Chemidoc Molecular Imager (Chemidoc; BioRad). Bands were quantified using the Quantity-One software (BioRad). The membranes were re-probed and tested for β-actin (1:1000, Sigma) and calnexin (1:1000, SICGEN, Cantanhede, Portugal) to compare and normalize the protein expression with the amount of protein loaded. The membranes immunoreactive for TH were stripped in order to make a re-probe for 5′-ectonucleotidase (CD73). Briefly, the membranes were incubated twice with a stripping buffer (6 M GnHCl, 0.2% Nonidet P-40 (NP-40), 0.1 M β-mercaptoethanol, 20 mM Tris-HCl, pH 7.5) for 5 min, washed with water and equilibrated with TBST. Afterwards the membranes were incubated with a primary antibody against CD73 (1:250, Abcam, Cambridge, UK) at room temperature and then conjugated with secondary and tertiary antibodies as described above.

Immunohistochemical analysis of type I and type II cell number, nestin-immune positive cells and CD73

After sectioning, CB sections were washed in PBS at room temperature (22–23°C) for 5 min and incubated in permeabilizing–blocking solution (PBS containing 0.1% Triton X-100 and 2% non-immunized goat serum) for 15 min. The incubation with the primary antibodies, rabbit anti-tyrosine hydroxylase (1:1000), nestin (1:250), CD73 (1:500) (Abcam, Cambridge, UK) and glial fibrillary acidic protein (GFAP) (1:250; Dako, Glostrup, Denmark) was done at 4°C and maintained overnight. After washing with PBS (3 × 10 min), the sections were incubated with the secondary antibody (goat anti-rabbit Alexa Fluor 594; Abcam, Cambridge, UK) at 1:2000 in permeabilizing–blocking solution for 1 h at room temperature (22–23°C). After washing with PBS (3 × 10 min), the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1}; Santa Cruz Biotechnology) for 5 min at room temperature (22–23°C). Finally, the sections were washed with PBS (4 × 5 min), and with distilled water, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Negative controls were similarly incubated but in the absence of primary antibody.

The sections were examined with a fluorescence microscope (ZEISS Axio Imager 2; ZEISS, Oberkochen, Germany) with the excitation and emission filters for Alexa Fluor 594. Images were captured using Axiocam 105 colour (ZEISS) and stored in a computer. With adequate software, Fiji app for Image J (https://imagej.nih.gov/ij/), tyrosine hydroxylase, GFAP, nestin and CD73 immune-positive areas and the entire area of the CB tissue in each section were measured and used to calculate the percentage area of immunoreactive cells.

Recording of CSN activity

The CSN recordings were performed as previously described by Conde *et al.* (2006*b*, 2012*a*) and Quintero *et al.* (2016). Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxia (normoxia: 20% O_2 + 5% O_2 + 75% N_2; hypoxia: 2%, 5% or 7% O_2 + 5% CO_2 + balanced N_2). The effects of hypoxia of several intensities: intense hypoxia (0% O_2, ∼20 mmHg), moderate hypoxia (5% O_2, 35–40 mmHg) and mild hypoxia (7% O_2, 50–55 mmHg); and the effect of hypercapnia (20% O_2 + 10% CO_2 + balanced N_2) were investigated in 3-month-old and 20- to 24-month-old rats, as well as the effects of suramin (50 μM) and ZM241385 (300 nM) on the CSN activity in normoxia, hypoxia (0 and 5% O_2) and hypercapnic-evoked CSN action potentials. Chemoreceptor activity was discriminated off-line for height and timing (Clampex 9.0, Molecular Devices, Winnersh, UK).

Effect of ageing on basal ventilation and on the ventilatory responses to hypoxic hypoxia and ischaemic hypoxia

Ventilation responses to hypoxic hypoxia were measured in conscious freely moving rats by whole body plethysmography as previously described (Conde *et al.* 2012*b*). Briefly, the rats were placed in the plethysmographic chamber and breathed room air for at least 30 min until they adapted to the chamber environment and acquired a standard resting behaviour. Specific protocols are provided in the Results section. All the gases were balanced with N_2 and applied at a flow of 2 l min^{-1}. The pressure change within the chamber reflecting tidal volume (V_T) was measured with a high-gain differential pressure transducer. Ideally, the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to V_T; a calibration of the system by injections of 5 ml of air into the chamber allowed a direct estimation of V_T. Pressure signals were fed to a computer for

visualization and storage for later analysis with EMKA software (Emka Technologies, Paris, France).

A detailed description of the methods to evaluate the response to ischaemic hypoxia in anaesthetized animals has been previously published (Monteiro *et al.* 2011). Evaluation of ventilatory parameters, respiratory frequency and tidal volume, in basal conditions and in response to ischaemic hypoxia (bilateral occlusion (5–15 s) of common carotid artery) were obtained by a pneumotachograph (Hugo SACHS Elektronik; Harvard Apparatus, Madrid, Spain) in anaesthetized and tracheostomized control and aged rats. Bilateral mid-cervical vagotomy was performed to abolish the role of vagal afferents innervating the lungs and the aortic chemoreceptors with a major influence on respiratory activity (Marek *et al.* 2008). Control experiments were performed in animals submitted to a bilateral cut of the CSN in order to distinguish central and peripherally mediated effects.

Effect of A_2 adenosine and P2X ATP receptor antagonists on ventilatory responses to ischaemic hypoxia

To investigate the role of adenosine and ATP on ventilatory responses evoked by ischaemic hypoxia, suramin (P2X ATP receptor antagonist, 14 μmol kg^{-1}), ZM241385 (A_2 adenosine receptor antagonist, 29 μmol kg^{-1}) and SCH58261 (A_{2A} adenosine receptor antagonist, 20 nmol kg^{-1}) were applied intravenously, separately or together, 3 min prior to occlusion of the common carotid artery in 3- and 24-month-old rats. The effect of A_2 and P2X antagonists was tested on occlusion of the common carotid artery of durations 5, 10 and 15 s.

Drugs and chemicals

Adenosine, ATP, SCH58621, sodium pentobarbital and suramin were obtained from Sigma (Madrid, Spain). Collagenase type I (292 U mg^{-1}) was obtained from Worthington (Lakewood, NJ, USA). ZM241385 was obtained from Tocris (Abingdon, UK). SCH58621 and ZM241385 were prepared as 5 mM stock solutions in dimethylsulfoxide (DMSO); the final concentration of DMSO was always below 1/500, which is too low to have any effect on our preparations.

Data analysis

The amount of adenosine released by the CBs quantified by HPLC was expressed in picomoles per CB after division of the absolute values obtained from the chromatograms by four (four CBs were used in each incubation) and correction to the volume used. Data were evaluated using GraphPad Prism Software, version 5 and were presented as mean ± SD. The significance of the differences between the means was calculated by unpaired Student's *t* tests, one-way analysis of variance (ANOVA) with Dunnett's and Tukey's multiple comparison tests and by two-way ANOVA with Bonferroni's multiple comparison test. *P* values of 0.05 or less were considered to represent significant differences.

Results

Effect of ageing on type I and type II cell number in the carotid body

It is generally agreed that ageing leads to an enlargement of CBs, which is concomitant with a decrease in the number of type I cells (for a review see Di Giulio, 2018). Our results obtained in Fig. 1*A* are in agreement with those previous studies. It is evident that the relative size of the CB, as well as the chemoreceptor clusters and the immunoreactivity for TH (red fluorescence, left panels, Fig. 1*A*), are smaller in the older animals (Fig. 1*B*). In agreement with the significant decrease of 49.04% in the immunoreactivity for TH (Fig. 1*B* upper panel), we also observed a significant decrease of 25.73% in TH expression in the whole CB (Fig. 1*B* lower panel). Ageing did not alter the percentage of cells immunoreactive to GFAP (Fig. 1*C* and *D*) and non-significantly decreased the cells immune-positive to nestin (Fig. 1*E* and *F*) by 12.80%.

Effect of ageing on ATP and adenosine release, and adenosine content, in the carotid body

ATP release from the CB of 3-month-old rats increased with hypoxic intensity (20% $O_2 = 5.11 \pm 2.38$ pmol CB^{-1}; 10% $O_2 = 9.60 \pm 6.15$ pmol CB^{-1}; 2% $O_2 = 18.00 \pm 7.12$ pmol CB^{-1}, Fig. 2*A*). This effect was similar to that described previously (Conde *et al.* 2012*a*). However, ageing significantly decreased the levels of ATP present extracellularly by 46.70%, 56.15% and 68.13% in response to 20%, 10% and 2% O_2, respectively (Fig. 2*A*). Similarly, the amount of adenosine present in CB extracellular medium from 3-month-old rats follows the same pattern described previously (Conde *et al.* 2012*a*), with the release of adenosine being higher at moderate hypoxias (20% $O_2 = 25.77 \pm 11.12$ pmol CB^{-1}; 10% $O_2 = 62.80 \pm 21.57$ pmol CB^{-1}; 2% $O_2 = 43.80 \pm 18.63$ pmol CB^{-1}). Ageing dramatically decreased the levels of adenosine present in CB extracellular medium by 97.45% in basal conditions, by 98.24% in response to 10% O_2 and by 98.13% in response to 2% O_2; nevertheless, the pattern of release remains the same (Fig. 2*B*). In contrast, the amount of adenosine quantified in homogenized CBs increased non-significantly by 73.20% in 20–24 months old animals

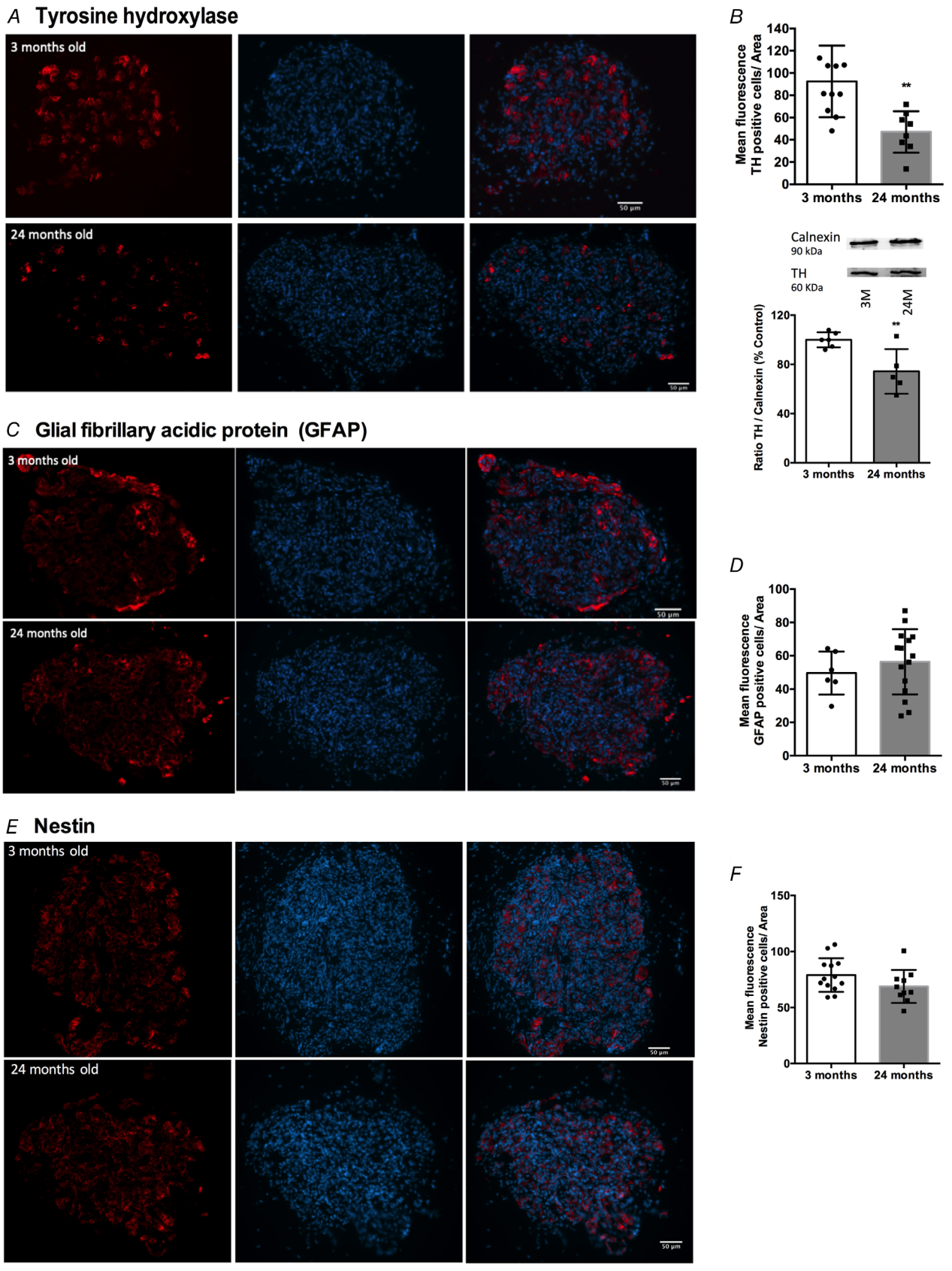

Figure 1. Effect of ageing on type I and type II cells in the carotid body (CB)

A, immunofluorescence images for tyrosine hydroxylase, a marker for CB type I cells (red fluorescence, left panels) and DAPI (blue fluorescence, middle panels) and its merge (panels on the right) in slices of 3-month-old (top) and 24-month-old (bottom) CBs. *B*, top graph represents mean fluorescence of tyrosine hydroxylase (TH)-positive areas present in serially sectioned CBs (four CBs from 3-month-old and 20- to 24-month-old rats); bottom panel shows a representative western blot comparing tyrosine hydroxylase immunoreactivity, corresponding to a 60 kDa band, in CBs of 3- and 24-month-old animals and its average expression of tyrosine hydroxylase in 3- and 24-month-old rats. Calnexin (90 KDa band) was used as loading protein. *C*, immunofluorescence images for glial fibrillary acidic protein (GFAP), a marker for CB type II cells (red fluorescence, left panels) and DAPI (blue fluorescence, middle panels) and its merge (panels on the right) in slices of 3-month-old (top) and 24-month-old (bottom) CBs. *D*, mean fluorescence of GFAP-positive areas present in serially sectioned CBs (five CBs from 3-month-old and 20- to 24-month-old rats). *E*, immunofluorescence images for nestin, a marker for neural stem cells (red fluorescence, left panels) and DAPI (blue fluorescence, middle panels) and its merge (panels on the right) in slices of 3-month-old (top) and 24-month-old (bottom) CBs. *F*, mean fluorescence of nestin-positive areas present in serially sectioned CBs (four CBs from 3-month-old and 20- to 24-month-old rats). Data represent means ± SD. $^{**}p < 0.01$, Student's *t* test.

from a control value of 2.66 ± 1.71 pmol (mg tissue)$^{-1}$ (Fig. 2*C*).

Effect of ageing on SNAP25, ENT1 and CD73 in the carotid body

Aiming to understand if the decreases in the amount of ATP and adenosine present extracellularly were due to altered exocytosis, we have analysed by western blot the expression of a key protein involved in the exocytotic machinery, SNAP25, a t-SNARE protein, as well as the equilibrative nucleoside transporter-1 (ENT1) responsible for the release of adenosine *per se* in moderate hypoxic conditions in the CB (Conde & Monteiro, 2004; Conde *et al.* 2012*a*), in 3- and 24-month-old animals. Surprisingly, ageing increased the SNAP-25 and ENT-1 expression by 83.71% and 400.52%, respectively, in the CB (Fig. 3*A* and *B*). Knowing that the extracellular conversion of ATP to adenosine is another important source of adenosine, especially in intense hypoxic conditions (Conde *et al.* 2012*a*), we have also evaluated the effect of ageing on the expression of ecto-5′-nucleotidase (CD73), the enzyme responsible for the conversion of AMP to adenosine, that is known to be present in the CB surrounding type I cells (Salman *et al.* 2017). It can be clearly noted CD73 has an extracellular localization, mainly, but not only, surrounding type I cells (Fig. 3*D*). Ageing significantly increased the expression of CD73 by 73.95% measured by western blot analysis (Fig. 3*C*) and by 87.12% measured by immunohistochemistry (Fig. 3*E*), suggesting an increased extracellular ATP catabolism to increase extracellular adenosine levels.

Effect of ageing on A_{2A} adenosine receptor and $P2X_2$ ATP receptor expression in the petrosal ganglion

In the present article we have only tested the effect of ageing on the expression of A_{2A} and $P2X_2$ receptors, since there is common agreement that the A_{2A} receptor subtype is responsible for the postsynaptic adenosine-mediated

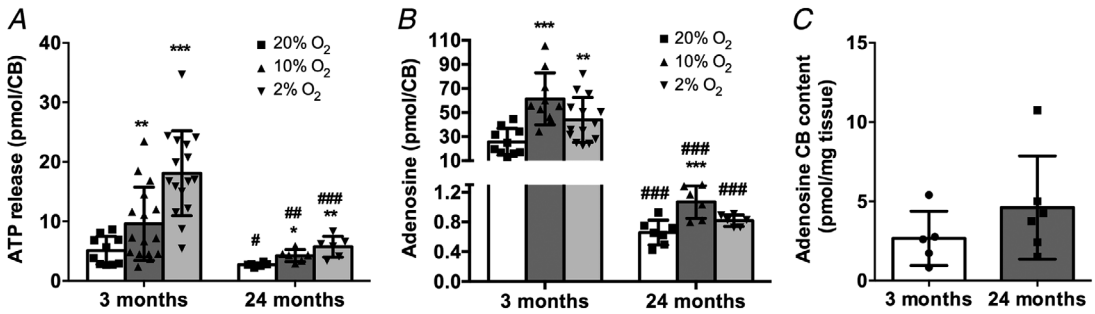

Figure 2. Effect of ageing on the release of ATP (*A*) and adenosine (*B*) in normoxic and hypoxic conditions and on adenosine content (*C*) in the rat carotid body (CB)
A, release of ATP from CB in normoxia (20% O_2) and by hypoxia of two intensities (10% and 2% O_2) in control (3-month-old rats; $n = 10$–16) and 24-month-old rats ($n = 6$). *B*, release of adenosine from CB in normoxia and by hypoxia of two intensities (10% and 2% O_2) in control (3-month-old rats; $n = 10$–14) and 24-month-old rats ($n = 6$–7). *C*, adenosine content in the rat CB in 3-month-old ($n = 5$) and 24-month-old animals ($n = 6$). Experiments were performed in the presence of EHNA and 5% CO_2. Vertical bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, one-way ANOVA with Dunnett's multiple comparison test between adenosine and ATP levels in response to different O_2 concentrations in comparison with levels in 20% O_2. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, two-way ANOVA with Tukey's multiple comparison test between adenosine and ATP levels in response to different O_2 concentrations between 3- and 24-month-old rats.

actions (Conde *et al.* 2006*b*, 2012*a*) and that $P2X_2$ is the receptor subtype involved in ATP-mediated actions in the carotid sinus nerve (Prasad *et al.* 2001; Rong *et al.* 2003). As it can be seen in Fig. 4, ageing decreases the expression of $P2X_2$ and A_{2A} receptors significantly by 52.55% and 42.17%, respectively, in the petrosal ganglion, in comparison with 3-month-old rats.

Contribution of adenosine and ATP antagonists to carotid sinus nerve activity in basal conditions and in response to hypoxia and hypercapnia

Figure 5*A* shows typical recordings of CSN chemosensory activity in young and aged animals. The left panel shows a typical CSN recording in response to intense hypoxia (perfusion with a 0% O_2-equilibrated solution) in young

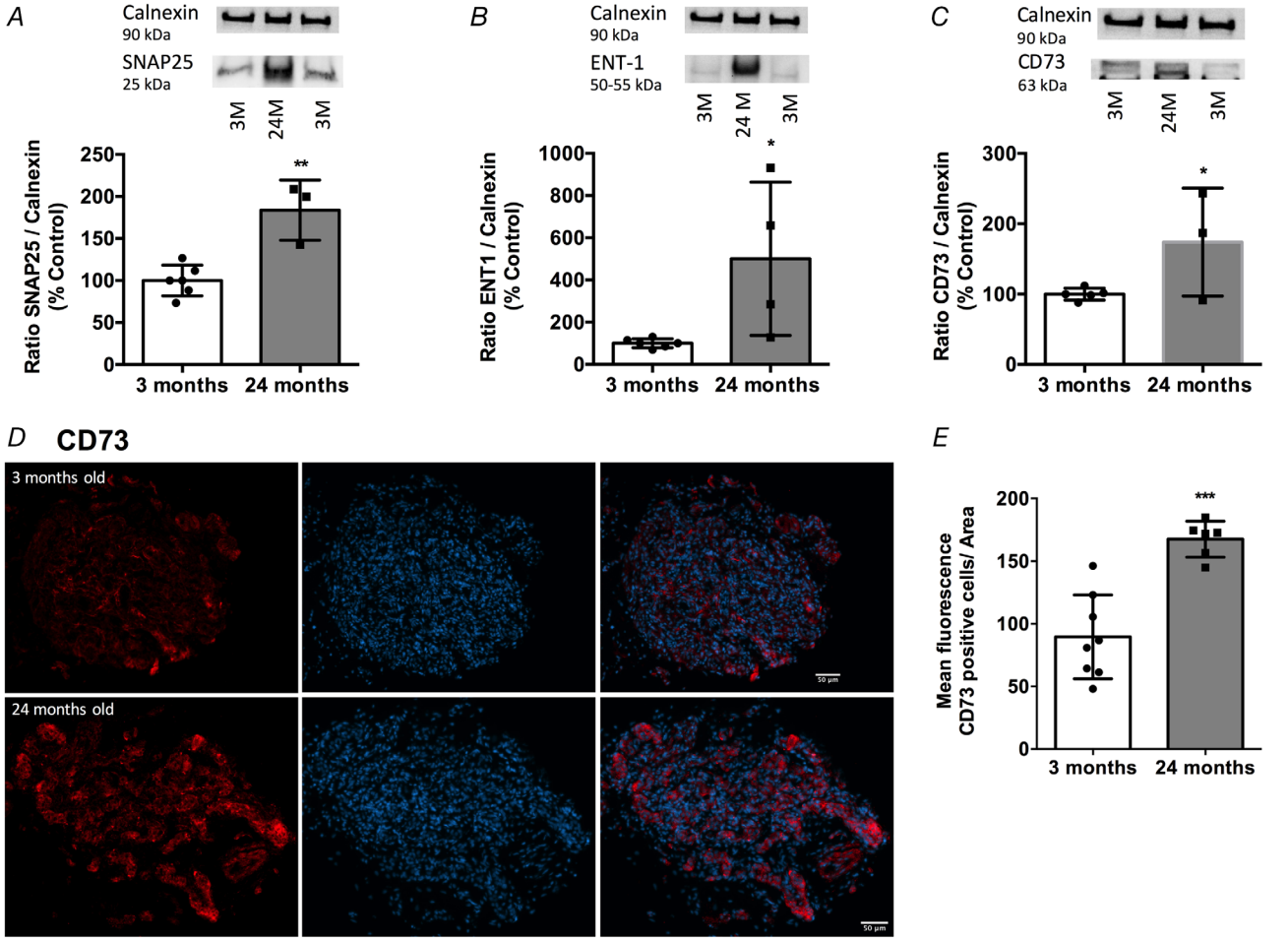

Figure 3. Effect of ageing on the levels of proteins involved in exocytosis machinery and on the levels of proteins involved in purinergic metabolism in the carotid body (CB)
A, effect of ageing on SNAP25 levels in the CB. Graph shows average expression of SNAP25, a t-SNARE protein involved in fusion of exocytotic vesicles with plasma membrane, in 3- and 24-month-old rats. At the top is depicted a representative western blot comparing SNAP25 immunoreactivity, corresponding to a 25 kDa band, in CBs of 3- and 24-month-old animals. A re-probing of the membranes with calnexin antibody, corresponding to the 90 kDa band is shown above the gel for SNAP25. *B*, effect of ageing on equilibrative nucleoside transporter type 1 (ENT1) levels in the CB. Graph shows average expression of ENT1 in 3- and 24-month-old rats. At the top is depicted a representative western blot comparing ENT1 immunoreactivity, corresponding to the 50–55 kDa band, in CBs of 3- and 24-month-old animals. A re-probing of the membranes with calnexin antibody, corresponding to the 90 kDa band is shown above the gel for ENT1. *C*, effect of ageing on ecto-5′-nucleotidase (CD73) levels in CBs. Graph shows average expression of CD73 in 3- and 24-month-old rats. Top image shows representative western blot for CD73 immunoreactivity, corresponding to 63 kDa, in young and old CBs. A re-probing of the membranes with calnexin antibody, corresponding to the 90 kDa band is shown above the gel for CD73. *D*, immunofluorescence images for CD73 (left panels) and DAPI (middle panels) and its merge (panels on the right) in slices of 3-month-old (top) and 24-month-old (bottom) CBs. Red fluorescence indicates immunoreactivity for CD73 and blue fluorescence indicates immunoreactivity for DAPI. *E*, mean fluorescence of CD73-positive areas present in serially sectioned CBs (five CBs from 3-month-old and 20- to 24-month-old rats). Data represent means ± SD. $^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t* test.

and old animals. Note that ageing produced an evident decrease in stimuli-evoked CSN activity. In the middle and right panels are represented typical CSN recordings evoked by 0% O_2 in young and in old animals, respectively, in drug-free conditions and in the presence of 300 nM ZM241385 at a concentration that blocks both A_{2A} and A_{2B} adenosine receptors (Conde *et al.* 2006*b*, 2012*a*) and in the presence of 50 μM suramin, a P2X ATP receptor blocker. As we can see in Fig. 5*B*, ageing did not decrease basal CSN activity. As previously described by others (see Gonzalez *et al.* 1994), we observed that CSN activity increased proportionally with hypoxic intensity, i.e. the lower the P_{O_2} the higher the CSN chemosensory activity, both in control and in aged animals (Fig. 5*C*). Ageing dramatically decreased hypoxic evoked-CSN activity by 77.76%, 67.79% and 51.86% in response to 7% O_2, 5% O_2 and 0% O_2, respectively (Fig. 5*C*). In aged rats, ZM241385 blocked the CSN chemosensory activity similarly in response to 0% O_2 and 5% O_2, by 50.35% and 53.05%, respectively (Fig. 5*D* and *E*). In agreement with what has been previously described for young rats (Conde *et al.* 2012*a*), in aged rats the effect of suramin, a P2X antagonist, on CSN chemosensory activity is as high as the hypoxic intensity (block of 74.71% in 0% O_2 and of 46.97% in 5% O_2) (Fig. 5*D* and *E*). Ageing was also unable to modify the responses to hypercapnia, as previously shown by Conde *et al.* (2006*b*) and here tested as the perfusion of the CB–CSN preparation with 10% CO_2 (Fig. 6*A*). A_2 adenosine and ATP receptor antagonists significantly decreased the CSN chemosensory response to hypercapnia by 68.97% and 62.54%, respectively (Fig. 6*B* and *C*). Ageing slightly decreased the contribution of adenosine and ATP to CSN Timiras hypercapnic responses, although without reaching statistical significance (Fig. 6*C*).

Effect of ageing on spontaneous ventilation and in the ventilatory responses to hypoxic and ischaemic hypoxia

Figure 7*A* and Table 1 show the ventilatory responses in freely moving animals in response to different levels of hypoxia (12%, 10% and 7% O_2). We can observe that age affects basal ventilation, both at the respiratory frequency and tidal volume level (Table 1), this being reflected at basal minute ventilation ($\dot{V}_E$) (Fig. 7*Aa*). Basal $\dot{V}_E$ in 24-month-old animals decreased by 39.10% compared with 3-month-old animals (3 months $\dot{V}_E = 535.81 \pm 111.89$ ml min^{-1} kg^{-1}), 24 months $\dot{V}_E = 363.44 \pm 94.40$ ml min^{-1} kg^{-1}); Fig. 7*Aa*). Also, we observed a smaller increase in $\dot{V}_E$ in the response to the different levels of hypoxia in the 24-month-old animals compared with the controls ($n = 8$, each group, Fig. 7*Aa* and *b*). Nevertheless, as Fig. 4*Ab* shows, this change expressed as a percentage over the control response was similar between both groups of animals for the

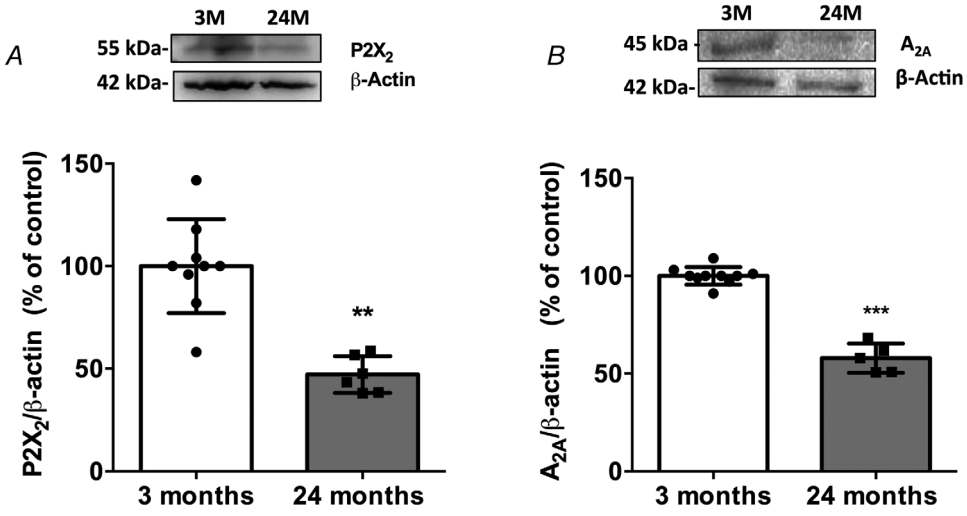

Figure 4. Effect of ageing on immunoreactivity of ATP $P2X_2$ and adenosine A_{2A} receptors in the carotid bodies (CBs)
A: upper panel, western blot comparing $P2X_2$ immunoreactivity, corresponding to the 55 kDa band, in CBs of 3- and 24-month-old animals. A re-probing of the membranes with β-actin antibody, corresponding to the 42 kDa band is shown above the gel for $P2X_2$; lower panel, effect of ageing on the average relative $P2X_2$ immunoreactivity ($n = 5$–9) in relation to β-actin immunoreactivity. *B*, upper panel, western blot comparing A_{2A} immunoreactivity, corresponding to the 45 kDa band, in CBs of 3- and 24-month-old rats. A re-probing of the membranes with β-actin antibody, corresponding to the 42 kDa band is shown above the gel for A_{2A}; lower panel, effect of ageing on the average relative A_{2A} immunoreactivity ($n = 5$–9) in relation to β-actin immunoreactivity. $^{**}p < 0.01$, $^{***}p < 0.001$; unpaired Student's *t* test comparing protein expression in 3- and 24-month-old rats. Data represent means ± SD.

Table 1. Effect of ageing on respiratory frequency and tidal volume in basal conditions and in response to acute hypoxic stimuli

	3 months old		24 months old	
	f_R (beats min^{-1})	Tidal volume (ml kg^{-1})	f_R (beats min^{-1})	Tidal volume (ml kg^{-1})
20% O_2	76.00 ± 9.38	7.05 ± 0.45	61.60 ± 9.26**	5.91 ± 1.64*
12% O_2	117.20 ± 14.70	7.62 ± 1.10	116.18 ± 16.26	6.20 ± 2.41
10% O_2	122.12 ± 18.92	8.21 ± 1.41	116.99 ± 27.21	6.56 ± 1.54*
7% O_2	114.82 ± 25.96	11.02 ± 2.55	114.22 ± 24.81	8.22 ± 2.46***

Data are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's t test comparing values of 3-month-old ($n = 8$) and 24-month-old ($n = 8$) animals. f_R, respiratory frequency.

milder hypoxic stimuli, 12% and 10% O_2, but significantly different for the more intense 7% O_2, that was unable to elicit a further response than the one observed for 10% O_2.

The same effect on basal ventilation was observed when ventilation was recorded in an anaesthetized animal setting. Age affected the ventilatory parameters in basal conditions, as we can see in Fig. 7*Ba*, representing the change in basal respiratory frequency, and Fig. 7*Bb* (left columns in the graph on the right) representing tidal volume (V_T). In 24-month-old animals the respiratory frequency (f_R) was smaller (13.6%), as was the tidal volume (V_T) (17.99%) ($n = 22$) (Fig. 7*Ba*). The adjusted minute ventilation ($\dot{V}_E$) was accordingly reduced in the old animals from 394.35 ± 181.67 to 269.97 ± 52.31 ml min^{-1} kg^{-1} (Fig. 7*Ba*, right columns in the graph on the right). This smaller ventilatory response in basal conditions is somehow more affected than the

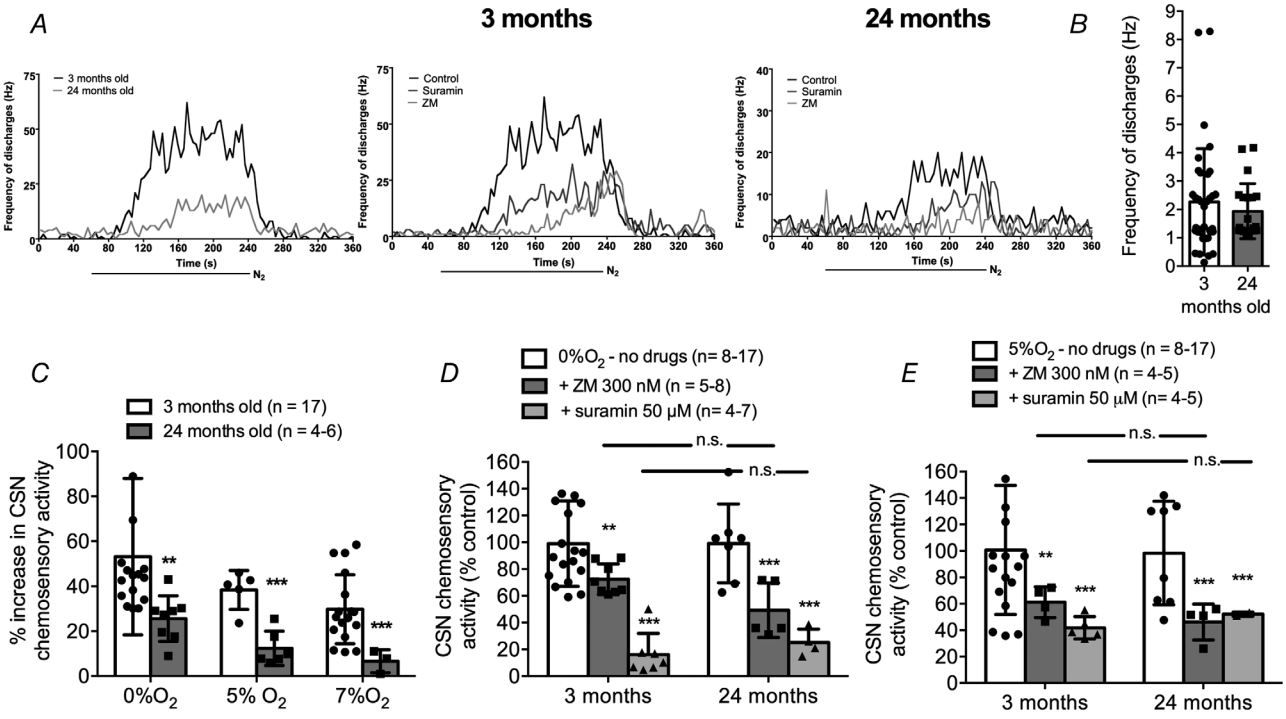

Figure 5. Effect of ageing and of A_2 adenosine and P2X ATP receptor antagonists on carotid sinus nerve (CSN) chemosensory activity in basal conditions and in response to hypoxia in the rat
A, typical CSN electrophysiological recording in 3- and 24-month-old rats. Left panel depicts a typical response to 0% O_2 in 3- and 24-month-old rats. Middle and right panels depict the effect of ZM241385, an adenosine A_2 receptor antagonist, and 50 μM suramin, a P2X ATP receptor antagonist, on the CSN chemosensory activity evoked by 0% O_2 in 3- and 24-month-old rats, respectively. Note the marked differences in the hypoxic responses recorded in preparations from control (3 months old) *vs.* aged (24 months old) rats. *B*, mean basal frequencies in 3- and 24-month-old rats. *C*, ageing decreases the augmentation of CSN activity induced by different hypoxic intensities (0%, 5% and 7% O_2). *D* and *E*, effect of 300 nM ZM241385 and 50 μM suramin on the CSN chemosensory activity evoked by 0% and 5% O_2, respectively, in 3- and 24-month-old rats **$p < 0.01$, ***$p < 0.001$ one- and two-way ANOVA with Tukey's multiple comparison test. Data represent means ± SD.

not significantly different decrease in the basal firing pattern of the CSN that we showed previously in the 24-month-old animals (see Fig. 5*B*). Note also that the ventilatory parameters are smaller in the anaesthetized setting than in the plethysmography recordings in freely moving animals, reflecting the depressor effect of the anaesthetic. Figure 7*Bb* shows the mean data for different durations of common carotid occlusion (CCO) (5, 10 and 15 s) on ventilatory response for 3- and 24-month-old animals. Longer occlusions caused higher increases over the baseline in control animals (CCO 5 s = 59.76 ± 4.20%, n = 20; CCO 10 s = 139.84 ± 10.58%, n = 20; CCO 15 s = 230.93 ± 15.19%, n = 20), this pattern being also maintained in the 24-month-old rats, although significantly smaller than controls for CCO with a duration of 10 s (103.40 ± 7.48%, n = 27) and 15 s (154.03 ± 10.32%, n = 27). When Fig. 7*Ab* and *Bb* are compared it is clear that the ventilatory responses to ischaemic hypoxia of different lengths correlate quite well with the ventilatory responses to hypoxic hypoxia and also that the effect that is mainly influenced by age is the response to high intensity stimuli.

Effect of adenosine and ATP antagonists on the ventilatory responses to ischaemic hypoxia

Subsequently we were prompted to determine the contribution of adenosine and ATP to the ventilatory responses to ischaemic hypoxia. To achieve this we studied the effects of P2X ATP and A_2 adenosine receptor antagonists on the ventilatory responses evoked by common carotid artery occlusions (CCOs) of 5, 10 and 15 s, as we had done previously in control conditions (without drugs). None of the drugs applied alone or concomitantly altered basal ventilation (data not shown). Figure 8 shows our results for both animal groups, expressed as percentage change in the $\dot{V}_E$ for each animal and inhibitor, or a combination of some of them. Suramin, the antagonist of P2X receptors decreased significantly, with a similar percentage, the ventilation in response to all ischaemic hypoxic intensities, both in 3-month-old animals as well as in 24-month-old animals (% decrease 3 months $\dot{V}_E$ CCO 5 s = 50.10%, CCO 10 s = 30.94%, CCO 15 s = 44.62%; % decrease 24 months $\dot{V}_E$ CCO 5 s = 44.53%, CCO 10 s = 44.65%, CCO 15 s = 50.47%; Fig. 8*A*–*C*). In contrast, both ZM241385 and SCH58261 when applied alone were more efficient in decreasing the ischaemic hypoxic ventilatory response in response to a low stimulus than to a stimulus of high ischaemic duration in both 3- and 24-month-old animals (3 months: % decrease $\dot{V}_E$ for ZM241385 applied alone CCO 5 s = 47.26%, CCO 10 s = 30.02%, CCO 15 s = 28.01%; 24 months: % decrease $\dot{V}_E$ for ZM241385 applied alone CCO 5 s = 40.23%, CCO 10 s = 29.06%, CCO 15 s = 26.94%; 3 months: % decrease $\dot{V}_E$ for SCH58261 applied alone CCO 5 s = 48.51%, CCO 10 s = 43.33%, CCO 15 s = 21.5%; 24 months: % decrease $\dot{V}_E$ for SCH58261 applied alone CCO 5 s = 48.35%, CCO 10 s = 21.05%, CCO 15 s = 21.17%, Fig. 8). ZM241385, the non-selective A_2 adenosine antagonist, when applied together with suramin did not significantly modify the suramin effect on the ventilation elicited by ischaemic hypoxia both in young and aged animals (see Fig. 8). In contrast, SCH58261, an A_{2A} selective adenosine antagonist, when applied together with suramin, potentiated the decrease

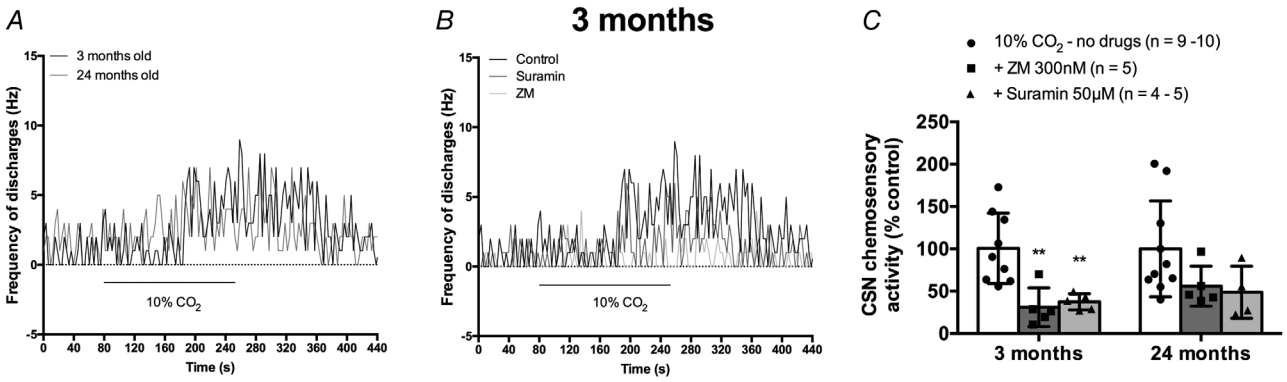

Figure 6. Contribution of adenosine and ATP to carotid sinus nerve (CSN) chemosensory activity in response to hypercapnia in 3- and 24-month-old rats
A, typical CSN electrophysiological recording in response to hypercapnia (20% O_2 + 10% CO_2) in 3- and 24-month-old rats. *B*, effect of ZM241385 (300 nM), an adenosine A_2 receptor antagonist, and suramin (50 µM), a P2X ATP receptor antagonist, on the CSN chemosensory activity evoked by 10% CO_2 in 3-month-old rats. *C*, ageing did not significantly change the effect of 300 nM ZM241385 or 50 µM suramin on the CSN chemosensory activity evoked by 10% CO_2, in 3- and 24-month-old rats. $^{**}p < 0.01$ two-way ANOVA with Tukey's multiple comparison test. Data represent means ± SD.

in ventilation produced by suramin both in 3- and 24-month-old animals, demonstrating that A_{2A} receptors are involved in the ventilatory responses to ischaemic hypoxia (see Fig. 8). The ventilatory inhibition produced by SCH58261 when applied with suramin was dependent on the lengthiness of the ischaemic hypoxia, and therefore its intensity. In fact, the inhibitory effect of SCH58261 in the presence of suramin is inversely correlated with hypoxic intensity, being higher with the less intense ischaemic hypoxias (CCO 5 s) (3 months: % decrease $\dot{V}_E$ suramin + SCH58261 CCO 5 s = 73.73%, CCO 10 s = 40.17%, CCO 15 s = 38.96% (24 months: % decrease $\dot{V}_E$ suramin + SCH58261 CCO 5 s = 81.04%, CCO 10 s = 61.28%, CCO 15 s = 46.22%). No significant differences were observed between the effects of SCH58261 applied with suramin in 3- and 24-month-old rats, meaning that the contribution of ATP and adenosine to the control of hypoxia is maintained in ageing.

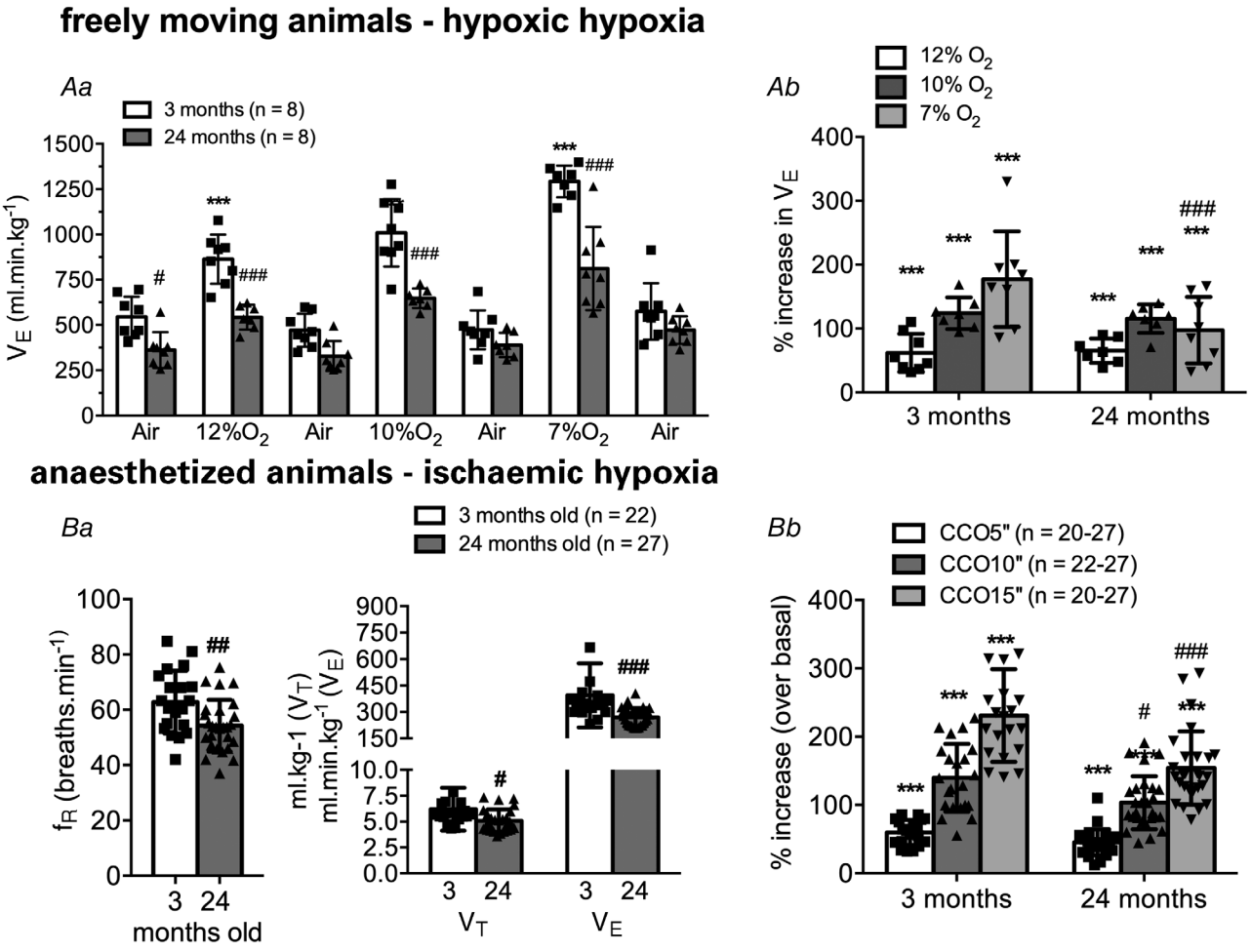

Figure 7. Effect of ageing on spontaneous ventilation and on the ventilatory responses induced by hypoxic hypoxia and ischaemic hypoxia

A, effect of ageing on the basal ventilation and on the ventilatory responses to acute hypoxia (12%, 10% and 7% O_2) in animals in whole body free movement. Ventilatory responses to acute hypoxia were represented as minute volume ($\dot{V}_E$). *Aa*, effect of ageing on the mean minute volume response to acute hypoxia. Acute hypoxias were applied 3 times for 10 min intervals with periods of 10 min in normoxia. For each animal and for the entire population of animals, minute ventilation data were normalized to unit body weight. Minute volume values were corrected to the rat's weight. *Ab*, % increase in $\dot{V}_E$ induced by acute hypoxia of different intensities in 3- and 24-month-old rats. *B*, effect of ageing on the basal ventilation and on the ventilator responses to ischaemic hypoxia, assessed as the occlusion of the common carotid artery (CCO) of different intensities (5, 10 and 15 s), in anaesthetized rats. *Ba*, mean basal respiratory frequency (f_R), and the mean basal tidal volume (V_T) and minute volume ($\dot{V}_E$) in 3- and 24-month-old rats. *Bb*, % increase in $\dot{V}_E$ induced by 5, 10 and 15 s of occlusion of the common carotid artery in 3- and 24-month-old rats. $^{***}p < 0.001$ comparing the effect of hypoxia with basal ventilation and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ comparing 3- and 24-month-old animals; one- and two-way ANOVA with Tukey's multiple comparison test. Data represent means ± SD.

Discussion

Here we show that the aged CB is characterized by an altered ratio in the number of CB type I and type II cells, and an altered release of ATP and adenosine neurotransmitters and its synapse dynamics. Additionally we confirmed our previous results by showing that ageing did not alter basal CSN activity and the responses to hypercapnia, but decreased CSN responses and ventilation in response to hypoxia. We also show here for the first time that both ATP and adenosine contribute to CB chemosensory activity in ageing and that although CB hypoxic response decreases with age, the relative contribution of both ATP and adenosine to CB activity is maintained through adjustments in CB purinergic metabolism.

Effects of ageing on CB cell dynamics

Ageing can be described as a sum of changes that occur in a living organism that progress to a functional impairment). It has been previously described that the function of the CB declines with age, this being associated with alterations in CB morphology (for a review see Di Giulio, 2018). In agreement with this, we described here an altered CB ratio of type I/type II cells in old animals, consistent with the decrease in 49.04% in the immunoreactivity for TH (Fig. 1*B* upper panel), and the observed decrease of 25.73% in TH expression by western blot analysis in the whole CB (Fig. 1*B* lower panel) and the absence of significant alterations in GFAP-immunoreactive cells (Fig. 1*C and D*). These alterations in CB morphology in ageing are in agreement with our previous results in which we found that the number of type I cells expressed by the total CB area progressively decrease during life, with animals of 12 months (50 years old in humans) exhibiting a 20% cell loss and 24-month-old animals exhibiting a loss of almost 50% (Conde *et al.* 2006*a*). However, little consensus exists on the morphological alterations in the aged CB as several reports have stressed the relative preservation of CB histology in elderly people (Heath *et al.* 1970; Di Giulio *et al.* 2012; Ortega-Sáenz *et al.* 2013). For example Heath *et al.* showed that people aged >80 years have a preserved CB histology in comparison with teenagers (Heath *et al.* 1970); however, this study was not designed to evaluate morphological differences among ages, and more recently Ortega-Sáenz *et al.* (2013) have shown similar glial cell-derived neurotrophic factor (GDNF) content and neurosecretory cell numbers in CBs from people older than 50 in comparison with younger controls. Many factors can account for the differences in morphological data in ageing, such as the animal species studied (rats in the present study and in Conde *et al.* 2006*a*, humans in Heath *et al.* 1970; Di Giulio *et al.* 2012 and Ortega-Sáenz *et al.* 2013) and the age of experimental groups (12- to 24-month-old animals, which corresponds to a range from 45 to 85 years of age in human studies in Conde *et al.* (2006*b*) or, for example, 50 years in the study of Ortega-Sáenz *et al.* 2013); however, other factors should be taken into consideration, such as the method of evaluating the percentage of cell loss. Note that here and in the past (Conde *et al.* 2006*a*) we have expressed TH-immunoreactivity in relation to the total area of the CB, which is known to enlarge with age (Arias-Stella & Valcarcel, 1973; Hurst *et al.* 1985; Conde *et al.* 2006*a*; DiGiulio, 2018) while other authors do not (Heath *et al.* 1970). In this regard, other studies in the literature that described marked changes in CB morphology are in agreement with ours, namely Hurst *et al.* (1985), where

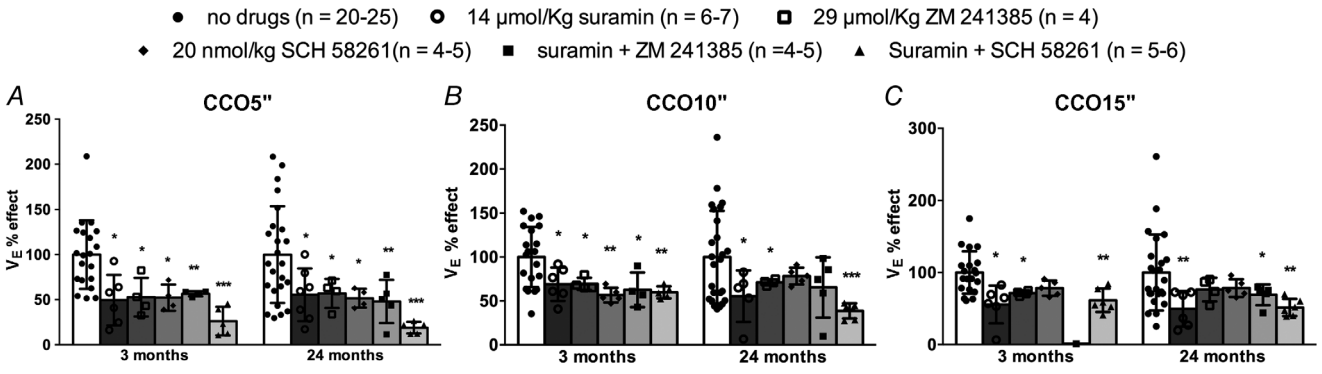

Figure 8. Effect of P2X ATP and A_2 adenosine receptor antagonists on the ventilatory responses evoked by carotid body (CB) ischaemia of 5 s (*A*), 10 s (*B*) and 15 s (*C*) in 3- and 24-month-old rats
CB ischaemia was assessed by occlusion of the common carotid artery (CCO) and ventilatory responses as minute volume ($\dot{V}_E$). The contribution of P2X ATP receptors to the ventilatory responses to ischaemic hypoxia was assessed by testing the effect of suramin, a non-selective P2X antagonist (14 µmol kg^{-1}). The contribution of A_2 adenosine receptors to the ventilatory responses to ischaemic hypoxia was assessed by testing the effect of ZM241385, a non-selective A_2 antagonist (29 µmol kg^{-1}) and SCH58261, an A_{2A} selective antagonist (20 nmol kg^{-1}). $^*P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA with Bonferroni's multiple comparison test. Data represent mean ± SD.

they showed a 36% loss in glomic tissue in 22 subjects between 66 and 100 years old, and Arias-Stella & Valcarcel (1973), where they showed a 28.36% glomic cell loss in subjects between 41 and 70 years old.

Apart from the decrease in the percentage of glomic tissue, other factors can contribute to a decreased CB functional activity in ageing, such as alterations in synapse dynamics, for example, alterations in vesicle size, alterations in fusion of vesicles during exocytosis, alterations in the levels of released neurotransmitters and a decrease in neurotransmitter signalling. In the present work we found that basal and hypoxic release of adenosine and ATP from CBs decreased with age (Fig. 2*A* and *B*). Previous studies on age-dependent changes in the release of adenosine and ATP in the hippocampus yielded the same results, since Cunha *et al.* (2001) showed that extracellular adenosine and ATP decreased to one-half in hippocampus of aged rats; however, this did not happen in the striatum. Pazzagli *et al.* (1995) showed that extracellular levels of adenosine are not affected by age, irrespective of the differences in adenosine deaminase activity. The decrease in adenosine and ATP released by the CBs observed in the present work can be due to the decreased number of chemoreceptor cells present in aged rats, as shown here and in Conde *et al.* (2006*a*). In fact, an age-decreased catecholamine release in response to hypoxia was previously described in the CB (Conde *et al.* 2006*a*). However, the reduced release of ATP observed here (Fig. 2*A*) is not consistent with the observed augmented levels of expression of SNAP25 (Fig. 3*A*), an essential protein for the Ca^{2+}-dependent exocytotic release of neurotransmitters. During ageing a decrease has been shown in the expression of SNAP25 in rat hippocampus (Chauhan *et al.* 2003) and decreased levels of expression of other synaptic proteins such as VAMP and Munc-18, essential components of the synaptic vesicle fusion protein complex in the pituitary (Jacobsson *et al.* 1998). Our results are in contrast with these findings, although we could postulate that the increased expression of SNAP25 in ageing observed here can reflect a compensatory mechanism to maintain the purinergic homeostasis within the CB even with low numbers of type I cells. Another possibility for this increased SNAP25 expression could be its presence in other types of cells in the CB, such as type II cells or macrophages whose infiltration within the CB probably increases with ageing. However, SNAP25 was not detected in macrophages, in contrast with other SNAREs, like SNAP23 (Logan *et al.* 2003; Jung *et al.* 2012). Consistent with the notion of the presence of a compensatory mechanism to maintain ATP and adenosine function in the aged CB, we found that the levels of the equilibrative nucleoside transporter type 1 (ENT1) are 4 times higher in aged CBs (Fig. 3*B*), this being consistent with the decreased levels of adenosine found extracellularly and the increased intracellular adenosine content (Fig. 2*C*). Therefore, we can postulate that in aged animals ENT1 is working to reuptake adenosine into type I cells and not to release adenosine *per se* as previously described (Conde & Monteiro, 2004; Conde *et al.* 2012*a*). Moreover, we found that the expression of CD73, an enzyme involved in the extracellular conversion of AMP to adenosine, doubled in aged animals (Fig. 3*C–E*), suggesting that in aged animals extracellular ATP is rapidly catabolized into adenosine, which can be immediately catabolized by adenosine deaminase or undergo the reuptake to type I cells via ENT1. Increased mRNA expression of CD73 has also been described in blood cells of older adults (>79 years old) when compared with young and middle-aged adults (Crooke *et al.* 2017). All together these results suggest that in ageing an increased SNAP25 expression will be associated with an increased exocytotic release of ATP, which we could not observe in the present work as it would undergo a quick catabolism into adenosine due to an increased expression of CD73 (Fig. 3). Extracellular adenosine will then undergo reuptake into CB cells by ENT1, whose expression is 400% increased in aged animals, increasing adenosine intracellular levels (Fig. 2*C*) that can be used to produce ATP.

These alterations in purinergic metabolism in aged CBs were accompanied by a postsynaptic decrease in the expression of A_{2A} adenosine and $P2X_2$ ATP receptors in the petrosal ganglion (Fig. 4). These results at the beginning were surprising since one would expect an up-regulation of these receptors due to the decreased release of these neurotransmitters (Fig. 2*A* and *B*). For example, Canas *et al.* (2009) demonstrated an up-regulation of A_{2A} receptors in ageing in the hippocampus, where those receptors are present presynaptically (Canas *et al.* 2009) while in the CB A_{2A} receptors are present postsynaptically (Conde *et al.* 2006*a*). In contrast with this up-regulation of A_{2A} receptors, other authors have shown that adenosinergic activities were unaffected by ageing in the cerebellum and substantia nigra (for a review see Burnstock & Dale, 2015). Additionally, a reduction in P2X receptor expression in ageing mouse brain has been described as their role in glial synaptic current generation (Lalo *et al.* 2011). This decrease in the expression of $P2X_2$ receptors was seen not only centrally but also in the periphery, as in the ageing rat the prostate exhibited a down-regulation of the $P2X_2$ (Slater *et al.* 2000). If our hypothesis is correct and if in fact we have increased levels of ATP being released and transformed into adenosine in aged animals, probably these results only reflect the postsynaptic adjustments in purinergic signalling.

Effects of ageing on CB output

In general, ageing leads to a decrease in the maximum number of impulses per minute in nerves (Rivner *et al.* 2001) and, in fact, we have previously demonstrated that

ageing decreased the hypoxia-evoked neural activity in the CSN in control and chronic intermittent hypoxic animals without affecting the CSN basal activity or the activity in response to hypercapnia/acidosis (Conde *et al.* 2006*a*; Quintero *et al.* 2016). In line with these results we found here that the basal level of CSN activity was unchanged with age, but when hypoxia was applied the response in the old animals was markedly decreased for all hypoxic levels tested (Fig. 5*B* and *C*). These results, together with the decrease in extracellular levels of purines in aged animals and with the decreased expression of A_{2A} and $P2X_2$ receptors in the petrosal ganglion, suggest that the decreased hypoxic responses exhibited in ageing are due to reduced signalling of adenosine and ATP in the CB in response to hypoxia.

In line with our previous findings, ATP and adenosine antagonists did not modify basal CSN activity in young (Conde *et al.* 2012*a*) and aged rats (Fig. 5*A*). The absence of effect of adenosine (McQueen & Ribeiro, 1986; Howell & Landrum, 1995; Conde *et al.* 2006*b*, 2012*a*) and ATP (Rong *et al.* 2003; Reyes *et al.* 2007) antagonists on basal CSN activity and spontaneous ventilation has been described in the past in different animal species, such as rats, cats and monkeys. However, some studies have suggested that adenosine and ATP can contribute to maintain basal CSN discharge. For example, Holmes *et al.* (2018) have shown that the pharmacological inhibition of ecto-5′-nucleotidase, and therefore the reduction of extracellular levels of adenosine coming from extracellular ATP, caused a reduction in basal CB sensory discharge. The differences between the Holmes *et al.* study in 2018 and the studies showing the absence of effect of adenosine antagonist on basal activity (McQueen & Ribeiro, 1986; Howell & Landrum, 1995; Conde *et al.* 2006*a*, 2012*a*) may rely on direct *vs.* indirect effects of adenosine on the output responses of the CB, as these studies have tested the direct blockade of adenosine receptors in CSN activity and ventilation, and Holmes *et al.* (2018) inhibited the degradation of AMP into adenosine. Knowing that CD73 is a cell surface enzyme mainly surrounding type I cells (Salman *et al.* 2017), we can postulate that during normoxia adenosine is acting mainly via adenosine receptors present in type I cells, and contributing to the release of other transmitter(s) that are important to fix CSN basal activity. In fact, we have previously described that caffeine, a non-selective antagonist of adenosine receptors, decreases the normoxic release of catecholamines from CBs without altering basal CSN discharges (Conde *et al.* 2006*b*).

In line with our previous findings (Conde *et al.* 2012*a*) we also observed here that P2X blocking in young animals almost completely blocked the CSN response to intense hypoxia, whereas it only partially decreased the response to moderate hypoxia (5% O_2). Also, ZM241385, in a concentration that blocked both A_{2A} and A_{2B} receptors, affected very similarly the hypoxic responses for 5% and 0% O_2 levels in young animals (Fig. 5*D* and *E*). These contributions of adenosine and ATP to the CSN chemosensory activity in response to moderate and intense hypoxia were maintained in aged rats (Fig. 5*D* and *E*), which likely reflects the requirement for the preservation of purinergic modulation in aged rats. These results are in agreement with the maintenance of adenosinergic control in the phrenic nerve in aged rats (Pereira *et al.* 2000), but contrast with the central nervous system where at least adenosinergic neuromodulation is modified in aged animals (Cunha *et al.* 2001; Canas *et al.* 2009; Burnstock & Dale, 2015).

Another finding of our paper was the fact that the basal respiratory frequency and tidal volume, measured both in freely moving animals and in anaesthetized animals, were reduced in old age (Table 1), with a resulting lower minute ventilation (Fig. 7*Aa* and *Ba*). However, the decrease in minute ventilation observed here and previously described (Monteiro *et al.* 2011; Quintero *et al.* 2016) does not mean hypoventilation, since it is well known that ageing is accompanied by a decrease in whole body O_2 consumption (Cerveri *et al.* 1995; Chan & Welsh, 1998). In the present work we also showed that the response to ischaemic hypoxia, achieved with common carotid occlusion, and hypoxic hypoxia, were significantly smaller for the intense hypoxias (10 and 15 s occlusions and 7% O_2) in the 24-month-old rats (Fig. 7). Finally, we have studied the relative contribution of each neurotransmitter to the ventilatory responses in carotid hypoxic ischaemias of 5, 10 and 15 s duration, and how it was influenced by age (Fig. 8). We have showed that when A_2 adenosine and P2X ATP blockers were applied alone they decreased the minute ventilation by approximately 50% in control or 24-month-old rats for all the occlusion times. These decreases in minute ventilation are in agreement with the role of adenosine and ATP in modulating ventilatory responses. Since the 1980s it has been generally agreed that adenosine has an excitatory effect on ventilation and that this effect is CB mediated as intracarotid injections of adenosine and its analogues increase ventilation in a dose-dependent manner, an effect that was abolished by CSN section and mediated by A_2 receptors (Monteiro & Ribeiro, 1987). Additionally, the decrease in ventilation by SCH58261 described in the present work (Fig. 8) was in line with the data previously obtained with CGS21680, which is a selective A_{2A} agonist at low doses and stimulates ventilation by ∼55% (Conde *et al.* 2009). Apart from adenosine, Rong *et al.* (2003) also demonstrated that hypoxic ventilatory responses are mediated by ATP since mice lacking ATP $P2X_2$ receptors exhibit attenuation of the ventilatory responses.

In this study we also found that the capacity of ZM241385 and SCH58261 (A_{2A} selective antagonist) to decrease minute ventilation decreased with the

hypoxic intensity. These results are on the same lines as the reduction of effect of ZM241385 on CSN activity in response to 0% O_2 in comparison with 5% O_2 (Fig. 5*D* and *E*) and with our previous findings for the effects of ZM241385 on CSN activity at distinct hypoxia intensities (Conde *et al.* 2012*a*). This effect was also observed in 24-month-old animals, meaning that the inverse relationship between adenosine action and hypoxia intensity is maintained in ageing. Another important finding was the fact that the ZM241385 did not potentiate the effect of suramin on minute ventilation in both control and 24-month-old rats, but SCH58261, the selective antagonist of A_{2A} receptors did, which suggests that probably A_{2B} receptors are having a distinct effect on ventilation. A_{2A} blockage, together with ATP blockade, decreased the minute ventilation by ∼75%, which is in agreement with our previous results on CSN activity (Conde *et al.* 2012*a*). This potentiation effect of SCH58261 was maintained in ageing and lost with hypoxic intensity, suggesting one more time that the contribution of adenosine and ATP to the ventilatory responses are maintained in ageing. In fact, Tubek *et al.* (2016) in a clinical study confirmed the excitatory role of adenosine in the CB function in humans, since the ventilatory and haemodynamic responses to adenosine were diminished following carotid body ablation and suggested that adenosine may be used for selective stimulation and assessment of CB activity. This study was performed in 11 individuals with ages between 56 and 73 years, which means that adenosine function in the CB in ageing is also maintained in humans.

Clinical importance of ATP and adenosine signalling in ageing

The potential clinical importance of modulating purines in the CB cannot be underestimated as in the last decade many pathologies that are aggravated by age, such as hypertension, diabetes, obstructive sleep apnoea and chronic heart failure have been associated with CB dysfunction (Peng *et al.* 2003; Abdala *et al.* 2012; Del Rio *et al.* 2013; Ribeiro *et al.* 2013). Recently we have described that adenosine receptor blockade with caffeine inhibits CSN activity in chronic intermittent hypoxic young animals, meaning that adenosine is involved in CB sensitization during chronic intermittent hypoxia (Sacramento *et al.* 2015) and therefore in the mechanisms underlying the pathophysiological alterations observed in obstructive sleep apnoea. Also, Pijacka *et al.* (2016) showed that $P2X_3$ purinergic receptor mRNA expression is up-regulated in petrosal sensory neurons in young rats with hypertension and that these neurons generate both tonic drive and hyper-reflexia in hypertensive rats, effects that were normalized by the blockade of $P2X_3$ receptors. However, at least in chronic intermittent hypoxia age exerts a neuroprotective effect against its harmful effects, since in aged rats chronic intermittent hypoxia does not alter carotid body responses, catecholamine-related parameters or redox status, and does not cause hypertension (Quintero *et al.* 2016). The lack of harmful effects observed in chronic intermittent hypoxia in ageing (Quintero *et al.* 2016) might be due to the decreased CB purinergic neurotransmission and therefore new insights into the CB purinergic mechanisms in pathological conditions during ageing are needed to completely understand if the CB overactivation seen in cardiometabolic diseases (Peng *et al.* 2003; Abdala *et al.* 2012; Ribeiro *et al.* 2013; Pijacka *et al.* 2016) persists in ageing. If so, modulation of purinergic signalling in the ageing and dysfunctional CB can be used therapeutically in the future.

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Additional information

Competing interests

The authors declare no conflicts of interest.

Author contributions

The experiments described in the present paper were performed in the CEDOC, NOVA Medical School/Faculty of Medical Sciences, NOVA University, and in the Department of Biochemistry, Molecular Biology and Physiology of the Faculty of Medicine of the University of Valladolid. The authors have contributed to the study as follows. Participated in research design: S.V.C. and C.G. Conducted experiments: J.F.S., S.V.C., M.J.R., E.O., B.F.M., F.O.M. Performed collection and data analysis: S.V.C., M.J.R., E.O., J.P.-L., B.F.M., E.C.M., F.O.M. Wrote or contributed to the writing of the manuscript: S.V.C., J.P.-L. All the authors have approved the final version of the manuscript, except Constancio Gonzalez, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

adenosine, ageing, ATP, carotid body, CD73, ENT1