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Purinergic signalling mediates bidirectional crosstalk between chemoreceptor type I and glial-like type II cells of the rat carotid body

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Key points

- Carotid body chemoreceptors are organized in clusters containing receptor type I and contiguous glial-like type II cells.
- While type I cells depolarize and release ATP during chemostimulation, the role of type II cells which express purinergic P2Y2 receptors (P2Y2Rs) and ATP-permeable pannexin-1 (Panx-1) channels, is unclear.
- Here, we show that in isolated rat chemoreceptor clusters, type I cell depolarization induced by hypoxia, hypercapnia, or high K^+ caused delayed intracellular Ca^{2+} elevations ($\Delta[Ca^{2+}]_i$) in nearby type II cells that were inhibited by the P2Y2R blocker suramin, or by the nucleoside hydrolase apyrase.
- Likewise, stimulation of P2Y2Rs on type II cells caused a delayed, secondary $\Delta[Ca^{2+}]_i$ in nearby type I cells that was inhibited by blockers of Panx-1 channels, adenosine A_{2A} receptors and 5′-ectonucleotidase.
- We propose that reciprocal crosstalk between type I and type II cells contributes to sensory processing in the carotid body via purinergic signalling pathways.

Abstract The mammalian carotid body (CB) is excited by blood-borne stimuli including hypoxia and acid hypercapnia, leading to respiratory and cardiovascular reflex responses. This chemosensory organ consists of innervated clusters of receptor type I cells, ensheathed by processes of adjacent glial-like type II cells. ATP is a major excitatory neurotransmitter released from type I cells and type II cells express purinergic P2Y2 receptors (P2Y2Rs), the activation of which leads to the opening of ATP-permeable, pannexin-1 (Panx-1) channels. While these properties support crosstalk between type I and type II cells during chemotransduction, direct evidence is lacking. To address this, we first exposed isolated rat chemoreceptor clusters to acute hypoxia, isohydric hypercapnia, or the depolarizing stimulus high K^+, and monitored intracellular $[Ca^{2+}]$ using Fura-2. As expected, these stimuli induced intracellular $[Ca^{2+}]$ elevations ($\Delta[Ca^{2+}]_i$) in type I cells. Interestingly, however, there was often a delayed, secondary $\Delta[Ca^{2+}]_i$ in nearby type II cells that was reversibly inhibited by the P2Y2R antagonist suramin, or by the nucleoside hydrolase apyrase. By contrast, type II cell stimulation with the P2Y2R agonist uridine-5′-triphosphate (100 μM) often led to a delayed, secondary $\Delta[Ca^{2+}]_i$ response in nearby type I cells that was reversibly inhibited by the Panx-1 blocker carbenoxolone (5 μM). This $\Delta[Ca^{2+}]_i$ response was also strongly inhibited by blockers of either the adenosine A_{2A} receptor (SCH 58261) or of the 5′-ectonucleotidase (AOPCP), suggesting it was due to adenosine arising from breakdown of ATP released through Panx-1 channels. Collectively, these data strongly suggest that purinergic signalling mechanisms mediate crosstalk between CB chemoreceptor and glial cells during chemotransduction.

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Abbreviations ADO, adenosine; AOPCP, α, β-methylene ADP; CB, carotid body; CBX, carbenoxolone; GFAP, glial fibrillary acid protein; MRS 1754, adenosine A_{2B} receptor antagonist; Panx-1, pannexin-1; P2Y2R, purinergic P2Y2 receptor; SCH 58261, adenosine A_{2A} receptor antagonist; UTP, uridine-5′-triphosphate.

Introduction

Peripheral chemoreceptors located in the carotid bodies (CBs) of mammals sense chemicals in arterial blood including O_2 and CO_2/H^+, and maintain homeostasis via activation of appropriate respiratory and cardiovascular reflex responses (Gonzalez *et al.* 1994; Kumar & Prabhakar, 2012). Within the CBs, innervated clusters of chemoreceptor type I cells are intimately ensheathed by thin processes of adjacent glial-like, sustentacular or type II cells (McDonald, 1981; Platero-Luengo *et al.* 2014). Chemostimuli such as hypoxia (low P_{O_2}) and acid hypercapnia (high CO_2/H^+) depolarize type I cells, leading to Ca^{2+}-dependent release of a variety of neurotransmitters and neuromodulators that help shape the afferent sensory discharge, carried by the carotid sinus nerve (Gonzalez *et al.* 1994; Peers & Buckler, 1995; Lopez-Barneo *et al.* 2008; Nurse, 2010; Kumar & Prabhakar, 2012; Nurse & Piskuric, 2013). Among these neurochemicals is the key excitatory neurotransmitter, ATP, which activates postsynaptic ionotropic P2X2/3 receptors on afferent nerve terminals (Nurse, 2010, 2014). Adenosine, generated via breakdown of extracellular ATP, or released from type I cells via equilibrative transporters (Conde & Monteiro, 2004), also contributes to CB excitation by activating adenosine A_2 receptors located presynaptically on type I cells and/or postsynaptically on afferent terminals (Conde *et al.* 2009, 2012).

It has often been hypothesized that the glial-like type II cells are not simply passive bystanders, but may play an active role in CB sensory processing via paracrine signalling pathways (Tse *et al.* 2012; Nurse & Piskuric, 2013; Murali *et al.* 2014, 2015; Nurse, 2014). This posit is based on the close juxtaposition between type I and type II cells coupled with the fact that type II cells express metabotropic receptors for ATP, as well as other type I cell neuromodulators including ACh and angiotensin II (Xu *et al.* 2003; Tse *et al.* 2012; Zhang *et al.* 2012; Murali *et al.* 2014, 2015). Moreover, multipotent stem cells of glial lineage have been reported to form synapse-like contacts with mature type I cells, which release endothelin-1 (ET-1) during chronic hypoxia and instruct the growth of these stem cells via stimulation of ET_B receptors (Platero-Luengo *et al.* 2014). In mature rat type II cells, exogenous application of agonists for purinergic P2Y2 receptors (-Rs), muscarinic AChRs, or angiotensin AT_1Rs causes a robust rise in intracellular Ca^{2+}, derived mainly from intracellular stores (Xu *et al.* 2003; Zhang *et al.* 2012; Murali *et al.* 2014). Activation of these signalling cascades leads to the opening of large-pore pannexin-1 (Panx-1) channels, which act as conduits for the further release of ATP (Bao *et al.* 2004; Locovei *et al.* 2006; Zhang *et al.* 2012; Murali *et al.* 2014). These data raise the attractive possibility that acute stimulation of type II cells by paracrine agents may contribute to CB excitation via ATP efflux through Panx-1 channels (Nurse, 2014). However, it remains debatable whether type I cells do actually communicate directly with type II cells by paracrine signals during sensory transduction, and a formal demonstration of this purported crosstalk during the processing of acute chemostimuli is still lacking.

In the present study, we sought evidence for crosstalk between type I and type II cells in isolated rat CB chemoreceptor clusters. As a first step, we investigated whether direct stimulation of type I cells with chemostimuli such as low O_2 (hypoxia) and high CO_2 (hypercapnia), or the depolarizing agent high K^+, might *indirectly* stimulate adjacent type II cells as a result of P2Y2R activation following ATP release. Secondly, we asked whether selective stimulation of type II cells with P2Y2R agonists could result in reciprocal crosstalk, leading to *indirect* responses in type I cells via signals released through Panx-1 channels. To address these questions, we applied Fura-2 ratiometric calcium imaging to dissociated rat CB preparations cultured for ∼2 days. In these preparations isolated cell clusters containing incompletely dissociated type I and type II cells, as well as dispersed isolated cells, are usually present. In summary, we obtained compelling evidence for paracrine signalling and reciprocal crosstalk between type I and type II cells involving purinergic mechanisms where both ATP and adenosine play key roles.

Methods

Ethical approval

All procedures for animal handling and tissue dissections were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC). These procedures were reviewed and approved by the McMaster's Animal Research Ethics Board (AREB). We understand the ethical principles under which the journal operates and our work complies with this animal ethics checklist.

Cell cultures of dissociated rat carotid body

Lactating female rats and their litters comprising 9- to11-day-old pups (Wistar, Charles River, Quebec, Canada) were purchased weekly and housed in our Central Animal Facility under veterinary supervision until ready for use, typically 2–4 days later. Animals were housed under a controlled light/dark cycle and had *ad lib* access to food and water. The pups, both males and females, weighed 20–30 g at the time their carotid bodies were removed. Procedures for preparing carotid body cultures were similar to those described in detail elsewhere (Zhang *et al.* 2000, 2012; Murali *et al.* 2014). Briefly, each pup from 1 litter (9–12 pups) was first rendered unconscious by a blow to the back of the head, and then killed immediately by decapitation; the entire procedure lasted ∼5 s. The carotid bifurcations were excised bilaterally and carotid bodies (CBs) were isolated and cleaned of surrounding tissue. The CBs were incubated for 1 h at 37°C in a balanced salt solution containing 0.1% trypsin (Sigma-Aldrich, Oakville, Ontario, Canada) and 0.1% collagenase (Gibco, Grand Isalnd, NY, USA). The tissues were then teased apart by mechanical dissociation and following trituration the resulting cell suspension was allowed to adhere to the central wells of modified tissue culture dishes, pre-coated with a thin layer of Matrigel (BD Biosciences, Mississauga, Ontario, Canada). For the first ∼24 h, the cells were cultured in basic growth medium (BGM) consisting of F-12 nutrient medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, 1% glutamine, 0.3% glucose and 3 μg ml^{-1} insulin. Then for the next 24–48 h, the medium was switched to Cosmic-BGM containing 50% BGM plus 50% modified BGM where 10% fetal bovine serum was replaced by 5% fetal bovine serum and 5% Cosmic calf serum (Hyclone Laboratories Inc., Logan, UT, USA), as previously described (Murali *et al.* 2014).

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the acetoxymethyl ester (AM) form of the fluorescent probe Fura-2 (Molecular Probes, Eugene, OR, USA), as previously described (Piskuric & Nurse, 2012; Zhang *et al.* 2012; Murali *et al.* 2014). Cells were first loaded with 2.5 μM Fura-2 AM in bicarbonate-buffered solution (BBS) for 30 min at 37°C, and then washed for ∼15 min to remove free dye. The BBS had the following composition (in mM): $NaHCO_3$, 24; NaCl, 115; glucose, 5; KCl, 5; $CaCl_2$, 2 and $MgCl_2$, 1; the pH was maintained at ∼7.4 by bubbling with a 5% CO_2–95% air mixture. Ratiometric Ca^{2+} imaging was performed using a Nikon Eclipse TE2000-U inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a Lambda DG-4 ultra-high-speed wavelength changer (Sutter Instrument Co., Novato, CA, USA), a Hamamatsu OCRCA-ET digital CCD camera (Hamamatsu, Sewickley, PA, USA) and a Nikon S-Fluor ×40 oil-immersion objective lens with a numerical aperture of 1.3. Dual images at 340 nm and 380 nm excitation (510 nm emission) were acquired every 2 s, with an exposure time of 100–200 ms. Pseudocolour ratiometric data were obtained using Simple PCI software version 5.3. All experiments were performed at 33–37°C.

The imaging system was calibrated using the Fura-2 Calcium Imaging Calibration Kit from Molecular Probes (Cat. No. F-6774). Photometric data at 340 nm and 380 nm excitation (510 nm emission) were obtained for 11 buffers of known Ca^{2+} concentrations from Ca^{2+}-free (0 μM) to saturating Ca^{2+} (39 μM). After correcting for background fluorescence, these values were used to calculate the following ratios as follows: 'R' is the 510 nm emission intensity at 340 nm excitation to 510 nm emission intensity at 380 nm excitation; R_{min}, the ratio at zero free Ca^{2+}; R_{max}, the ratio at saturating Ca^{2+}; and β, the fluorescence intensity with excitation at 380 nm for zero free Ca^{2+} (F_{380max}), to the fluorescence intensity at saturating free Ca^{2+} (F_{380min}). The intracellular free $[Ca^{2+}]$ was obtained after substituting these ratios into the Grynkiewicz equation (Grynkiewicz *et al.* 1985) as follows:

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})}{(R_{max} - R)}$$

where R_{min} = 0.18, R_{max} = 7.81, β = 12.29, K_d = 225 nM and R is the ratio obtained during the experiment for a given cell. Statistical analysis of three or more groups was performed using repeated measures ANOVA with Tukey's multiple comparison *post hoc* test or the Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test (depending on whether the data were matched observations). Statistical analysis of two unmatched groups such as type I *versus* type II cells was performed using the Mann–Whitney test. Graphpad Prism 5 was used to perform the statistical analysis and all tests were for non-parametric data. The 'n' values indicated in the text and figures represent the number of sampled dishes, from which 20–30 randomly chosen cells were imaged per dish, unless otherwise noted.

Immunofluorescence

Double label immunofluorescence was used to visualize the relationship between chemoreceptor type I and glial-like type II cells in dissociated CB cultures grown under the conditions described above. Cultures were rinsed in pre-warmed phosphate buffered saline (PBS) and then fixed for 1 h in 95% methanol–5% acetic acid at −20°C. After washing 3× for 3 min each, cultures were incubated overnight in PBS containing anti-tyrosine

hydroxylase (TH) antibody (1:2500 dilution; rabbit polyclonal AB 152; EMD Millipore, Temecula, CA, USA) plus anti-glial fibrillary acid protein (GFAP) antibody (1:100 dilution; mouse monoclonal MAB 360; EMD Milllipore) at 4°C. Cultures were then washed 3× in PBS (10 min each) and incubated in PBS containing Alexa 488-congugated goat anti-mouse IgG (1:400; A11029, Molecular Probes) and Alexa 594 goat anti-rabbit IgG (1:400; A11037, Molecular Probes) for 1 h at room temperature in the dark. After a final wash in PBS (3×, 3 min each) cultures were mounted in Vectashield and viewed under a Zeiss IM35 inverted microscope equipped with epifluorescence optics.

Reagents and drugs

Cells were perfused with BBS containing (in mM): 24 $NaHCO_3$, 115 NaCl, 5 glucose, 5 KCl, 2 $CaCl_2$, and 1 $MgCl_2$, and pH was maintained at ∼7.4 by bubbling a 5% CO_2–95% air mixture. For hypoxia solutions the BBS was aerated with a 5% CO_2–95% N_2 gas mixture for ∼60 min before use; for isohydric hypercapnia the $NaHCO_3$ concentration in BBS was increased to 48 mM (maintaining osmolarity by reducing NaCl to 91 mM) and the solution was aerated with 10% CO_2–90% air (pH ∼7.4). The following reagents and drugs were obtained from Sigma-Aldrich: uridine-5′-triphosphate (UTP), carbenoxolone (CBX), suramin, SCH 58261 (A_{2A} receptor antagonist), MRS 1754 (A_{2B} receptor antagonist), apyrase and adenosine-5′-*O*-(α,β-methylene)-diphosphonate sodium salt (AOPCP).

Results

The experiments described below were performed mainly on 2-day-old cultures of dissociated rat carotid bodies prepared as described in Methods. Ratiometric Ca^{2+} imaging was routinely performed on isolated chemoreceptor clusters and/or neighbouring type II cells. These clusters typically contain a mixture of type I cells and a lower proportion of contiguous type II cells whose processes intermingle with the type I cells. That feature is illustrated in Fig. 1*A*, where type I and type II cells are immuno-labelled for tyrosine hydroxylase (TH) and glial fibrillary acid protein (GFAP), respectively. In the following figures, sample traces of intracellular Ca^{2+} responses monitored simultaneously in type I and type II cells are often shown, while revealing latency differences between the stimulated 'driver' and adjacent 'follower' cell. It should be noted, however, that the trace corresponding to the 'driver' cell is only a representative example from the stimulated population. The actual 'driver' cell(s) that elicited the 'follower' response in each case is/are unknown, and could be any other member(s) of the cell cluster.

Indirect 'follower' responses in type II cells mediated by chemostimulation or by high K^+-evoked depolarization of nearby type I cells

In general, type II cells can be uniquely identified by the presence of a rapid, robust rise in intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$) during application of the P2Y2R agonist UTP (100 μM) (Xu *et al.* 2003; Zhang *et al.* 2012). Solitary type II cells, well isolated from chemoreceptor clusters, do not normally respond directly to chemostimuli such as hypoxia and acid hypercapnia (Piskuric & Nurse, 2012; Tse *et al.* 2012), or the depolarization stimulus high K^+ (30 mM), as exemplified in Fig. 1*B* and *C*. To determine whether type I cells can communicate with nearby type II cells, we first imaged cells within and near the edge of chemoreceptor clusters while applying the chemostimuli hypoxia (P_{O_2} ∼25 mmHg) or isohydric hypercapnia (10% CO_2; pH = 7.4). As expected from previous studies (Buckler & Vaughan-Jones, 1994*a*,*b*; Piskuric & Nurse, 2012), and exemplified in Figs 1*D* and *E*, 2*B* and 3*A*, type I cells frequently responded to the chemostimuli hypoxia and isohydric hypercapnia with a rise in intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$). Interestingly, Fig. 1*D* and *E* (blue arrow and trace) also illustrate that, in contrast to their 'solitary' counterparts, type II cells situated near a chemoreceptor cell cluster may respond to these chemostimuli with a significant $\Delta[Ca^{2+}]_i$. Data pooled from many similar examples revealed that for hypoxia the mean $\Delta[Ca^{2+}]_i$ response (∼50 nM) of type I cells was significantly greater than that (∼25 nM) of type II cells (Mann–Whitney test, $P < 0.001$; Fig. 2*C*). Similarly, for isohydric hypercapnia the mean $\Delta[Ca^{2+}]_i$ response (∼73 nM) of type I cells was significantly greater than that (∼33 nM) of type II cells (Mann–Whitney test, $P < 0.001$; Fig. 2*D*). Notably for both stimuli, the type II cell response was always delayed relative to that of the type I cell, consistent with cross-talk from type I to type II cells (Figs 1*E* and 2*Ba*). A comparison of the latency differences between the type I *versus* type II cell responses for hypoxia and isohydric hypercapnia is shown in Fig. 2*F*. The 'follower' type II cell responses were delayed by 17.0 ± 3 s relative to the 'driver' type I cell responses for hypoxia ($n = 9$ dishes), and by 10.5 ± 2.7 s for isohydric hypercapnia ($n = 10$ dishes; Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test, $P < 0.05$). In summary, stimulation of CB receptor type I cells with a chemical stimulus can lead to a delayed, secondary activation of neighbouring type II cells.

We next determined whether a general, sustained depolarization of type I cells with high K^+ (30 mM) could similarly elicit 'indirect', delayed intracellular $\Delta[Ca^{2+}]$ responses in nearby type II cells. As exemplified in Fig. 2*Bb*,

high K^+ elicited robust $\Delta[Ca^{2+}]_i$ responses in type I cells as expected; however, smaller but delayed $\Delta[Ca^{2+}]_i$ responses were frequently observed in neighbouring type II cells. A scatterplot of $\Delta[Ca^{2+}]_i$ in neighbouring type I and type II cells during high K^+ stimulation is shown in Fig. 2*E* for one experimental series, which combines pooled data from many similar experiments ($n = 17$). Comparison of response latencies revealed that for high K^+ the 'follower' type II cell responses were delayed by 8.8 ± 1.8 s relative to the 'driver' type I cell responses.

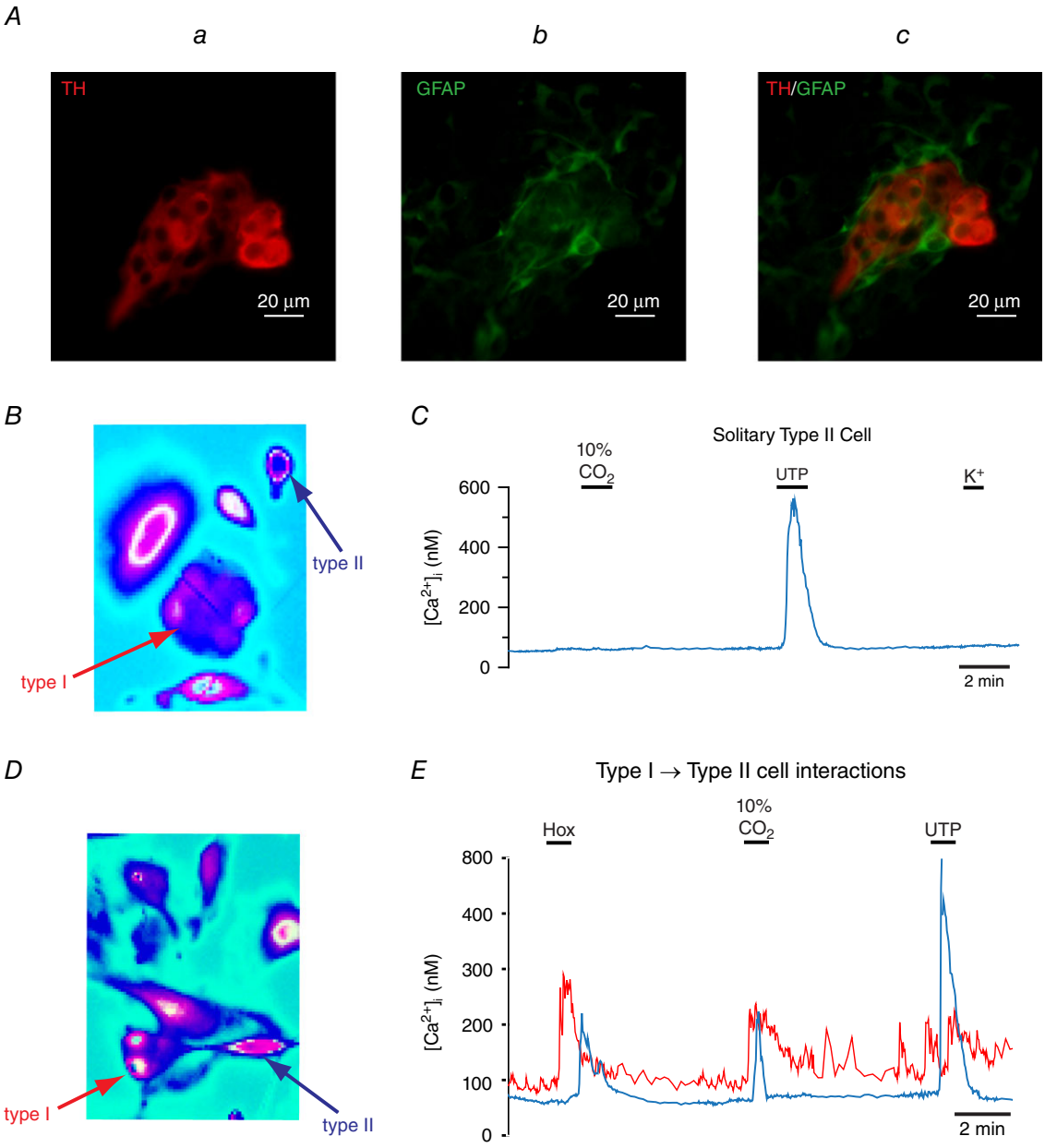

Figure 1. Intracellular Ca^{2+} responses in carotid body type I *versus* type II cells during chemostimulation, and evidence for crosstalk
A, immunofluorescence staining of rat carotid body culture showing typical chemoreceptor cluster containing tyrosine hydroxylase (TH)-positive type I cells (red; *Aa*), contiguous GFAP-positive type II cells/processes (green; *Ab*), and merge (*Ac*). 'Solitary' type II cell (blue arrow), well isolated from a type I cluster (red arrow) in *B*, fails to respond directly to the chemostimulus isohydric hypercapnia (10% CO_2; pH = 7.4) or the depolarizing stimulus high K^+, but elicits a robust rise in intracellular Ca^{2+} to the P2Y2R agonist UTP (*C*). By contrast, type II cells located *near* a type I cluster as in *D* may respond *indirectly* to chemostimuli such as hypoxia (Hox) (P_{O_2} ~25 mmHg) and isohydric hypercapnia as shown in *E* (blue trace); note the delay in type II cell response relative that of a type I cell (*E*; red trace) in the cluster (*D*), consistent with crosstalk from type I to type II cells.

This was significantly shorter than the delay seen with hypoxia and isohydric hypercapnia (Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test, $P < 0.05$), probably because membrane depolarization and release of the 'paracrine signal' from type I cells (see later) occurred more rapidly during high K^+ application.

Inhibition of delayed Ca^{2+} responses in 'follower' type II cells by the P2Y2 receptor blocker suramin and the nucleoside hydrolase apyrase

The delayed or indirect Ca^{2+} responses recorded in 'follower' type II cells as shown in Figs 1 and 2 could be due to paracrine activation of P2Y2 receptors (P2Y2Rs) by ATP released during stimulation of nearby type I cells. To test this, we investigated whether type II cell Ca^{2+} responses triggered by isohydric hypercapnia or high K^+ could be inhibited by suramin, a blocker of P2Y2Rs. As exemplified in Fig. 3*A*, the indirect type II cell $\Delta[Ca^{2+}]_i$ response due to isohydric hypercapnia was completely inhibited by 100 μM suramin in most cases; ∼90% (122/136) of type II cells that initially responded to this stimulus failed to do so in the presence of suramin. The scatterplot in Fig. 3*B* shows that the mean $\Delta[Ca^{2+}]_i$ induced in 'follower' type II cells by isohydric hypercapnia was 42.3 ± 4.1 nM before, 7.0 ± 4.6 nM during, and 33.9 ± 4.7 nM after washout of suramin, corresponding to ∼84% inhibition ($P < 0.001$; $n = 12$ dishes; Friedman test with Dunn's multiple comparison *post hoc* test). In Fig. 3*B*, suramin also appeared to cause a significant inhibition of the $\Delta[Ca^{2+}]_i$ response induced by isohydric hypercapnia in the 'driver' type I cells; the mean (±SEM) $\Delta[Ca^{2+}]_i$ response in type I cells was 69.2 ± 5.5 nM for isohydric hypercapnia, 40.9 ± 8.7 nM for isohydric hypercapnia plus suramin (100 μM), and 59.2 ± 9.1 nM for isohydric hypercapnia after washout of suramin (repeated measures ANOVA with Tukey's multiple comparison *post hoc* test; $P < 0.05$, $n = 12$ dishes). Suramin also appeared to inhibit the proportion of type I cells that was responsive to isohydric hypercapnia by ∼30%. Possible reasons for this will be discussed later.

Similarly, as illustrated in Fig. 3*C* and *D*, suramin either completely or partially inhibited the high K^+-induced indirect Ca^{2+} responses in 'follower' type II cells and the effect was reversible (Fig. 3*C* and *D*); the mean $\Delta[Ca^{2+}]_i$ was 76.0 ± 10.0 nM before, 20.0 ± 4.1 nM during, and 66.4 ± 10.4 nM after wash-out of suramin (Fig. 3*D*; $n = 13$ dishes; Friedman test with Dunn's multiple comparison *post hoc* test, $P < 0.05$).

To confirm a role for ATP in the mediation of type I to type II cell crosstalk we used the nucleoside hyrolase apyrase, which generally degrades extracellular ATP, for example ATP released during chemostimulation of the carotid body (Buttigieg & Nurse, 2004) and taste (Dando & Roper, 2009) receptor cells. Indeed, as illustrated in Fig. 3*E*, the delayed $\Delta[Ca^{2+}]_i$ in 'follower' type II cells induced by hypercapnia was reversibly inhibited by apyrase (100 units); data from nine similar experiments are summarized in Fig. 3*F*, where the mean $\Delta[Ca^{2+}]_i$ in 'follower' type II cells was 34.9 ± 4.03 nM before, 1.29 ± 1.88 nM during, and 25.01 ± 3.59 nM after apyrase. By contrast, apyrase had no significant effect on the type I cell $\Delta[Ca^{2+}]_i$ responses during hypercapnia. Taken together, these data suggest that the rise in $[Ca^{2+}]_i$ seen in type II cells during isohydric hypercapnia and high K^+ is due predominantly to the paracrine action of ATP, released from type I cells.

Evidence for crosstalk from type II to type I cells: role of pannexin-1 channels

During the course of these studies we routinely applied the selective P2Y2R agonist UTP to confirm the identity of type II cells in or near chemoreceptor clusters (Xu *et al.* 2003; Zhang *et al.* 2012). Interestingly, in these experiments a delayed Ca^{2+} response was often seen in nearby type I cells, consistent with crosstalk from type II to type I cells (see Figs 3*C* and 4*B*). A scatterplot showing a comparison of $\Delta[Ca^{2+}]_i$ responses in type II *versus* type I cells from such experiments ($n = 15$) is shown in Fig. 4*D*; the mean $\Delta[Ca^{2+}]_i$ response for the 'driver' type II cells was ∼175 nM compared to ∼43 nM for the 'follower' type I cells during exposure to 100 μM UTP. A comparison of the relative latencies during UTP application revealed that the mean latency difference between 'driver' type II and 'follower' type I cell responses was 11.5 ± 1.7 s ($n = 17$ dishes; Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test, $P < 0.05$). Note in Fig. 4*B*, the reciprocal or bidirectional nature of crosstalk between type I and type II cells is evident during direct stimulation of type I cells with high CO_2 or high K^+.

Because activation of P2Y2 receptors on type II cells with UTP leads to the opening of large-pore, pannexin-1 (Panx-1) channels (Zhang *et al.* 2012), we tested whether crosstalk from type II to type I cells involved release of factor(s) via Panx-1 channels. As shown in Fig. 4*C* and *D*, addition of the Panx-1 channel blocker carbenoxolone (CBX; 5 μM) resulted in the reversible inhibition of the UTP-evoked delayed responses in 'follower' type I cells, with negligible effect on the Ca^{2+} responses in 'driver' type II cells. Data from a total of 160 'follower' type I cells that initially responded during UTP application revealed that only 18 (∼11%) showed any detectable $\Delta[Ca^{2+}]_i$ responses in the presence of UTP plus CBX. Summary data from this experimental series are shown in Fig. 4*D*; the mean $\Delta[Ca^{2+}]_i$ for type I cells in the presence of UTP was 48.1 ± 6.0 nM, compared to −2.6 ± 3.2 nM in the presence of UTP plus CBX, and 39.8 ± 6.1 nM in the presence of UTP after washout of CBX ($n = 15$

dishes; Friedman test with Dunn's multiple comparison *post hoc* test, $P < 0.001$). Notably, CBX had no significant effect on UTP-evoked Ca^{2+} responses in type II cells; the mean $\Delta[Ca^{2+}]_i$ responses for control UTP, UTP plus CBX, and recovery with UTP alone were 171.5 ± 14.7 nM, 115.9 ± 11.2 nM, and 110.0 ± 8.2 nM, respectively (Friedman test with Dunn's multiple comparison *post hoc* test, $P > 0.05$). These data are consistent with the notion

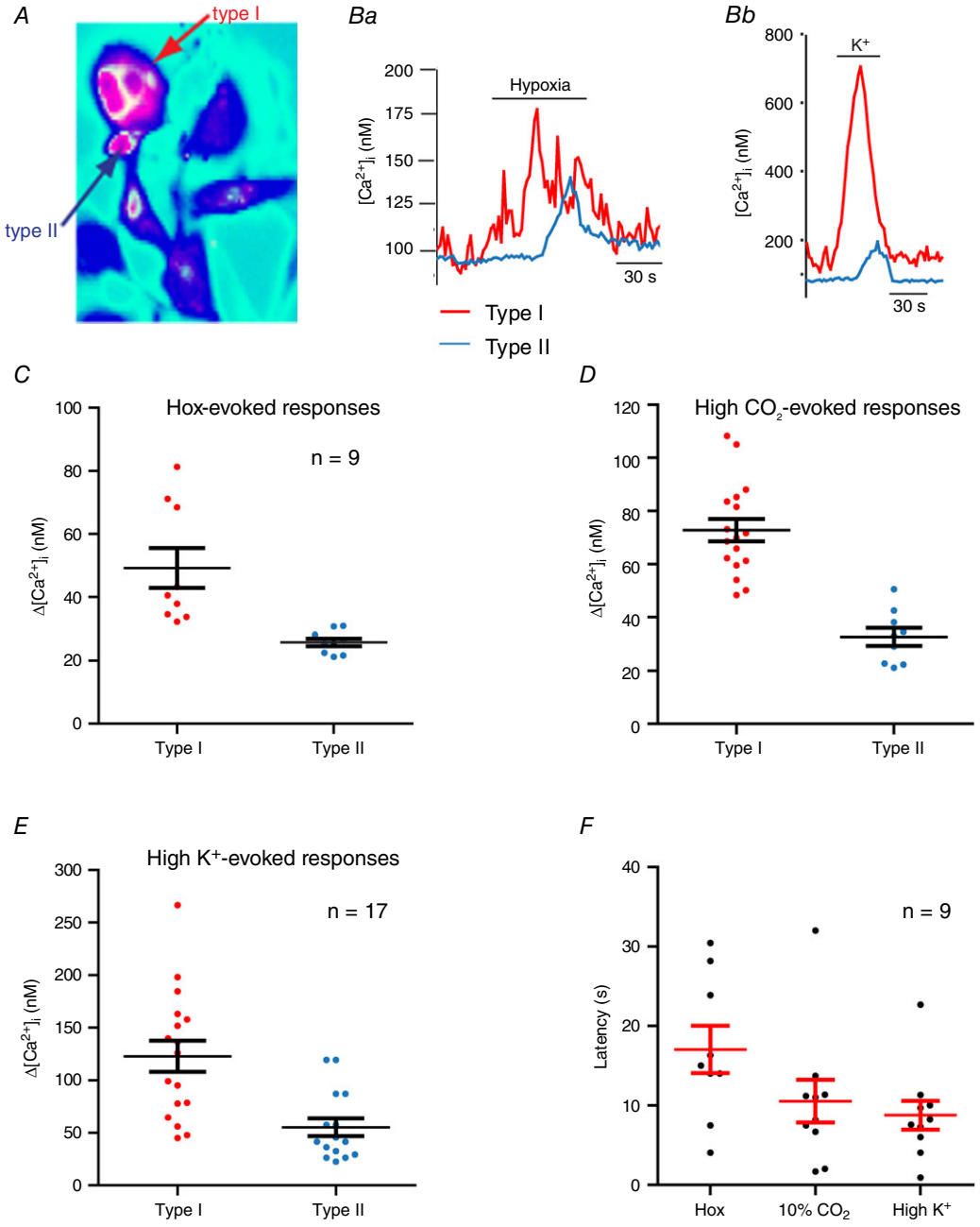

Figure 2. Comparison of the magnitude and relative latency of 'driver' type I *versus* 'follower' type II cell Ca^{2+} responses to chemostimuli and high K^+
A and *B*, intracellular Ca^{2+} responses from a type I cell within a cluster (red arrow; *A*) and a type II cell (blue arrow; *A*) adjacent to the cluster; in *B*, type II cell response follows that of the type I cell by several seconds during hypoxia (*Ba*) and high K^+ (*Bb*) application. *C–E*, a comparison of the magnitude of the increase in intracellular Ca^{2+} ($\Delta[Ca^{2+}]_i$) in 'driver' type I *versus* 'follower' type II cells during hypoxia, hypercapnia and high K^+. Data represent mean ± SEM where n = number of dishes sampled; each dot is the mean $\Delta[Ca^{2+}]_i$ for one dish from which 3–12 cells were randomly sampled. *F*, comparison of mean (±SEM) latency of 'follower' type II cell Ca^{2+} responses relative to that of the 'driver' type I cells during hypoxia, hypercapnia and high K^+.

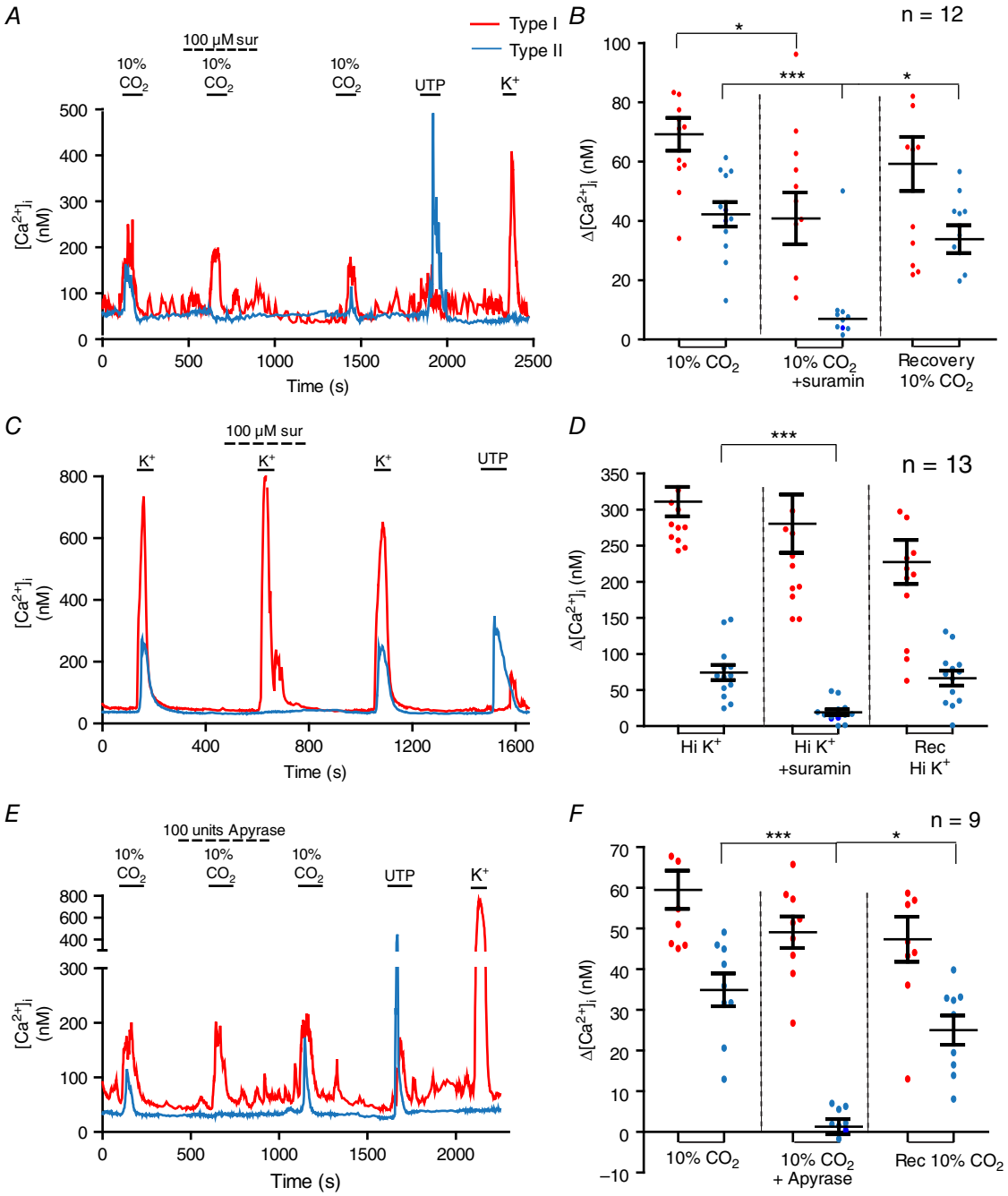

Figure 3. Blockade of purinergic P2Y2 receptors with suramin, or hydrolysis of extracellular ATP with apyrase, inhibits crosstalk from type I to type II cells
Example traces (blue) showing the reversible inhibition of the delayed or indirect Ca^{2+} responses in type II cells by the P2Y2R blocker suramin (100 μM) during hypercapnia (*A*) and high K^+ (*C*). Note in each case that the type I cell response persists in the presence of suramin. Failure of high K^+ to evoke a secondary Ca^{2+} response in *A* may be due to 'run down' over long times (~40 min), arising from various factors including receptor desensitization and/or Ca^{2+} store depletion. Summary data of the Ca^{2+} responses in type I *versus* type II cells before, during, and after suramin are shown for hypercapnia (*B*) and high ('Hi') K^+ (*D*). As exemplified in *E*, the indirect Ca^{2+} response evoked by hypercapnia in type II cells is also reversibly inhibited by the nucleoside hydrolase apyrase (100 units); note apyrase has no effect on the type I cell Ca^{2+} response during hypercapnia. Summary data of Ca^{2+} responses in type I *versus* type II cells before, during and after apyrase are shown in *F*. Data represent mean ± SEM, *n* = number of dishes; $^*P < 0.05$, $^{***}P < 0.001$.

that type II cells may communicate with type I cells via open Panx-1 channels.

Adenosine A_{2A} receptor antagonist SCH 58261 inhibits UTP-evoked indirect Ca^{2+} responses in type I cells

Panx-1 channels have pores large enough to allow release of molecules < 1 kDa such as ATP and glutamate from a variety of cell types (Locovei *et al.* 2006; MacVicar & Thompson, 2010). We previously provided evidence that Panx-1 channels in rat CB type II cells act as conduits for ATP release (Zhang *et al.* 2012). However, it was unlikely that ATP was the *direct* mediator of the delayed or secondary type I cell Ca^{2+} responses following stimulation of type II cells with UTP because ATP is known to *inhibit* type I cells via P2Y1 receptors (Xu *et al.* 2005). Nonetheless, because adenosine (ADO) derived from breakdown of ATP can elicit $\Delta[Ca^{2+}]_i$ responses in type I cells via ADO A_{2A} receptors (Xu *et al.* 2006), and both A_{2A} and A_{2B} receptors are expressed in rat type I cells (Conde *et al.* 2009), we tested the effects of A_2 antagonists on the Ca^{2+} responses. As illustrated in Fig. 5*A* and *B*, application of the selective A_{2A} antagonist (SCH 58261; 5 nM) led to an almost complete and reversible inhibition of the UTP-evoked secondary Ca^{2+} responses in type I cells. On the other hand, application of the selective A_{2B} antagonist (MRS 1754; 100 nM) had little or no effect (Fig. 5*C* and *D*); the secondary UTP-evoked $\Delta[Ca^{2+}]_i$ values before, during and after MRS 1754 were 33.8 ± 2.8 nM, 28.8 ± 1.9 nM, and 31.0 ± 3.7 nM, respectively ($P > 0.05$; $n = 11$ dishes). A comparison of the relative effects of A_{2A} *versus* A_{2B} receptor blockers on the percentage inhibition of UTP-evoked $\Delta[Ca^{2+}]_i$ responses in type I cells is shown in Fig. 5*E*. As expected, ADO blockers had no significant effect on the UTP-evoked Ca^{2+} responses in type II cells. The above data suggest that ADO acting predominantly via A_{2A} receptors on type I cells is the principal mediator of crosstalk from type II to type I cells.

Role of extracellular 5′-nucleotidase in type II to type I cell crosstalk

The above data raised the question concerning the source of ADO mediating type II to type I crosstalk. Did it arise directly from ADO release through Panx-1 channels in type II cells, or rather from ATP release, followed by its subsequent breakdown to ADO by extracellular 5′-ectonucleotidase? To distinguish between these two possibilities we tested the effects of α,β-methylene ADP (AOPCP), a blocker of 5′-ectonucleotidase (Conde & Monteiro, 2004). As exemplified in Fig. 6*A*, AOPCP (150 μM) had no significant effect on the UTP-evoked Ca^{2+} response in type II cells; however, the delayed type I cell Ca^{2+} response during UTP application was largely abolished and the effect was reversible. Quantitative data on the effects of AOPCP on the UTP-evoked Ca^{2+} responses in type II *versus* type I cells are summarized in Fig. 6*B*. In type I cells AOPCP inhibited the $\Delta[Ca^{2+}]_i$ response by ∼70%. These data suggest that the ADO mediating the delayed 'follower' response in type I cells during stimulation of type II cells arose predominantly from the extracellular breakdown of ATP released from type II cells.

Discussion

The present study provides direct evidence for cell–cell communication between carotid body (CB) type I and type II cells and, more importantly, we show that this crosstalk is likely to occur during chemotransduction. Type II cells have been described as glia-like, sheath and/or sustentacular cells that form intimate morphological associations with chemoreceptor type I cells that are usually organized in cell clusters (McDonald, 1981; Kondo, 2002; Platero-Luengo *et al.* 2014). However, until now there has been no direct evidence that they play an active role during acute chemosensing, though growth of CB type II-like stem cells of glial origin were recently shown to be stimulated by endothelin-1 released from type I cells during chronic hypoxia (Platero-Luengo *et al.* 2014). Moreover, previous studies in this laboratory demonstrated that type II cells express gap junction-like pannexin-1 (Panx-1) channels which act as conduits for ATP release following P2Y2 receptor activation and intracellular Ca^{2+} mobilization (Locovei *et al.* 2006; Zhang *et al.* 2012; Murali *et al.* 2014). This led to the proposal that release of the primary excitatory neurotransmitter ATP from type I cells during chemotransduction resulted in paracrine activation of type II cells and amplification of the ATP signal via the mechanism of ATP-induced ATP release (Zhang *et al.* 2012).

In the present study, an *in vitro* model of rat CB chemoreceptor clusters in short-term (∼2 day old) culture, combined with ratiometric calcium imaging, allowed us to uncover evidence for crosstalk between type I and type II cells. We found that membrane depolarization induced by natural chemostimuli such as hypoxia and high CO_2, as well as by high K^+, caused a rise in intracellular Ca^{2+} in type I cells as expected, but this was frequently followed by a delayed (several seconds) rise in intracellular Ca^{2+} in neighbouring type II cells. This was attributable largely to activation of P2Y2Rs on type II cells by ATP released from adjacent type I cells. We also provide evidence that communication between type I and type II cells can be bidirectional, because selective stimulation of P2Y2Rs on type II cells with UTP led to a secondary, delayed rise in intracellular Ca^{2+} in nearby type I cells. This latter

response was attributable to stimulation of A_{2A} receptors by adenosine, generated from the breakdown of ATP released from type II cells via Panx-1 channels.

The magnitude of the delays in these secondary Ca^{2+} signals was quite variable ranging from approximately 2 s to as long as 30 s. In addition to the fact that slow-acting G-protein-coupled receptors mediated the secondary responses in all cases, there were other potential factors that contributed to the long delays. These include: (i) a long separation distance between the follower cell and the source of the paracrine signal; (ii) the variable time required for the paracrine signal to reach a concentration sufficient to activate its receptors; and (iii) flow parameters during perfusion of the culture. Additionally, it should be noted that 'follower' type II cells situated near the edge of a receptor cluster were usually selected for study because their Ca^{2+} signals could be more easily resolved. Such cells, however, may lack the normally close membrane associations with type I cells compared to their counterparts buried within the chemoreceptor cluster (Kondo, 2002; Platero-Luengo *et al.* 2014). In this regard, our previous electrophysiological studies on type II cells suggested that the time to activation of the Panx-1 current by P2Y2R agonists occurred up to 10 times more slowly in 'isolated' type II cells (≥6 s) compared to those present in their 'native' configuration within receptor cell clusters

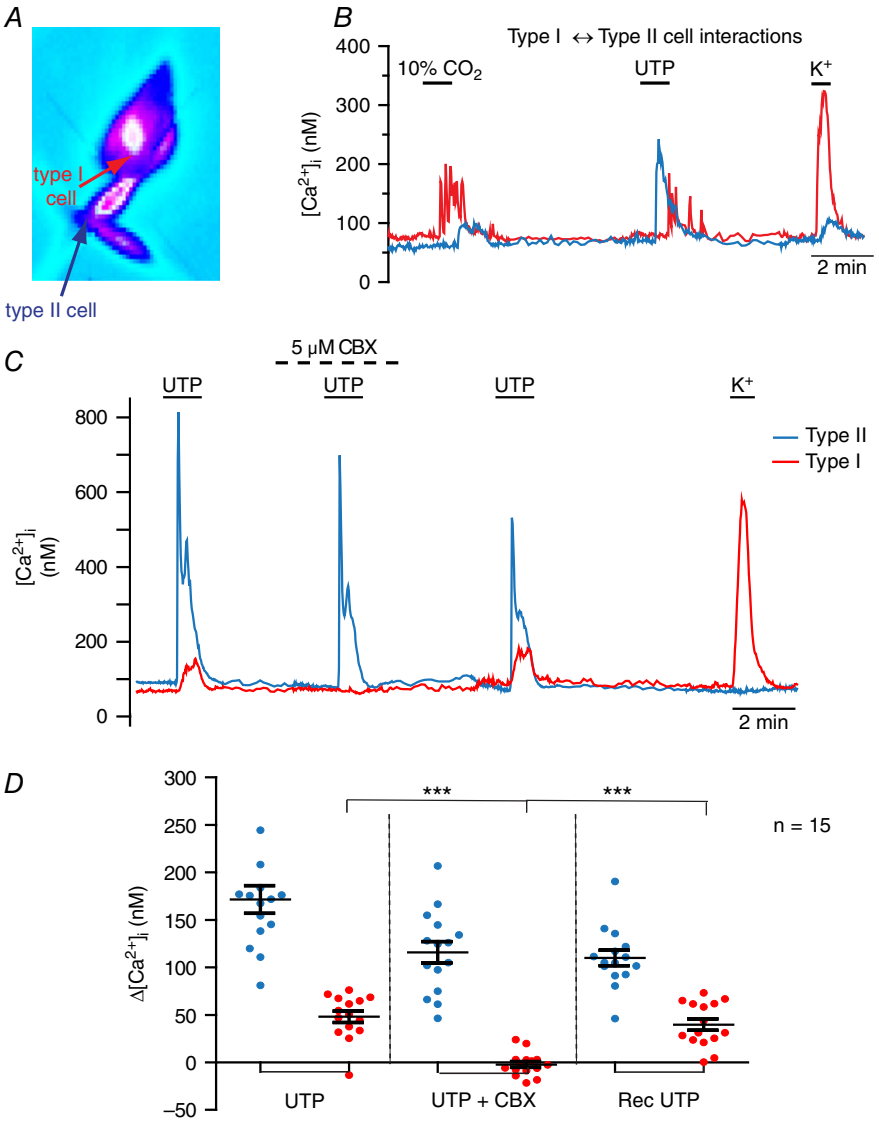

Figure 4. Crosstalk from type II to type I cells is mediated via pannexin-1 (Panx-1) channels
Stimulation of P2Y2Rs on type II cells with UTP (100 μM), as exemplified for the cell in *A* and *B* (blue trace), elicits a delayed Ca^{2+} response in nearby type I cell (red trace); note in these traces, stimulation of the type I cell cluster with high CO_2 (isohydric hypercapnia) or high K^+ elicits a delayed Ca^{2+} response in the same type II cell, indicating that communication between the type II cell and type I cluster is bidirectional. In *C*, the UTP-evoked delayed, indirect response in the 'follower' type I cell (red trace) was reversibly abolished by the pannexin-1 channel blocker, carbenoxolone (CBX; 5 μM). *D*, summary data (mean ± SEM) showing the reversible blockade of the delayed, indirect UTP-evoked Ca^{2+} responses in type I cells by CBX (***$P < 0.001$); note CBX had no significant effect on UTP-evoked responses in 'driver' type II cells in *C* (blue trace) and *D*.

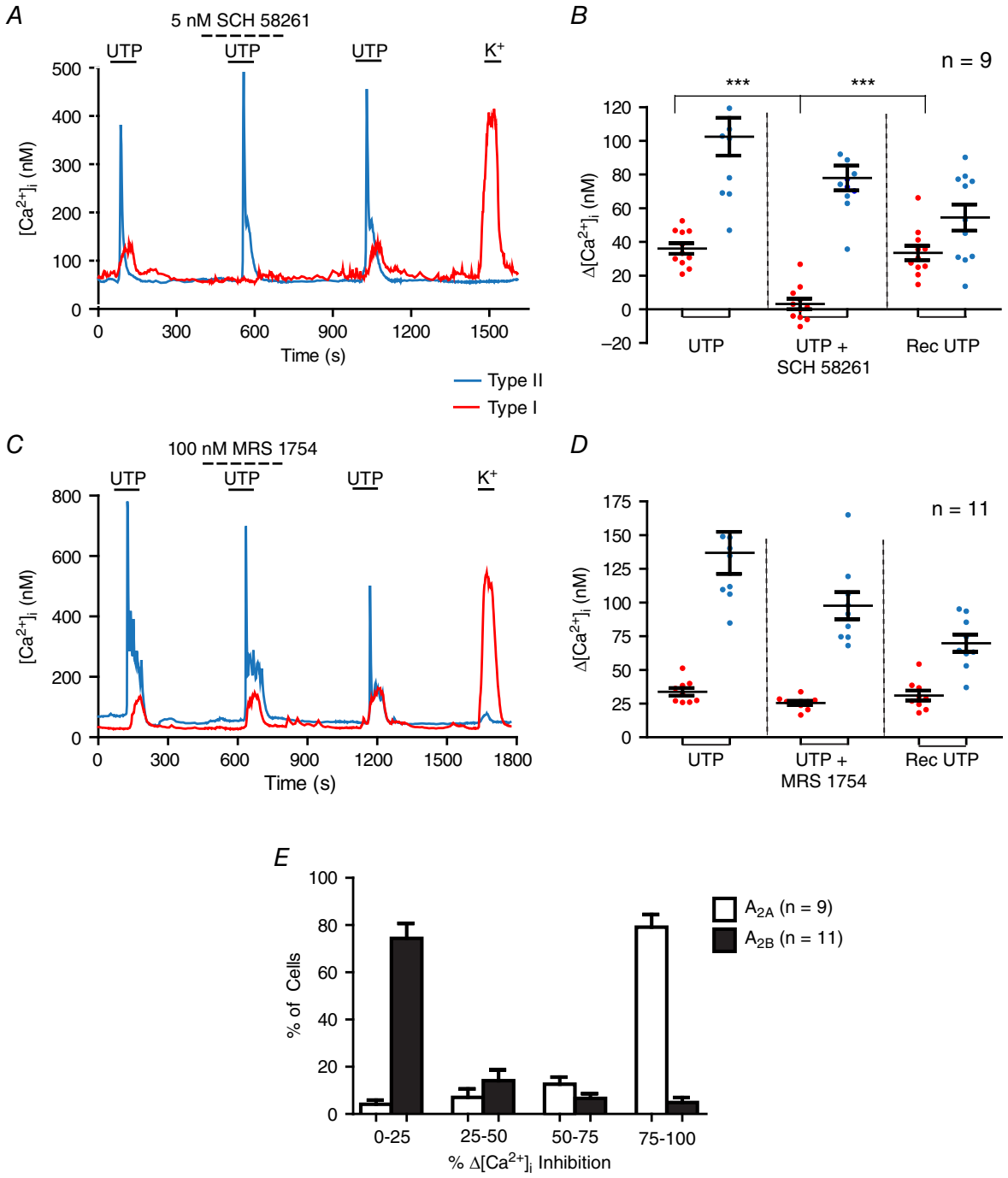

Figure 5. Adenosine acting via A_{2A} receptors mediates crosstalk from type II to type I cells
In *A* and *B*, the UTP-evoked delayed responses in 'follower' type I cells are almost completely inhibited by the selective adenosine A_{2A} receptor blocker SCH 58261 (5 nM) and the effect is reversible (***$P < 0.001$); notably SCH 58261 had no significant effect on the UTP-evoked responses in 'driver' type II cells. By contrast, the selective adenosine A_{2B} blocker MRS 1754 (100 nM) had little or no effect in comparable experiments (*C* and *D*). A histogram of the percentage of type I cells sensitive to the A_{2A} and A_{2B} receptor blockers *versus* percentage inhibition of intracellular Ca^{2+} rise is shown in *E* (*n* = number of dishes sampled; 5–20 cells sampled per dish).

(Zhang *et al.* 2012). Therefore it is likely that *in situ*, i.e. within the 3-dimensional ovoid structure of the carotid body, the latency of the secondary responses arising from crosstalk may be more rapid than that observed under the present experimental conditions. Taken together, these data add further support to the posit that during sensory transduction type II cells participate in a 'tripartite synapse' with type I cells and petrosal nerve endings as summarized in Fig. 7, thereby contributing to sensory integration in the CB by releasing 'gliotransmitters', especially ATP (Nurse & Piskuric, 2013; Nurse, 2014).

Type I cells communicate with type II cells during chemotransduction using ATP as a paracrine signal

The chemostimuli hypoxia and hypercapnia (10% CO_2) depolarize and increase intracellular Ca^{2+} in type I cells (Buckler & Vaughan-Jones, 1994*a*,*b*), leading to release of multiple neurotransmitters of which ATP plays a major excitatory role (Gonzalez *et al.* 1994; Nurse, 2010, 2014). While this released ATP was previously thought to stimulate mainly postsynaptic ionotropic P2X2/3 receptors on afferent nerve terminals (Zhang *et al.* 2000; Prasad *et al.* 2001), the present study strongly suggests an additional paracrine role in stimulating G-protein-coupled P2Y2 receptors (P2Y2Rs) on neighbouring glia-like type II cells. This conclusion is based on the observation that the P2Y2R antagonist suramin partially or completely inhibited the delayed Ca^{2+} response in type II cells following depolarization of type I cells by hypercapnia or high K^+. Confirmation that ATP was the predominant mediator was obtained in additional experiments showing this crosstalk was also inhibited by the nucleoside hydrolase apyrase, which degrades extracellular ATP. Interestingly, suramin had no significant effect on the intracellular Ca^{2+} responses elicited in type I cells by high K^+; however, it appeared to inhibit partially the Ca^{2+} responses elicited by isohydric hypercapnia. The reason for this preferential effect of suramin is presently unclear but a possible explanation is that high K^+ and high CO_2 may not release identical neurotransmitters from type I cells. However, because any autocrine–paracrine action of ATP on type I cells is thought to involve inhibitory P2Y1 receptors (Xu *et al.* 2005), it is also possible that suramin blocked the excitatory effects of a paracrine signal derived from the stimulation of P2Y2Rs on type II cells by ATP (see below).

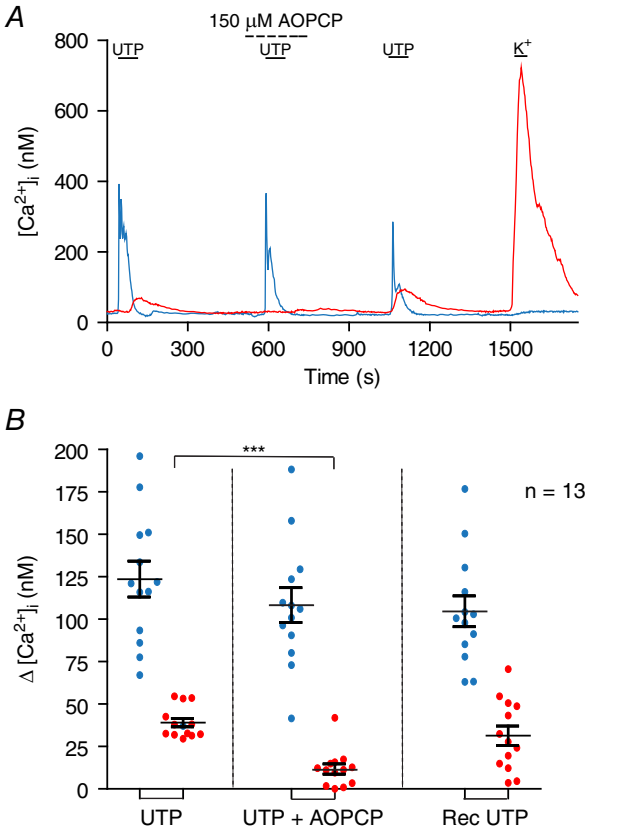

Figure 6. Role of 5′-ectonucleotidase in type II to type I cell crosstalk
A, the delayed, indirect Ca^{2+} response in a type I cell (red trace) evoked by stimulating type II cells with UTP (e.g. blue trace) was reversibly inhibited by the 5′-ectonucleotidase blocker, α,β-methylene ADP (AOPCP; 150 μM). Pooled data from several similar experiments are summarized in *B* ($n = 13$ dishes). Note AOPCP had no significant effect on the UTP-evoked Ca^{2+} response in type II cells in *A* and *B* (***$P < 0.001$).

Type II cells communicate with type I cells via Panx-1 channels: role of adenosine generated from ATP breakdown

Application of the P2Y2R agonist UTP evoked a rise in intracellular Ca^{2+} in type II cells as expected (Xu *et al.* 2003; Zhang *et al.* 2012), but this was often followed by a secondary, delayed rise in intracellular Ca^{2+} in neighbouring type I cells. These data are consistent with the involvement of Panx-1 channels in this crosstalk because the secondary type I cell Ca^{2+} responses were completely inhibited by 5 μM carbenoxolone, a Panx-1 channel blocker (Ma *et al.* 2009). Interestingly, the principal candidate mediating type II to type I cell crosstalk appeared to be adenosine because the secondary type I cell Ca^{2+} response was almost completely inhibited by the specific adenosine A_{2A} receptor antagonist SCH 58261 (5 nM), whereas the A_{2B} receptor antagonist MRS 1754 (100 nM) had little or no effect. Given the presence of both A_{2A} and A_{2B} receptors on rat CB type I cells (Gauda, 2002; Conde *et al.* 2006, 2009; Livermore & Nurse, 2013), it would appear that after the opening of Panx-1 channels by P2Y2R ligands adenosine was produced in a spatially restricted zone near high affinity A_{2A} receptors, and/or the concentration of adenosine

reached was not sufficiently high to activate low affinity A_{2B} receptors (Fredholm *et al.* 2001). Moreover, we demonstrated that the principal source of adenosine mediating this crosstalk is not via direct release from type II cells, but rather from extracellular breakdown of ATP released through Panx-1 channels. In particular, we found that inhibition of 5′-ectonucleotidase with α,β-methylene ADP (AOPCP) largely prevented the indirect UTP-evoked Ca^{2+} responses in type I cells. Thus, it appears that ATP released from type II cells via Panx-1 channels is catabolized extracellularly by 5′-ectonucleotidases into adenosine, which (at least partly) stimulates A_{2A} receptors on type I cells leading to a rise in intracellular Ca^{2+} (Xu *et al.* 2006). However, we cannot rule out the possibility that small amounts of adenosine may also be released directly from type II cells via Panx-1 channels because residual Ca^{2+} responses in type I cells sometimes persisted in the presence of AOPCP. Interestingly, in a recent study, AOPCP almost completely abolished the basal normocapnic single unit CB sinus nerve discharge and diminished the chemosensory response evoked by hypercapnia (Holmes *et al.* 2015).

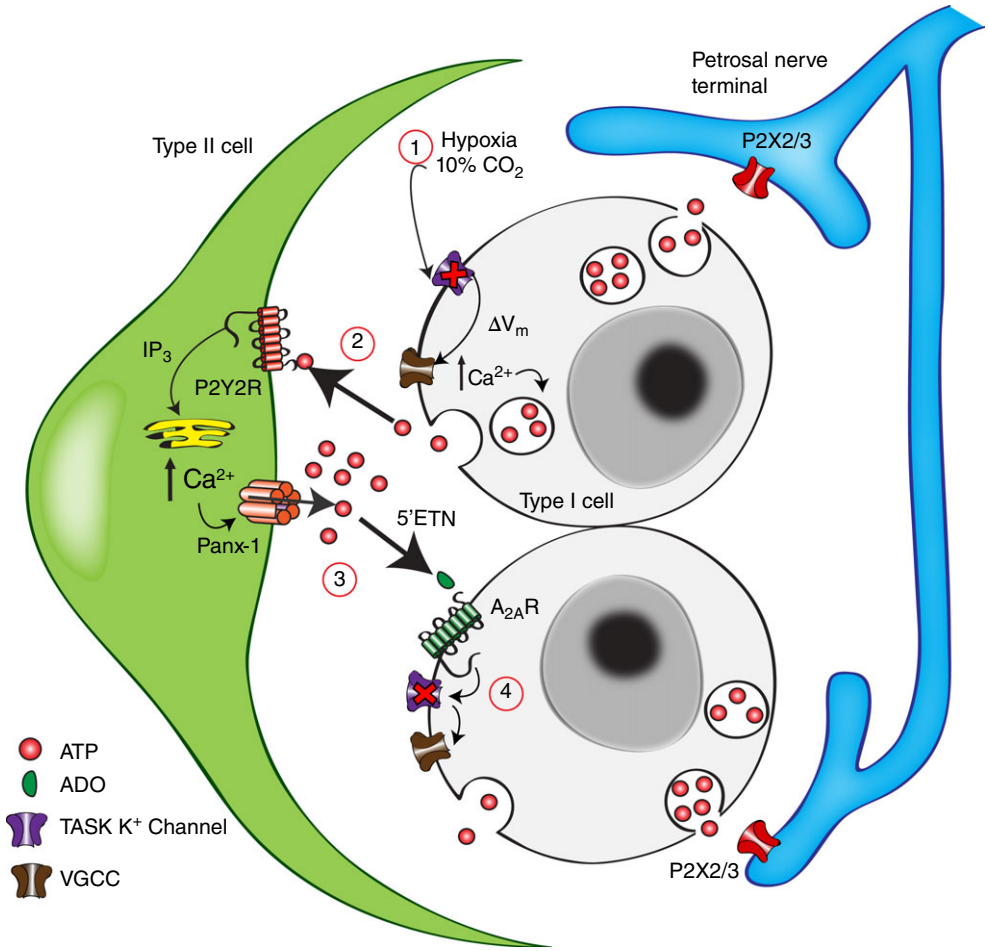

Figure 7. Schematic representation of the role of purinergic signalling mechanisms in cell–cell interactions at the rat carotid body 'tripartite' synapse
The chemostimuli hypoxia and hypercapnia depolarize type I cells through inhibition of TASK1/3 K^+ channels (1), leading to Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCC) and ATP release (2). ATP stimulates postsynaptic P2X2/3 receptors on petrosal afferent nerve terminals causing excitation, as well as P2Y2 receptors (P2Y2R) on adjacent glial-like type II cells. P2Y2R stimulation leads to Ca^{2+} release from intracellular stores via inositol trisphosphate (IP_3) signalling pathways and opening of pannexin-1 channels, allowing further release of ATP into the synaptic cleft. ATP (from both type II and type I cells) is broken down by extracellular 5′-ectonucleotidase (5′ENT) into adenosine (ADO) (3), which activates primarily ADO A_{2A} receptors ($A_{2A}R$) on type I cells. Activation of A_{2A} receptors leads to inhibition of TASK1/3 channels to further enhance type I cell depolarization (4) (Xu *et al.* 2006), and therefore ATP release. Omitted for clarity is the pathway by which hypoxia stimulates ADO release from type I cells via a plasma membrane nucleoside transporter, and negative feedback pathways by which ATP inhibits type I cells via P2Y1 receptors (Xu *et al.* 2005) and pannexin-1 channels in type II cells (Nurse, 2014).

Is there a physiological role for bidirectional crosstalk between type I and type II cells?

It has been proposed that both ATP and adenosine (ADO) are key neurotransmitters that contribute to the CB sensory discharge during hypoxia (Conde *et al.* 2009; Nurse & Piskuric, 2013), with a preference for ADO during mild hypoxia and ATP during severe hypoxia (Conde *et al.* 2012). The present studies suggest that in addition to hypoxic intensity, the occurrence of bidirectional crosstalk between type I and type II cells might also help determine the relative levels of these two purines in the synaptic cleft. Figure 7 summarizes a proposed model illustrating how crosstalk might contribute to sensory processing at the CB 'tripartite' synapse via purinergic signalling pathways. There is a consensus that during hypoxia and acid hypercapnia rat type I cells depolarize due to the closing of TASK1/3 background K^+ channels (Buckler, 2015; Kim & Kang, 2015). This leads to voltage-gated Ca^{2+} entry and release of multiple neurotransmitters including ATP and adenosine (Prasad *et al.* 2001; Buttigieg & Nurse, 2004; Conde *et al.* 2012; Nurse & Piskuric, 2013; Nurse, 2014). Levels of ATP and adenosine may be further controlled by crosstalk between type I and type II cells as follows. First, stimulation of P2Y2Rs on type II cells by ATP released from type I cells provides an additional source of ATP, via the mechanism of 'ATP-induced ATP release' through pannexin-1 channels (Zhang *et al.* 2012). Second, type II cell-derived ATP (released through pannexin-1 channels) can be broken down by 5′-ectonucleotidase to adenosine, which in turn can activate A_{2A} receptors on type I cells, leading to a rise in intracellular Ca^{2+}. Notably, adenosine has been previously shown to depolarize and increase intracellular Ca^{2+} in rat type I cells via A_{2A} receptors, due to adenylate cyclase/protein kinase A-dependent inhibition of TASK-like background K^+ channels (Xu *et al.* 2006). Thus, crosstalk during hypoxia/hypercapnia might lead to a positive feedback inhibition of TASK1/3 channels via the ATP/adenosine couple so as to enhance type I cell depolarization and neurotransmitter release. Given the heterogeneity among type I cells with respect to their hypoxic sensitivity (Bright *et al.* 1996), crosstalk could help coordinate or synchronize activity among type I cells within a cluster. For example, though type I cells outnumber type II cells by a ratio of approximately 4:1 (McDonald, 1981), the long processes of type II cells could ensure that any given stimulated type II cell could in turn recruit several type I cells through adenosine-mediated TASK1/3 inhibition, thereby enhancing neurotransmitter release from the chemoreceptor cluster.

Comparison with other chemosensitive sites

Despite variations in some of the details, the adoption of purinergic mechanisms, gap junctional-like channels, and gliotransmission appears to be a general strategy for signalling at chemoreceptive sites in mammals. For example, in another chemosensory organ, i.e. the mammalian taste bud, it is proposed that gustatory stimulation of receptor cells leads to the release of ATP via Panx-1 channels and excitation of both postsynaptic afferent fibres and presynaptic (type III) cells (Roper, 2013). ATP can also boost its own secretion via positive feedback mechanisms and can be degraded to ADP and adenosine by ectonucleotidases to further regulate gustatory neurotransmission (Bartel *et al.* 2006; Dando & Roper, 2009; Roper, 2013). On the other hand, at medullary central chemoreceptors, elevation in CO_2 or acidity is thought evoke ATP release mainly from glial cells, i.e. astrocytes (Gourine *et al.* 2010). Following release, ATP can be further broken down to ADP, AMP and adenosine by a variety of ectonucleotidases to further shape central respiratory responses to elevations in CO_2/H^+ (Funk, 2013). ATP release from astrocytes may occur in part via CO_2-sensitive gap junction (connexin or Cx26) hemichannels (Huckstepp *et al.* 2010), which are structurally similar to the pannexin family. To our knowledge Cx26 expression has not been identified in the carotid body; however, other gap junction proteins including Cx43 and Cx36 are expressed (Chen *et al.* 2002; Frinchi *et al.* 2013). Though the cellular localization of Cx36 has not been clearly defined (Frinchi *et al.* 2013), it is likely that Cx43 functions mainly to mediate electrical coupling between neighbouring receptor cells (Chen *et al.* 2002; Eyzaguirre, 2007). Our data in the CB, together with those from other laboratories (Conde *et al.* 2012; Tse *et al.* 2012; Holmes *et al.* 2015), are consistent with a model where ATP release may occur from both receptor (type I) and glial (type II) cells during chemostimulation, and sensory transmission is further shaped by the paracrine actions of adenosine generated, at least in part, via breakdown of ATP by ectonucleotidases (Fig. 7).

In summary, we demonstrate for the first time that rat CB glial-like type II cells can respond indirectly to acute chemostimuli such as hypoxia and hypercapnia with an increase in cytosolic $[Ca^{2+}]$. Moreover, we have contributed novel information on the role of purinergic signalling mechanisms in mediating bidirectional crosstalk between type I and type II cells. Further studies are required to identify other neuromodulators that are likely to be involved in this crosstalk and therefore contribute to sensory integration at the CB 'tripartite synapse', especially in pathophysiological conditions where CB chemosensitivity is altered (Kumar & Prabhakar, 2012).

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Additional information

Competing interests

None declared.

Author contributions

S.M. performed all Ca^{2+} imaging experiments, data analysis and preparation of figures, and wrote the manuscript. C.A.N. was involved in the planning and designing of the all the experiments, helped interpret the data, and edited and approved the final version of this manuscript. Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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