

Evidence that 5-HT stimulates intracellular Ca^{2+} signalling and activates pannexin-1 currents in type II cells of the rat carotid body

Sindhubarathi Murali*, Min Zhang* and Colin A. Nurse 

Department of Biology, McMaster University, Hamilton, Ontario, Canada

Key points

- 5-HT is a neuromodulator released from carotid body (CB) chemoreceptor (type I) cells and facilitates the sensory discharge following chronic intermittent hypoxia (CIH).
- In the present study, we show that, in addition to type I cells, adjacent glial-like type II cells express functional, ketanserin-sensitive 5-HT₂ receptors, and their stimulation increases cytoplasmic Ca^{2+} derived from intracellular stores.
- In type II cells, 5-HT activated a ketanserin-sensitive inward current ($I_{5\text{-HT}}$) that was similar to that (I_{UTP}) activated by the P2Y2R agonist, UTP.
- As previously shown for I_{UTP} , $I_{5\text{-HT}}$ was inhibited by BAPTA-AM and carbenoxolone ($5\ \mu\text{M}$), a putative blocker of ATP-permeable pannexin (Panx)-1 channels; I_{UTP} was reversibly inhibited by the specific Panx-1 mimetic peptide channel blocker, ¹⁰Panx peptide.
- Paracrine stimulation of type II cells by 5-HT, leading to ATP release via Panx-1 channels, may contribute to CB excitability, especially in pathophysiological conditions associated with CIH (e.g. obstructive sleep apnoea).

Abstract Carotid body (CB) chemoreceptor (type I) cells can synthesize and release 5-HT and increased autocrine–paracrine 5-HT₂ receptor signalling contributes to sensory long-term facilitation during chronic intermittent hypoxia (CIH). However, recent studies suggest that adjacent glial-like type II cells can respond to CB paracrine signals by elevating intracellular calcium ($\Delta[\text{Ca}^{2+}]_i$) and activating carbenoxolone-sensitive, ATP-permeable, pannexin (Panx)-1-like channels. In the present study, using dissociated rat CB cultures, we found that 5-HT induced $\Delta[\text{Ca}^{2+}]_i$ responses in a subpopulation of type I cells, as well as in most (~67%) type II cells identified by their sensitivity to the P2Y2 receptor agonist, UTP. The 5-HT-induced Ca^{2+} response in type II cells was dose-dependent ($\text{EC}_{50} \sim 183\ \text{nM}$) and largely inhibited by the 5-HT_{2A} receptor blocker, ketanserin ($1\ \mu\text{M}$), and also arose mainly from intracellular stores. 5-HT also activated an inward current ($I_{5\text{-HT}}$) in type II cells ($\text{EC}_{50} \sim 200\ \text{nM}$) that was reversibly inhibited by ketanserin ($1\text{--}10\ \text{nM}$), the Ca^{2+} chelator BAPTA-AM ($5\ \mu\text{M}$), and low concentrations of carbenoxolone ($5\ \mu\text{M}$), a putative Panx-1 channel blocker. $I_{5\text{-HT}}$ reversed direction at approximately $-11\ \text{mV}$ and was indistinguishable from the UTP-activated current (I_{UTP}). Consistent with a role for Panx-1 channels, I_{UTP} was reversibly inhibited by the specific Panx-1 mimetic peptide blocker ¹⁰Panx ($100\ \mu\text{M}$), although not by its scrambled control peptide (^{sc}Panx). Because ATP is an excitatory CB neurotransmitter, it is possible that the contribution of enhanced 5-HT signalling to the increased sensory discharge during CIH may occur, in part, by a boosting of ATP release from type II cells via Panx-1 channels.

*These authors contributed equally to this work.

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Corresponding author C. A. Nurse: Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada. Email: nursec@mcmaster.ca

Abbreviations CB, carotid body; CIH, chronic intermittent hypoxia; 5-HT_{2A}R, 5-HT_{2A} receptor; Panx, pannexin.

Introduction

The mammalian carotid bodies (CB) are sensory organs that monitor acutely blood chemicals such as O₂, CO₂ and pH and maintain homeostasis in the cardiovascular and cardiorespiratory systems via the initiation of appropriate reflex responses (Gonzalez *et al.* 1994; Kumar & Prabhakar, 2012). Dysfunction of the CB chemoreflex occurs in several pathophysiological conditions associated with sympathetic overactivity, including hypertension, heart failure, diabetes and sleep apnoea (Kumar & Prabhakar, 2012, 2015; Schultz *et al.* 2015; Iturriaga *et al.* 2016). The mechanisms underlying these alterations in CB function are incompletely understood but have commonly been attributed to changes in chemoreceptor (type I) cell function, including expression levels of their secreted neurotransmitters and neuromodulators, as well as their cognate receptors within the CB (Iturriaga & Alcayaga, 2004; Kumar & Prabhakar, 2012; Nurse, 2014; Prabhakar *et al.* 2015). Thus, clarification of the roles of these neuroactive agents is critical for understanding the integrated function of the CB in both normal and pathophysiological conditions. This task has recently become even more challenging subsequent to the demonstration that, in addition to type I cells, neighbouring sustentacular type II cells may also express functional G-protein coupled receptors for type I cell paracrine signals including ATP, ACh and angiotensin II (Xu *et al.* 2003; Tse *et al.* 2012; Zhang *et al.* 2012; Murali *et al.* 2014, 2015). Stimulation of these receptors in type II cells causes a rise in intracellular calcium that is considered to be necessary for the activation of an inward current carried by non-selective ion channels (Murali *et al.* 2014). In a previous study (Zhang *et al.* 2012), these channels appeared to be ATP-permeable and their sensitivity to low doses of carboxoxolone (5 μ M), together with immunolocalization evidence, led us to propose they were similar to pannexin (Panx)-1 channels, which are known to act as conduits for ATP release in various cell types (Locovei *et al.* 2006; Huang *et al.* 2007; Zhang *et al.* 2012; Murali *et al.* 2014). However, there is increasing skepticism over the reliability of pharmacological agents such as carboxoxolone with respect to distinguishing Panx-1 channels from gap junctional connexin hemichannels (Lohman & Isakson, 2014). Nevertheless, given that ATP is a major excitatory neurotransmitter in the CB (Nurse, 2014), these findings have led to the proposal that type II cells may also contribute to the CB afferent discharge via paracrine signalling pathways (Tse *et al.* 2012; Zhang *et al.* 2012; Nurse, 2014).

The monoamine 5-HT is among the less well-studied CB neuromodulators. However, there is abundant evidence that chemoreceptor type I cells can synthesize and store 5-HT (Gronblad *et al.* 1983; Oomori *et al.* 1994; Zhang & Nurse, 2000; Liu *et al.* 2011; Yokoyama *et al.* 2013) and, at least in some studies, 5-HT can be released in response to acute hypoxia (Zhang *et al.* 2003; Peng *et al.* 2009; Ramirez *et al.* 2012). Moreover, exogenous 5-HT elicits complex physiological responses in the CB involving multiple receptor types located on type I cells, sensory nerve endings and/or blood vessels (Kirby & McQueen, 1984). Importantly, a critical role proposed for 5-HT in the CB is to promote long-term facilitation of the sensory discharge during exposure to chronic intermittent hypoxia (CIH), a situation observed in pathophysiological conditions that lead to autonomic imbalances (e.g. central or obstructive sleep apnoea) (Peng *et al.* 2009; Prabhakar, 2011). The actions of 5-HT result in the modulation of the CB sensory response and have been largely attributed to the presence of 5-HT_{2A} receptors (5-HT_{2A}R) localized mainly (but not exclusively) to type I cells (Kirby & McQueen, 1984; Zhang *et al.* 2003; Jacono *et al.* 2005; Yokoyama *et al.* 2015). However, RT-PCR analysis suggests that, in addition to 5-HT_{2A}R, and controversially 5-HT_{5A}R, mRNAs, at least seven more 5-HT receptor mRNAs are expressed in extracts of rat CB (Wang *et al.* 2000; Zhang *et al.* 2003; Yokoyama *et al.* 2015). Taken together, these studies indicate a complex role of 5-HT and its receptors in paracrine signalling pathways in the CB.

In the present study, we tested the hypothesis that rat CB type II cells may participate in the paracrine actions of 5-HT via expression of functional 5-HT receptors. We found that 5-HT, acting mainly via ketanserin-sensitive 5-HT₂ receptors, evoked robust intracellular Ca²⁺ responses in a significant proportion of type II cells and, surprisingly, these responses were detected more frequently than corresponding ones in neighbouring type I cells. Previous studies have demonstrated that stimulation of Ca²⁺ signalling pathways in type II cells by other CB neuromodulators (e.g. ATP and angiotensin II) leads to activation of a similar inward current carried by non-selective cation channels (Zhang *et al.* 2012; Murali *et al.* 2014) and we found that this was also the case for 5-HT. Finally, to strengthen the evidence for a role of Panx-1 channels in the activation of the inward current, we compared the effects of a specific mimetic peptide inhibitor of Panx-1 channels,

¹⁰Panx peptide, with its scrambled control peptide, ^{sc}Panx (Thompson *et al.* 2008; Lohman & Isakson, 2014).

Methods

Ethical approval

Procedures for animal handling and tissue removal followed the guidelines of the Canadian Council on Animal Care and were approved by the McMaster's Animal Research Ethics Board. Animals were housed in the McMaster Central Animal Facility under a constant 12:12 h light/dark cycle with access to food and water available *ad libitum*. We understand the ethical principles of the journal with respect to studies on animals and our work complies with its animal ethic checklist.

Carotid body cultures

The procedures for preparing CB cultures were similar to those described in detail previously (Zhang *et al.* 2000, 2012). Briefly, rat pups 9–14 days old (Wistar, Charles River, Quebec, Canada) were rendered unconscious by a blow to the back of the head and then killed immediately by decapitation. The carotid bifurcations were excised and the CBs were isolated, cleaned of surrounding tissue and then exposed to a physiological salt solution containing 0.1% trypsin (Sigma-Aldrich, Oakville, Ontario, Canada) and 0.1% collagenase (Gibco, Grand Island, NY, USA). The tissue was then mechanically dissociated and the resulting cell suspension plated on modified tissue culture dishes pre-coated with a thin layer of Matrigel (BD Biosciences, Mississauga, Ontario, Canada). The cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 2–7 days. The growth medium consisted of F-12 nutrient medium supplemented with 1% penicillin-streptomycin, 1% glutamine, 0.3% glucose, 3 μg ml⁻¹ insulin and a series of serum additives, as described previously (Zhang *et al.* 2012; Murali *et al.* 2014). The Ca²⁺ imaging experiments were usually performed on cultures that were ~48 h old, whereas patch clamp recordings were obtained from 5–7-day-old cultures to facilitate optimal recordings from isolated type II cells (Zhang *et al.* 2012).

Fura-2 intracellular Ca²⁺ measurements

Fura-2 measurements of intracellular Ca²⁺ concentration ([Ca²⁺]_i) were obtained from both type I and type II cells using procedures similar to those described in detail elsewhere (Piskuric & Nurse, 2012; Zhang *et al.* 2012; Murali *et al.* 2014). Briefly, cells were loaded with 2.5 μM fura-2 AM diluted in a physiological bicarbonate-buffered solution for 30 min at 37°C. After washing for ~15 min to remove free dye, cells were imaged using an Eclipse TE2000-U inverted microscope (Nikon,

Mississauga, ON, Canada) equipped with 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 40x oil-immersion objective lens with a numerical aperture of 1.3. The perfusion (extracellular) solution was maintained at ~35 °C and contained (mM): 24 NaHCO₃, 115 NaCl, 5 glucose, 5 KCl, 2 CaCl₂ and 1 MgCl₂ (pH ~7.4), as maintained by bubbling a 5% CO₂/95% air mixture. Images were acquired every 2 s at 340 nm and 380 nm excitation (510 emission), with an exposure time of 100–200 ms. Pseudocolour ratiometric data were obtained using Simple PCI, version 5.3 (Hamamatsu Photonics, Shizuoka, Japan). The procedures for instrument calibration and calculation of intracellular free [Ca²⁺] were identical to those described previously (Piskuric & Nurse, 2012; Zhang *et al.* 2012). For most experiments statistical analysis was performed using repeated measures ANOVA with Tukey's *post hoc* multiple comparison test, as indicated.

Whole-cell recordings from type II cells

Whole-cell currents were recorded from type II cells using the nystatin perforated-patch technique as described previously (Zhang *et al.* 2000, 2012). The cells were perfused with bicarbonate-buffered saline warmed to ~35°C and contained (in mM): 24 NaHCO₃, 115 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and 12 and sucrose (pH 7.4), as maintained by bubbling with 5% CO₂/95% air mixture. The pipette solution contained (mM): 115 potassium gluconate, 25 KCl, 5 NaCl, 1 CaCl₂, 10 Hepes and 200 μg ml⁻¹ nystatin (pH 7.2). A fast perfusion system utilizing a double-barreled pipette assembly was used for rapid application of 5-HT to the cells (Zhang *et al.* 2000, 2003, 2012). Voltage clamp data were obtained with the aid of a MultiClamp 700A patch clamp amplifier and a Digidata 1322A analog-to-digital converter (Axon Instruments Inc., Union City, CA, USA) and stored on a personal computer. Data acquisition and analysis were performed using pCLAMP, version 9.0 (Axon Instruments Inc.). Multiple comparisons of ionic currents or current density (pA/pF), obtained by dividing peak current by whole cell capacitance, were performed using ANOVA. *P* < 0.05 was considered statistically significant.

Reagents and drugs

Reagents and drugs were obtained from Sigma-Aldrich (Oakville, ON, Canada): UTP, 5-HT, ketanserin, carbenoxolone, SB 69955 and BAPTA-AM. The specific mimetic peptide inhibitor of Panx-1 channels, ¹⁰Panx peptide, and the ¹⁰Panx scrambled control peptide, ^{sc}Panx (Thompson *et al.* 2008), were kindly provided by

Dr Roger Thompson (University of Calgary, Calgary, Alberta, Canada).

Results

The reported results pertain mainly to isolated 'solitary' type II cells to eliminate or minimize cross-talk as a result of direct stimulation of neighbouring type I cells by 5-HT (Zhang *et al.* 2003; Murali & Nurse, 2016). In some cases, intracellular Ca^{2+} responses were monitored simultaneously in type I cells that were members of a cell cluster and nearby type II cells. In addition to their characteristic spindle shape, type II cells were routinely identified by the presence of a rise in intracellular Ca^{2+} ($\Delta[\text{Ca}^{2+}]_i$) following stimulation with the P2Y2R agonist, UTP (Xu *et al.* 2003; Zhang *et al.* 2012). The absence of cross-talk from type I to type II cells was supported by the lack of a $\Delta[\text{Ca}^{2+}]_i$ response in type II cells during exposure to the depolarizing stimulus, high K^+ (30 mM) (Murali & Nurse, 2016), which stimulates Ca^{2+} -dependent neurosecretion from type I cells. For Ca^{2+} imaging experiments, the reported 'n' values refer to the number of culture dishes sampled; typically, 15–30 cells were sampled per dish. In voltage clamp experiments, the 'n' value represents the number of cells sampled.

5-HT evokes intracellular Ca^{2+} responses in both type I and type II cells

In 2-day-old CB cultures, application of 5-HT (10 μM) via the perfusate elicited a rise in intracellular Ca^{2+} ($\Delta[\text{Ca}^{2+}]_i$) in subpopulations of type I and type II cells (Fig. 1A). In one experimental series, ~40% (77/190 cells) of type I cells, identified by the presence of a Ca^{2+} response to the depolarizing stimulus high K^+ (Fig. 1A), were sensitive to 10 μM 5-HT. This proportion is comparable to that previously reported in electrophysiological studies on the 5-HT modulation of K^+ currents in rat type I cells (~40%) (Zhang *et al.* 2003). By contrast, a larger proportion (~67%; 208/310 cells) of UTP-sensitive type II cells responded to 10 μM 5-HT. In general, the peak Ca^{2+} response in type II cells was more robust than that in type I cells (Fig. 1A); the relative magnitude of the mean peak $\Delta[\text{Ca}^{2+}]_i$ evoked by 5-HT was ~229 nM in type II cells compared to ~54 nM in type I cells (Mann–Whitney *t* test; $P < 0.01$) (Fig. 1B). Despite the larger peak, Ca^{2+} responses in type II cells decayed more rapidly compared to the longer-duration responses in type I cells; when averaged over the time of 5-HT application, the mean $\Delta[\text{Ca}^{2+}]_i$ was slightly higher in type II than type I cells (Fig. 1C).

The effect of 5-HT on $\Delta[\text{Ca}^{2+}]_i$ in type II cells was dose-dependent (Fig. 2A). A plot of the dose–response curve for [5-HT] vs. $\Delta[\text{Ca}^{2+}]_i$ is shown in Fig. 2B where the estimated EC_{50} is ~183 nM; this value is higher than that reported for 5-HT acting at 5-HT_{2A} receptors in other glial

cell types (e.g. C6 glioma cells) ($\text{EC}_{50} = 100$ nM) (Muraoka *et al.* 1998). There was broad variability in the $\Delta[\text{Ca}^{2+}]_i$ responses among cells in a given dish. For example, in three randomly chosen cells exposed to 10 μM 5-HT, the mean $\Delta[\text{Ca}^{2+}]_i$ responses were 95.3 ± 5.3 nM (range 64–134 nM; $n = 18$), 126.1 ± 5.8 nM (range 58–174 nM; $n = 25$) and 96.5 ± 5.4 nM (range 65–134 nM; $n = 15$). The 5-HT receptors on type II cells displayed slight desensitization on repeated application of 10 μM 5-HT spaced ~10 min apart; however, only the first and third responses were significantly different from one another (Friedman test, $P < 0.001$; $n = 10$ dishes) (Fig. 2C and D).

Evidence for a role of 5-HT_{2A} receptors in the 5-HT-evoked intracellular Ca^{2+} responses in both type I and type II cells

Previous studies using different techniques demonstrated that rat type I cells express 5-HT_{2A}R (Zhang *et al.* 2003; Peng *et al.* 2009; Liu *et al.* 2011; Yokoyama *et al.* 2015). As expected, the 5-HT-induced Ca^{2+} responses in type I cells were sensitive to the selective 5-HT_{2A}R antagonist, ketanserin (Fig. 3A). A scatter plot of $\Delta[\text{Ca}^{2+}]_i$ responses induced by 10 μM 5-HT in type I cells before, during and after ketanserin is shown in Fig. 3E. Ketanserin inhibited the mean $\Delta[\text{Ca}^{2+}]_i$ response in type I cells by ~54%; the mean \pm SEM $\Delta[\text{Ca}^{2+}]_i$ response was 28.4 ± 4.8 nM for 5-HT (10 μM), 13.0 ± 2.26 nM for 5-HT plus ketanserin (1 μM) and 18.0 ± 6.2 nM for 5-HT after washout of ketanserin (repeated measures ANOVA, $P < 0.01$, $n = 9$ dishes). In previous studies, ketanserin also blocked the 5-HT-induced inhibition of K^+ currents in rat type I cells (Zhang *et al.* 2003).

By contrast to the present findings, 5-HT-evoked Ca^{2+} responses were not detectable in clustered rat type I cells in a recent study by Yokoyama *et al.* (2015). Given the possibility of cross-talk between type II and type I cells (Murali & Nurse, 2016), we considered whether the type I cell Ca^{2+} responses seen in the present study arose indirectly (i.e. consequent to type II cell stimulation). This did not appear to be the case because perfusion with 5 μM carbenoxolone, a Panx-1 channel inhibitor that blocks type II to type I cross-talk (Murali & Nurse, 2016), did not abolish the 5-HT-evoked Ca^{2+} responses in type I cells, although the mean response was slightly reduced (Fig. 3B and F). Note that carbenoxolone did not significantly affect 5-HT-evoked Ca^{2+} responses in type II cells (Fig. 3B and D). These data support the notion that 5-HT can evoke direct Ca^{2+} responses in at least a subpopulation of type I cells via 5-HT_{2A} receptors.

Because 5-HT_{2A} receptors are commonly linked to the generation of intracellular Ca^{2+} signals in glial cells (Verkhatsky & Kettenmann, 1996), we tested whether those receptors might also contribute to the type II cell Ca^{2+} responses using ketanserin. Indeed,

the 5-HT-evoked $\Delta[\text{Ca}^{2+}]_i$ was reversibly inhibited by ketanserin ($1 \mu\text{M}$) in the majority of responsive type II cells (180/252; 70%) (Fig. 3A). The mean $\Delta[\text{Ca}^{2+}]_i$ response induced by $10 \mu\text{M}$ 5-HT in the absence and presence of ketanserin was 52 nM and 15 nM, respectively, corresponding to $\sim 71\%$ inhibition ($n = 14$ dishes; Friedman test with Dunn's *post hoc* multiple comparison test; $P < 0.01$) (Fig. 3C). In general, there was only partial recovery of 5-HT-induced responses after washout of ketanserin; the mean $\Delta[\text{Ca}^{2+}]_i$ response after ketanserin

was 28.5 nM compared to 52 nM before ($n = 14$ dishes; Friedman test with Dunn's *post hoc* multiple comparison test, $P < 0.001$). This effect may be related to the gradual run-down in 5-HT-evoked responses with repeated 5-HT application (Fig. 2C and D).

The persistence of a residual Ca^{2+} response in some type II cells exposed to 5-HT plus ketanserin, together with the observation that ketanserin had a negligible effect on a minority population of type II cells, raised the question of whether other 5-HT receptors were

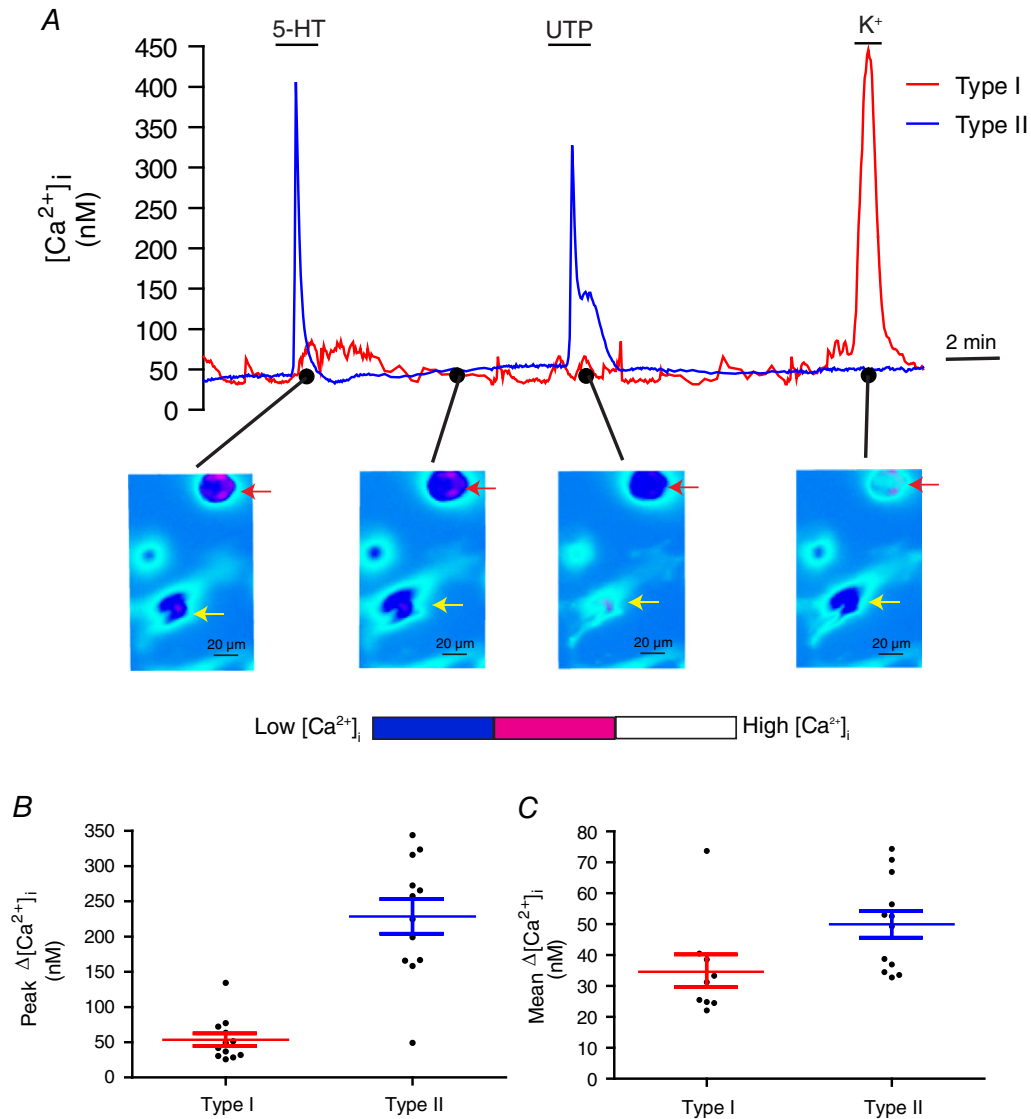


Figure 1. 5-HT-evoked intracellular Ca^{2+} responses in type I vs. type II cells

A, 5-HT ($10 \mu\text{M}$) causes a rise in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in both a type I cell and a type II cell in the same culture. Note that the type II cell (indicated by the yellow arrow, lower) responds to UTP but not high K^+ and elicits a more robust Ca^{2+} response than the type I cell (indicated by the red arrow, lower); the type I cell responds to high K^+ but not UTP. Comparison of the peak (mean \pm SEM) $\Delta[\text{Ca}^{2+}]_i$ responses in type I vs. type II cells after exposure to $10 \mu\text{M}$ 5-HT is shown in (B) ($n = 12$ dishes; 20–30 cells sampled per dish); the mean $\Delta[\text{Ca}^{2+}]_i$ responses averaged over the time of 5-HT application are shown in (C) (type I, $n = 9$ dishes; type II, $n = 12$ dishes). [Colour figure can be viewed at wileyonlinelibrary.com]

involved. The expression of mRNA for a broad variety of G-protein coupled 5-HT receptors was recently reported in extracts of the rat CB (Yokoyama *et al.* 2015). Although controversial (Yokoyama *et al.* 2015), expression of 5-HT_{5A} receptor mRNA was reported in the rat CB and 5-HT_{5A}R immunoreactivity has been localized to type I clusters (Wang *et al.* 2000). Moreover, rat 5-HT_{5A} receptors typically display submicromolar affinity for 5-HT ($EC_{50} \sim 0.6 \mu\text{M}$) (Goodfellow *et al.* 2012), which is not dissimilar to that observed for the 5-HT-induced Ca^{2+} responses in type II cells ($EC_{50} \sim 0.2 \mu\text{M}$) (Fig. 2B). In the present study, the putative 5-HT_{5A}R blocker SB 699551 at $10 \mu\text{M}$, a concentration expected to inhibit rat 5-HT_{5A} receptors (Thomas *et al.* 2006; Goodfellow *et al.* 2012), had no significant effect on the 5-HT-induced Ca^{2+} response; the mean $\Delta[\text{Ca}^{2+}]_i$ response induced by $10 \mu\text{M}$ 5-HT was $50.9 \pm 4.9 \text{ nM}$ (control), $45.3 \pm 5.5 \text{ nM}$ in the presence of SB 699551 and $34.2 \pm 4.6 \text{ nM}$ after washout of SB 699551 ($P > 0.05$; $n = 11$ dishes). These data do not support a

significant contribution of 5-HT_{5A}R to the intracellular Ca^{2+} responses induced by 5-HT in type II cells.

5-HT-induced Ca^{2+} transients in type II cells originate from intracellular stores

In most cases, 5HT_{2A}R activation leads to an increase in Ca^{2+}_i from an intracellular source via the $G\alpha_q$ -PLC-IP₃ signalling pathway (Peng *et al.* 2009; Liu *et al.* 2011). Therefore, to confirm that the 5-HT-evoked $[\text{Ca}^{2+}]_i$ transients in type II cells arose from an intracellular source, we monitored Ca^{2+} responses in a nominally 'Ca²⁺-free' solution (0 Ca²⁺ plus 1 mM EGTA). In some experiments, the order of presenting normal Ca²⁺ and 0 Ca²⁺ solutions was reversed to account for the desensitization properties of the receptor. A high proportion (86%; $n = 133/154$) of type II cells that initially responded to 5-HT in normal Ca²⁺ continued to do so in Ca²⁺-free solution. Indeed, as shown in Fig. 4A, type II cell Ca²⁺-transients evoked by

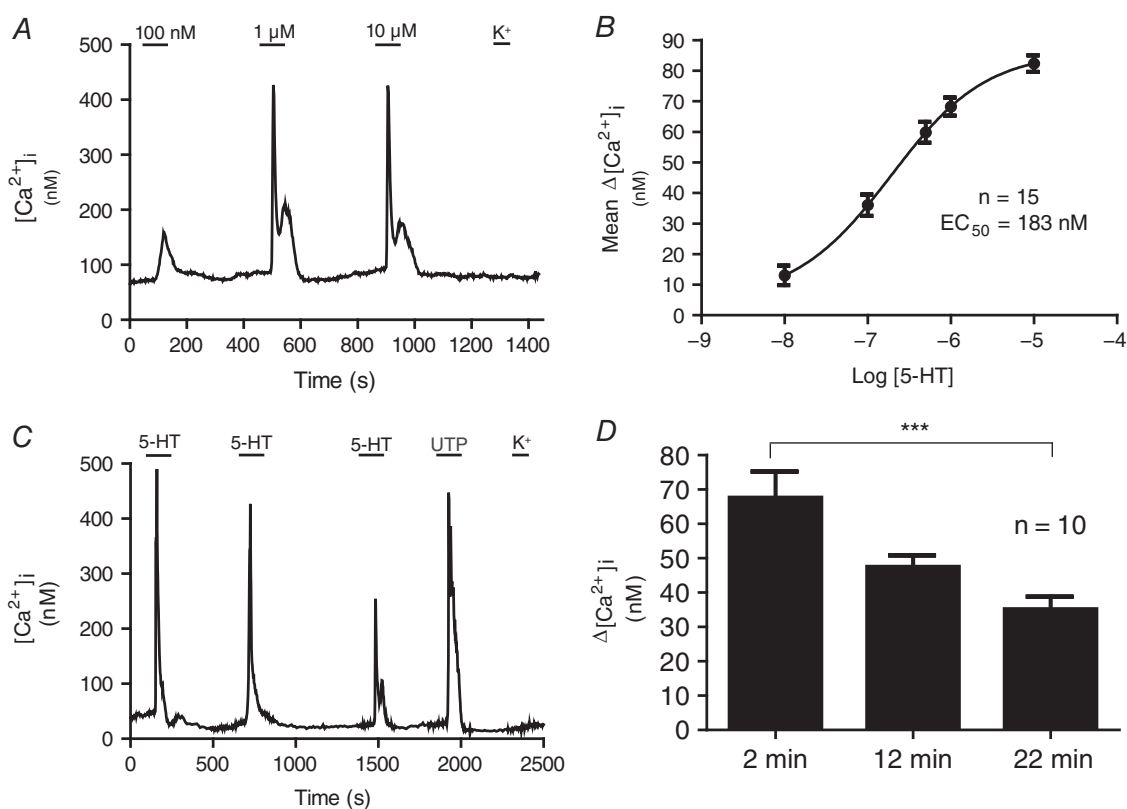


Figure 2. Dose–response curve and time-dependent effects of 5-HT on intracellular calcium responses in type II cells

The effect of increasing doses of 5-HT (100 nM, $1 \mu\text{M}$ and $10 \mu\text{M}$) on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is shown in (A), for a type II cell. B, dose–response relation for mean 5-HT-induced $\Delta[\text{Ca}^{2+}]_i$ in type II cells; the dose–response curve was fitted with the Hill equation and yielded an $EC_{50} = 183 \text{ nM}$ ($n = 15$ dishes). C, repeated exposure to $10 \mu\text{M}$ 5-HT caused a gradual decrease in $[\text{Ca}^{2+}]_i$ responses in type II cells, presumably as a result of receptor desensitization. D, time-course of the reduction in $\Delta[\text{Ca}^{2+}]_i$ responses in type II cells after 2, 12 and 22 min exposure to $10 \mu\text{M}$ 5-HT; $n = 10$, $***P < 0.001$, Friedman test with Dunn's multiple comparison *post hoc* test.

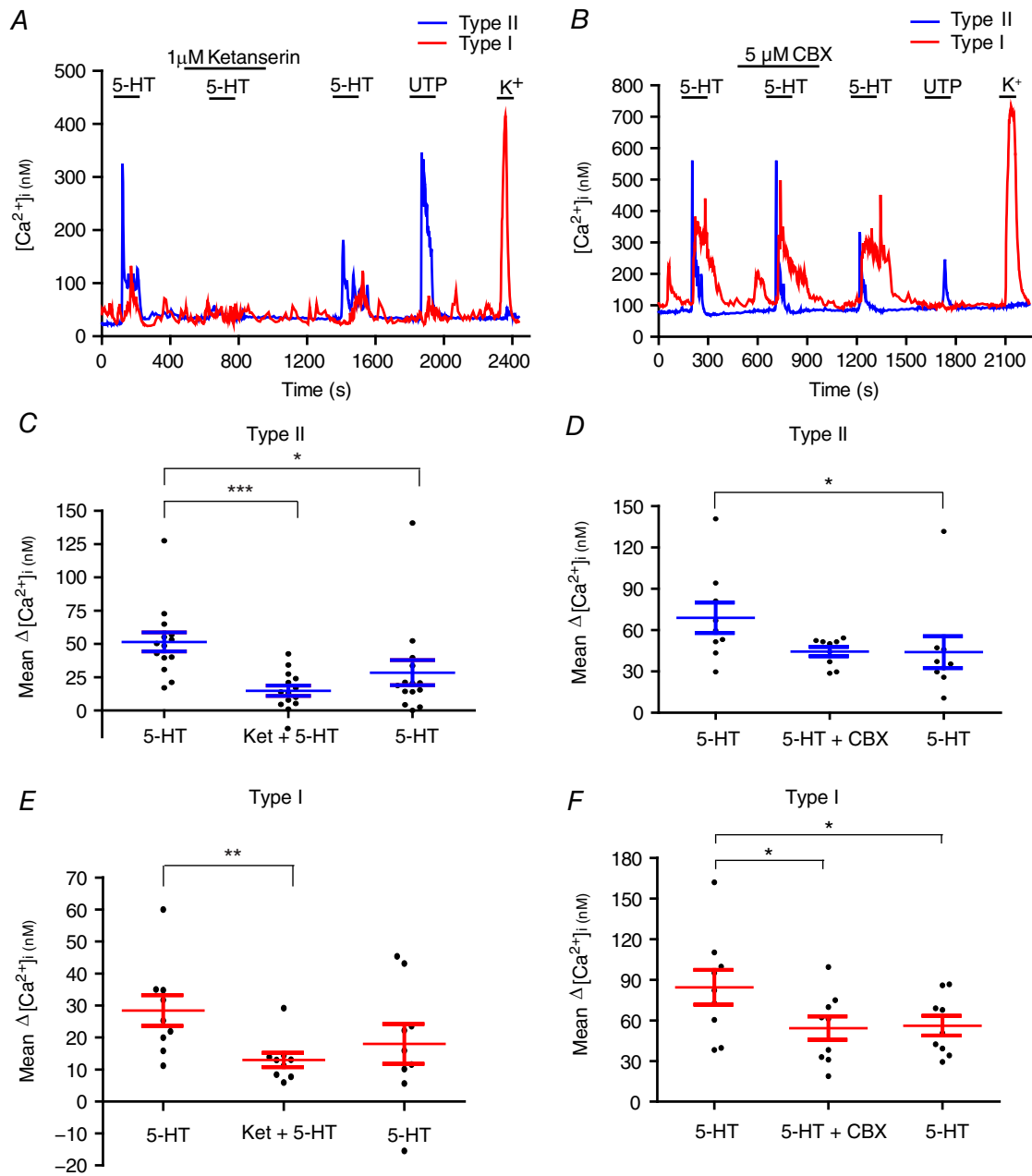


Figure 3. Effects of ketanserin and carbenoxolone on the 5-HT-evoked intracellular Ca^{2+} responses in type I and type II cells

In some cases, as shown in (B), ketanserin (1 μ M), a selective 5-HT_{2A} receptor antagonist, completely blocked the 5-HT-induced Ca^{2+} transients in type I and type II cells within the same cell cluster; note the type I cell was sensitive to high K^+ but not UTP, whereas the converse was true for the type II cell. In other cases, inhibition by ketanserin was incomplete, as shown in the summary data for both cell types in (C) and (E). In (B) and (F), the Panx-1 channel inhibitor carbenoxolone (CBX; 5 μ M) failed to block the 5-HT-evoked intracellular Ca^{2+} response in type I cells, although the overall mean response was reduced (F; $n = 9$). This suggests that the type I cell response to 5-HT was not primarily a result of cross-talk between type II and type I cells, which can occur via Panx-1 channels (Murali & Nurse, 2016). Note that carbenoxolone had no significant effect on the mean 5-HT-evoked ΔCa^{2+} responses in type II cells (D; $n = 9$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

10 μM 5-HT were not significantly altered on switching from normal to Ca^{2+} -free medium, suggesting the predominant source of Ca^{2+} was intracellular. Figure 4B provides a scatter plot comparison of 5-HT-induced $\Delta[\text{Ca}^{2+}]_i$ in normal (2 mM) and Ca^{2+} -free solutions; the mean \pm SEM $\Delta[\text{Ca}^{2+}]_i$ in normal calcium was 77.5 ± 7.2 nM compared to 74.8 ± 13.4 in zero calcium (unpaired *t* test with Welch's correction, $P = 0.8644$; $n = 9$ dishes). In support of an intracellular Ca^{2+} source, application of the store depleting agent thapsigargin (1 μM) prevented the 5-HT-induced $\Delta[\text{Ca}^{2+}]_i$ (Fig. 4C). Of 431 5-HT-responsive type II cells tested, only nine cells (i.e. $\sim 2\%$) elicited a significant rise in Ca^{2+} in the presence of 5-HT plus thapsigargin; in Fig. 4D, thapsigargin dramatically reduced the mean $\Delta[\text{Ca}^{2+}]_i$ from 77.8 ± 6.98 to -16.1 ± 3.61 nM ($n = 12$ dishes; Friedman test with Dunn's *post hoc* multiple comparison test, $P < 0.001$). In these experiments, the effects of thapsigargin were not reversible (Fig. 4C and D).

5-HT activates a carbenoxolone-sensitive inward current in type II cells

We previously demonstrated that other CB neurotransmitters such as ATP and angiotensin II activated an inward current in type II cells via P2Y2 and AT_1 receptors, respectively (Zhang *et al.* 2012; Murali *et al.* 2014); the sensitivity of this current to low doses of carbenoxolone (5 μM) was consistent with the proposed involvement of Panx-1 channels. Similarly, in voltage clamp studies, 5-HT activated a dose-dependent inward current ($\text{EC}_{50} = 0.21$ μM) at a holding potential of -60 mV in responsive type II cells (Fig. 5A and B). The latency of the current response decreased with increasing 5-HT concentrations over the range 0.02–10 μM and varied typically between 4 and 12 s (Fig. 5A and C). Consistent with activation of a non-selective cation current, the 5-HT-induced current showed a mean \pm SEM reversal potential of -10.8 ± 3.2 mV ($n = 5$) (Fig. 6A and B).

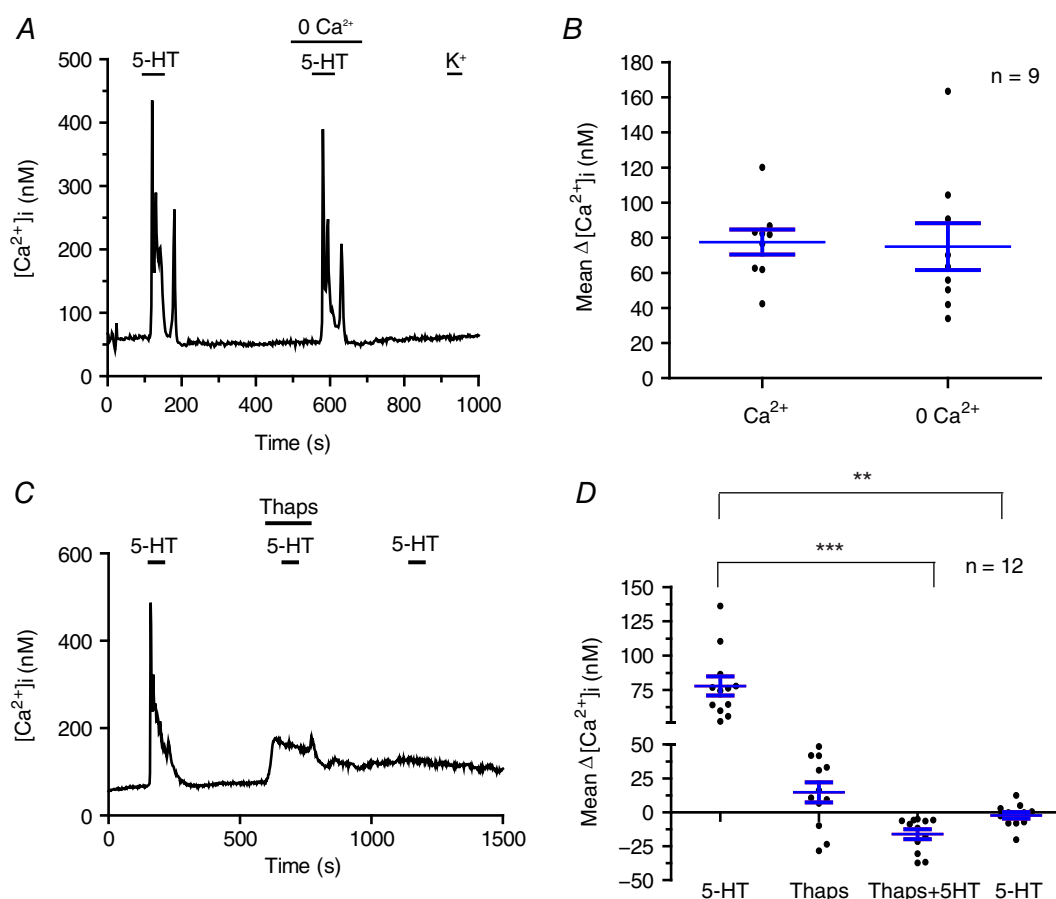


Figure 4. Role of intracellular stores in the 5-HT-induced rise in $[\text{Ca}^{2+}]_i$ in type II cells

A, 5-HT-induced rise in $[\text{Ca}^{2+}]_i$ persisted in solution containing nominally-free extracellular Ca^{2+} (0 Ca^{2+}); summary data of the mean \pm SEM $\Delta[\text{Ca}^{2+}]_i$ responses from nine dishes are shown in (B). Consistent with an intracellular source of Ca^{2+} , the 5-HT-induced $\Delta[\text{Ca}^{2+}]_i$ was inhibited in the presence of the store-depleting agent, thapsigargin (Thaps; 1 μM) in (C) and (D); note the effects of thapsigargin were irreversible in (C) and (D) ($n = 12$ dishes; $**P < 0.01$; $***P < 0.001$). [Colour figure can be viewed at wileyonlinelibrary.com]

We next investigated whether the 5-HT-induced inward current in type II cells was sensitive to the putative Panx-1 channel inhibitor, carbenoxolone. As shown in Fig. 7A, the 5-HT-induced inward current (at -60 mV holding potential) was reversibly inhibited by $5 \mu\text{M}$ carbenoxolone, which is an effect similar to that previously observed when ATP/UTP or ANG II was used to stimulate type II cells (Zhang *et al.* 2012; Murali *et al.* 2014). The onset of current blockade was slow, developing gradually over a period of 6 min (Fig. 7A); summary data for a group of four cells treated in this way are illustrated in Fig. 7B.

Sensitivity of the inward current in type II cells to the specific Panx-1 channel blocker, $^{10}\text{Panx}$ peptide

There is increasing skepticism over the use of carbenoxolone, even at low concentrations, as a reliable tool for the positive identification of Panx-1 channels

(Lohman & Isakson, 2014). Therefore, to obtain more compelling evidence for Panx-1 channel involvement, we tested the effects of the specific mimetic peptide inhibitor of Panx-1 channels, $^{10}\text{Panx}$ peptide, and its scrambled control peptide, $^{\text{sc}}\text{Panx}$ (Thompson *et al.* 2008). Figure 8A illustrates the similarity of the carbenoxolone-sensitive, inward current activated by $50 \mu\text{M}$ UTP and $5 \mu\text{M}$ 5-HT in the same type II cell. Perfusion with an extracellular solution containing $^{10}\text{Panx}$ peptide ($100 \mu\text{M}$) resulted in a reversible blockade of the UTP-activated inward current in this cell (Fig. 8Aa). On the other hand, perfusion with scrambled control peptide $^{\text{sc}}\text{Panx}$ ($100 \mu\text{M}$) was ineffective in the same cell (Fig. 8Ab); $^{\text{sc}}\text{Panx}$ was similarly ineffective on the UTP-activated inward current in two other type II cells (data not shown). As shown in Fig. 8B for a different cell, blockade of the UTP-activated inward current with $^{10}\text{Panx}$ peptide developed gradually over several minutes, similar to the effect of carbenoxolone

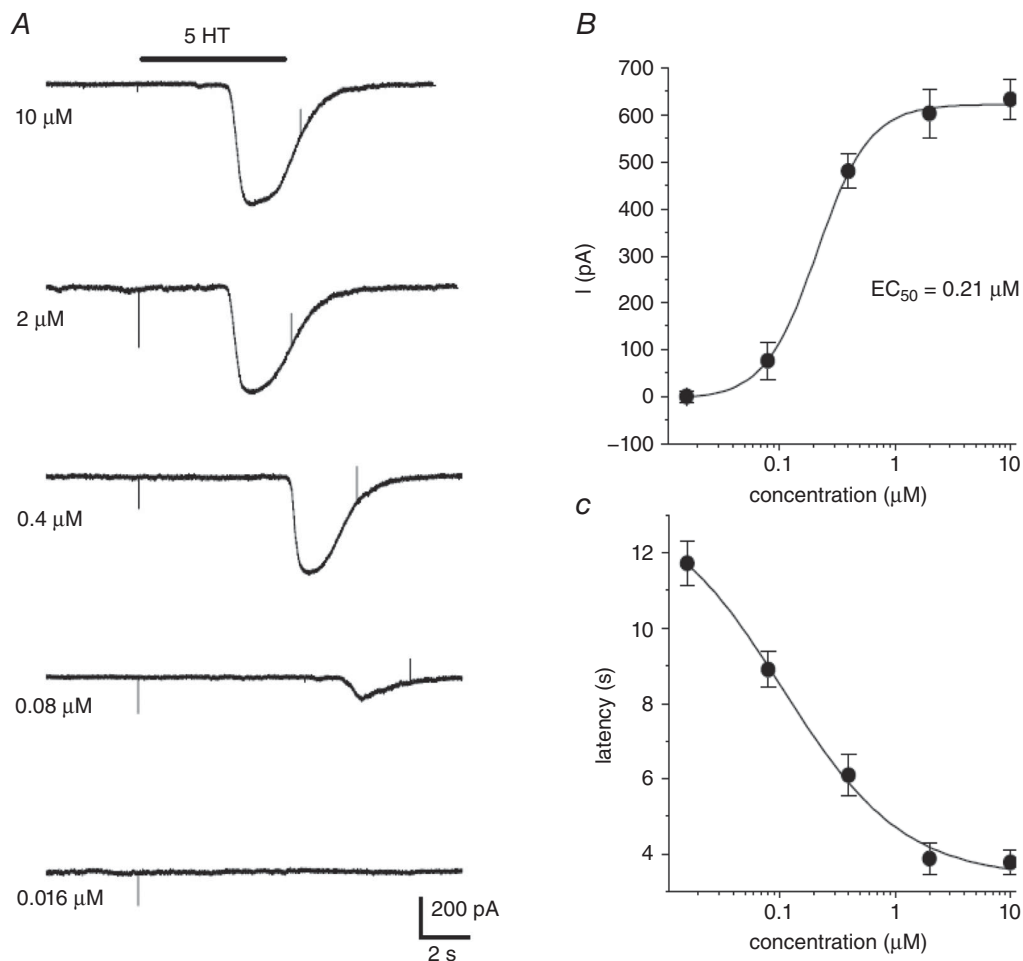


Figure 5. Dose- and time-dependent activation of an inward current in type II cells by 5-HT

Application of varying doses of 5-HT by rapid perfusion during the period indicated by upper horizontal bar evokes a delayed inward current in a type II cell (A); holding potential = -60 mV. B, a fit of the dose-response curve for the effects of 5-HT on the peak inward current yields an EC_{50} of $0.21 \mu\text{M}$ using the Hill equation (data points represent the mean \pm SEM; $n = 5$ cells). The mean \pm SEM latency of the current response vs. 5-HT concentration is shown in (C) ($n = 5$ cells).

(Fig. 7A). A histogram of UTP-activated inward current density measurements for three different type II cells before, during and after exposure to 10 Panx peptide is shown in Fig. 8C; note almost complete inhibition of the current by 10 Panx peptide in all cases and a full recovery of the response after washout of the blocker. Taken together, these data strongly support a role of Panx-1 channels in the generation of the inward current in type II cells.

Effects of 5-HT receptor blockers on the 5-HT-activated inward current in type II cells

Given the sensitivity of the 5-HT-induced Ca^{2+} transients to the 5-HT $_{2A}$ receptor blocker ketanserin (Fig. 3), we first tested the effects of ketanserin on the inward current induced by 5 μM 5-HT in type II cells. As shown in Fig. 9A, low doses of ketanserin (1–10 nM) (i.e. values near its IC_{50} at 5-HT $_{2A}$ receptors;

$\text{IC}_{50} = 1.58$ nM) (Barnes & Sharp, 1999) reversibly inhibited the 5-HT-induced inward current at -60 mV holding potential. An illustration of the concentration-dependent inhibition of this inward current (plotted as current density, pA/pF) is shown in Fig. 9B ($n = 5$ cells).

Interestingly, in contrast to the Ca^{2+} imaging data (see above), application of the 5-HT $_{5A}$ receptor antagonist SB 699551 at concentrations of 1, 5 and 10 μM caused ~4%, 50% and 97% inhibition of the inward current, respectively; for a group of seven cells, the mean peak current evoked 5 μM 5-HT was 379 ± 47 pA initially, compared to 364 ± 52 pA for 5-HT plus 1 μM SB 699551 ($P > 0.05$), 193 ± 38 pA for 5-HT plus 5 μM SB 699551 ($P < 0.05$), 13 ± 9 pA for 5-HT plus 10 μM SB 699551 ($P < 0.001$) and 359 ± 43 pA for 5-HT alone ($P > 0.05$), after washout of the drug. Although these data are consistent with the blockade of rodent 5-HT $_{5A}$ R

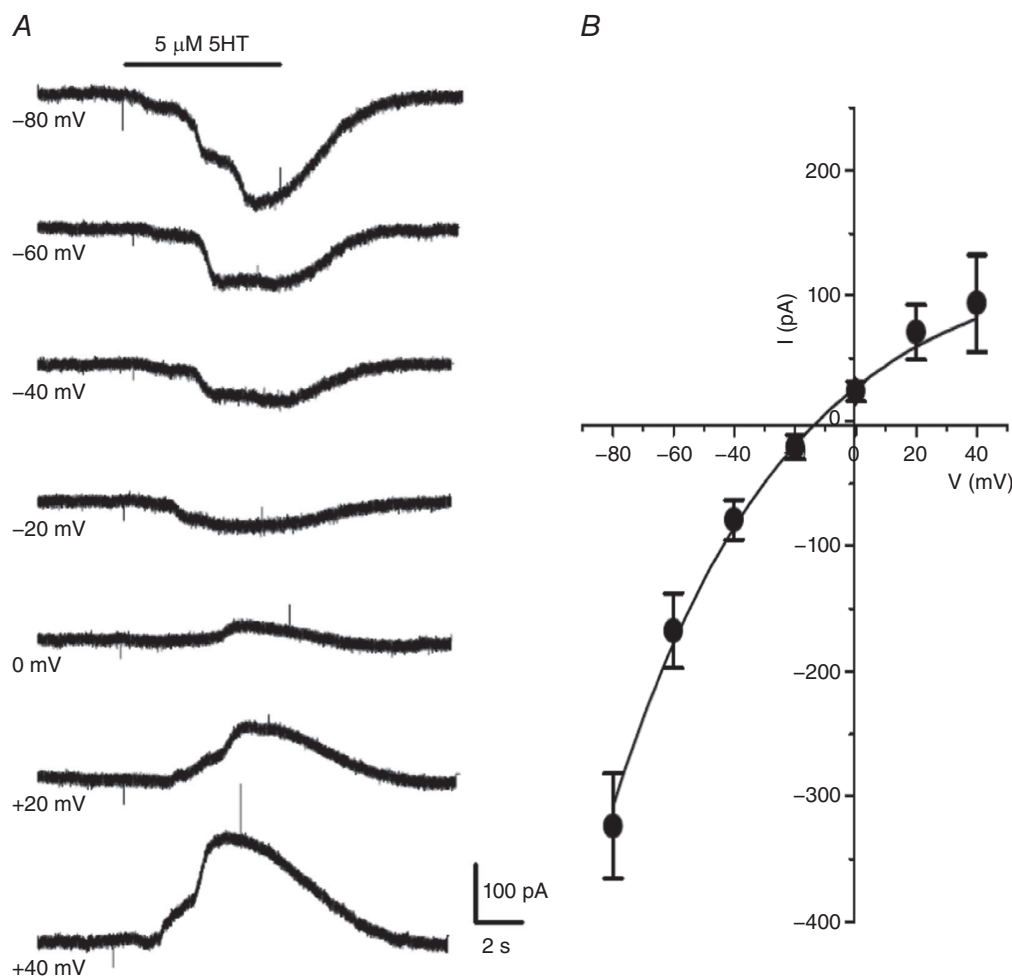


Figure 6. Reversal potential of the 5-HT-activated current in type II cells

A, the current evoked by 5 μM 5-HT ($I_{5\text{-HT}}$) was inward at more negative potentials and outward at more positive potentials; note that $I_{5\text{-HT}}$ sometimes showed multiple peaks (e.g. at -80 and $+40$ mV), which may reflect different conductance states. The reversal potential of $I_{5\text{-HT}}$ for a group of five cells was approximately -11 mV (B), which is consistent with activation of non-selective ion channels.

(Thomas *et al.* 2006), they are inconclusive because, in parallel experiments, we found that 10 μM SB 699551 caused > 90% blockade of the inward current when it was activated by ATP. In the latter experiments, the mean peak current evoked by 20 μM ATP was 716 ± 78 pA before, 43 ± 41 pA during ($P < 0.001$) and 634 ± 83 pA ($P > 0.01$) after perfusion with 10 μM SB 699551, corresponding to ~95% inhibition ($n = 8$). These data suggest that SB 699551 may cause non-specific blockade of the Panx-1-like channels and, together with the Ca^{2+} imaging data (see above), do not support a significant

contribution of 5-HT_{5A} receptors in the type II cell response.

Role of intracellular Ca^{2+} in the activation of the inward current by 5-HT in type II cells

We previously showed that, in type II cells, a rise in cytosolic Ca^{2+} was necessary for the activation of Panx-1 current by ATP and angiotensin II (Murali *et al.* 2014). To determine whether this was also the case for 5-HT, we used the membrane-permeable Ca^{2+} chelator,

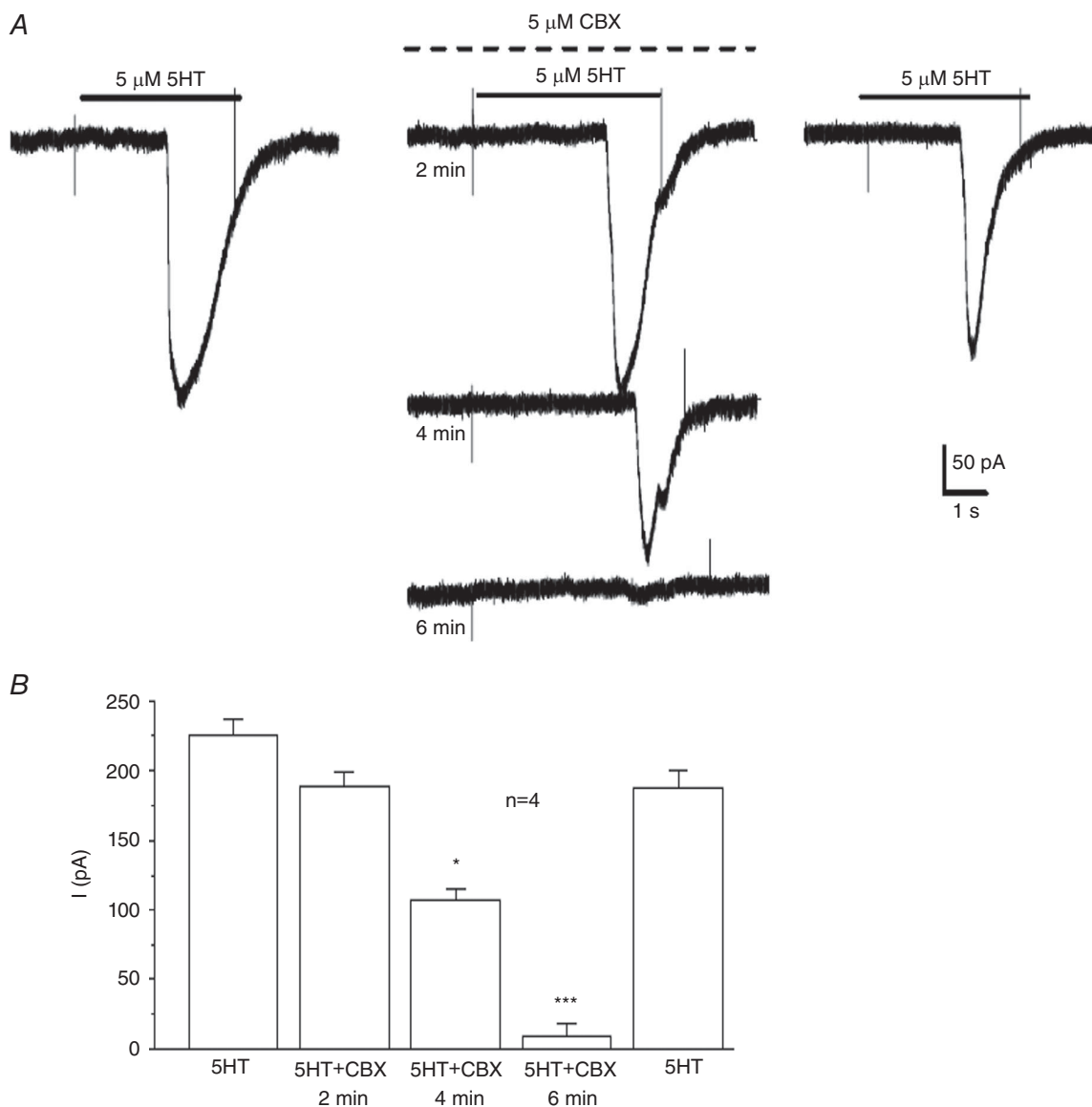


Figure 7. Inhibition of the 5-HT-induced inward current in type II cells by the Panx-1 channel blocker carbenoxolone

A, the 5-HT-induced inward current was reversibly inhibited by 5 μM carbenoxolone (CBX), a Panx-1 channel blocker (upper dotted line above middle traces); note the block developed gradually over a period of ~6 min (middle traces). *B*, summary data for a group of four cells exposed to 5-HT before, during and after CBX; * $P < 0.05$; *** $P < 0.001$.

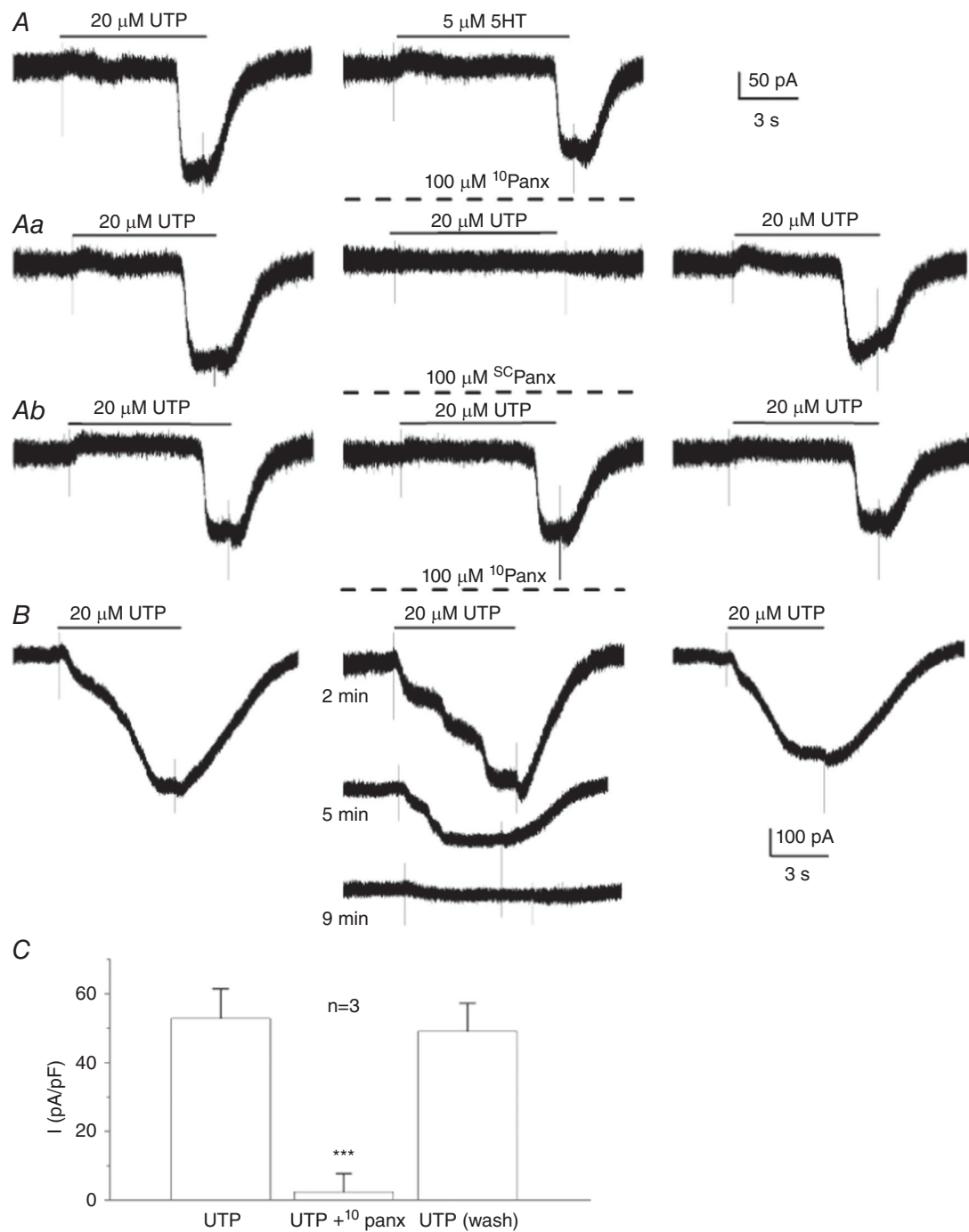


Figure 8. Effects of the specific mimetic peptide inhibitor of Panx-1 channels (¹⁰Panx peptide) and of scrambled control peptide (^{sC}Panx) on the inward current in type II cells

A, showing the similarity of the inward current activated by UTP and 5-HT in the same cell; holding potential -60 mV. The UTP-activated inward current was reversibly inhibited by ¹⁰Panx peptide ($100 \mu\text{M}$) in (*Aa*) but not by $100 \mu\text{M}$ ^{sC}Panx peptide in (*Ab*); all recordings from in (*A*), (*Aa*) and (*Ab*) were from same cell. The time course of current blockade by ¹⁰Panx peptide is shown in (*B*) for a different cell. *C*, showing UTP-activated inward current (plotted as current density pA/pF) for three cells before, during and after (wash) treatment with $100 \mu\text{M}$ ¹⁰Panx peptide; *** $P < 0.001$.

BAPTA-AM ($5 \mu\text{M}$). As shown in Fig. 10, the inward current evoked by $5 \mu\text{M}$ 5-HT was almost completely inhibited in the presence of BAPTA-AM and the effect was reversible ($\sim 95\%$ inhibition; $P < 0.001$). This blockade by BAPTA-AM typically occurred within 4–5 min of exposure and recovery of the initial response was complete some 10–14 min after washout of the drug. These data suggest that a rise in intracellular Ca^{2+} is necessary for activation of the inward current by 5-HT.

Discussion

In the present study, we demonstrate for the first time that glial-like sustentacular or type II cells of the juvenile rat CB express functional receptors for 5-HT, an endogenous neuromodulator known to be synthesized and released by neighbouring chemoreceptor (type I) cells. This finding adds further support to the proposal that several paracrine

signalling pathways help shape the integrated sensory response of the CB during chemotransduction (Nurse, 2010; Kumar & Prabhakar, 2012; Tse *et al.* 2012; Nurse, 2014). Using ratiometric fura-2 imaging, we found that 5-HT evoked a marked elevation in intracellular Ca^{2+} ($\Delta[\text{Ca}^{2+}]_i$) in $\sim 67\%$ of type II cells and the source of this Ca^{2+} was mainly intracellular. By comparison, a smaller proportion ($\sim 40\%$) of type I cells responded to 5-HT with an elevation of intracellular Ca^{2+} and their peak responses were generally smaller, although longer lasting, than those seen in type II cells. In voltage clamp studies, we found a subpopulation of isolated type II cells in which 5-HT activated a delayed, inward current ($I_{5\text{-HT}}$) at holding voltages near the resting potential. This current had a reversal potential of approximately -11 mV and was reversibly inhibited by the Panx-1 channel blocker, carbenoxolone ($5 \mu\text{M}$), and was also indistinguishable from the current that was activated during ATP/UTP

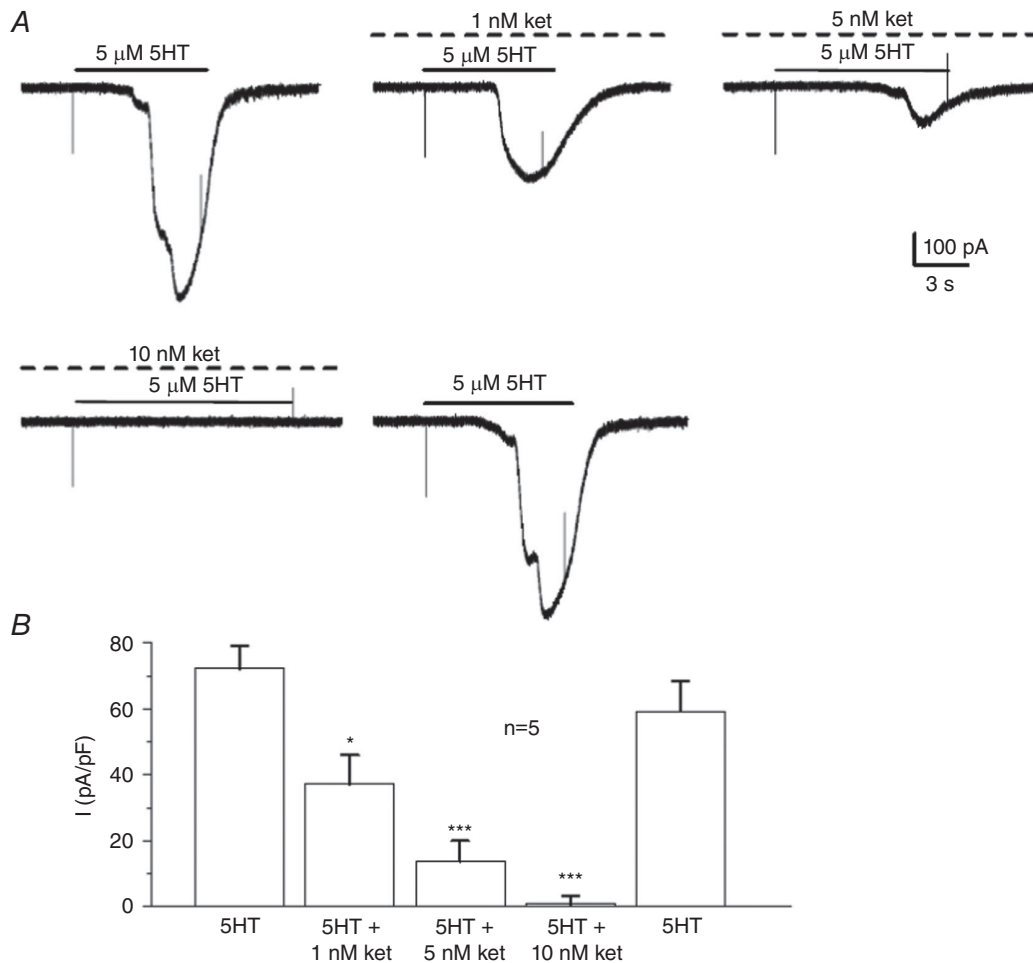


Figure 9. Sensitivity of the 5-HT-induced inward current in type II cells to ketanserin

A (upper and lower traces), increasing doses of ketanserin (ket), a selective blocker of 5HT_{2A} receptors, over the dose range 1, 5 and 10 nM, caused a progressive inhibition of the 5-HT-induced inward current in type II cells; note complete blockade was apparent at 10 nM ket and that the 5-HT-induced response recovered completely after washout of ket. B, summary data for a group of five cells; * $P < 0.05$; *** $P < 0.001$.

and angiotensin II stimulation of type II cells in previous studies (Zhang *et al.* 2012; Murali *et al.* 2014). Consistent with a role for intracellular Ca^{2+} , application of the Ca^{2+} chelator BAPTA-AM ($5 \mu\text{M}$) prevented activation of the inward current by 5-HT, which is a property similar to that previously reported for ATP and angiotensin II (Murali *et al.* 2014). The effect of BAPTA-AM was probably not the result of non-specific external blockade of the underlying channels because intracellular application of BAPTA via the patch pipette during whole-cell recording also caused inhibition of the ATP-activated inward current (M. Zhang and C. A. Nurse, unpublished observations). Both the 5-HT-evoked $\Delta[\text{Ca}^{2+}]_i$ and inward current were sensitive to low doses of ketanserin, suggesting involvement of the 5-HT₂ receptor. More compelling evidence for the role of Panx-1 channels as the carriers of the inward current was obtained in experiments using UTP. Although the UTP-activated current (I_{UTP}) in type II cells was previously shown to be sensitive to low doses of carbenoxolone ($5 \mu\text{M}$) (Zhang *et al.* 2012), the ability of this drug to distinguish Panx-1 channels from connexin hemichannels is increasingly being challenged (Lohman & Isakson, 2014). In the present study, we found that I_{UTP} was also reversibly abolished by the specific mimetic peptide inhibitor of Panx-1 channels, ¹⁰Panx peptide (Thompson *et al.* 2008; Lohman & Isakson, 2014), whereas its scrambled control peptide ^{sc}Panx was ineffective. These data, together with the previous immunohistochemical evidence that Panx-1 is expressed in rat type II cells, provide strong support for the proposal that Panx-1 channels are the mediators of the inward current and

the conduits for ATP release from these cells, as reported previously (Zhang *et al.* 2012).

Evidence for a role of 5-HT_{2A} receptors in the type II cell response

Expression of mRNA for multiple G-protein coupled 5-HT receptor subtypes has been detected in the rat CB, although most functional studies have implicated a role for 5-HT_{2A}R, localized primarily to type I cells (Wang *et al.* 2000; Zhang *et al.* 2003; Jacono *et al.* 2005; Peng *et al.* 2006; Liu *et al.* 2011; Yokoyama *et al.* 2015). In the present study, the 5-HT_{2A}R subtype also appeared to be a major contributor to both the 5-HT-induced Ca^{2+} rise and the 5-HT-activated inward current in type II cells. Accordingly, both responses were sensitive to low concentrations of the 5-HT_{2A}R blocker ketanserin and had a submicromolar affinity for 5-HT ($\text{EC}_{50} \sim 200 \text{ nM}$), consistent with that for 5-HT_{2A}R (Muraoka *et al.* 1998; Okoro, 1999). Minor contributions from other 5-HT receptors known to be expressed in the rat CB could not be ruled out (Yokoyama *et al.* 2015) because residual intracellular Ca^{2+} responses to 5-HT persisted in the presence of ketanserin in some experiments. However, pharmacological tests did not support a significant contribution from 5-HT_{5A}R, which display submicromolar affinity for 5-HT (Noda *et al.* 2003; Goodfellow *et al.* 2012), can also signal via IP₃-sensitive Ca^{2+} stores (Noda *et al.* 2003) and have been localized to type I cell clusters in a previous study (Wang *et al.* 2000). In particular, the selective 5-HT_{5A}R blocker SB 699551

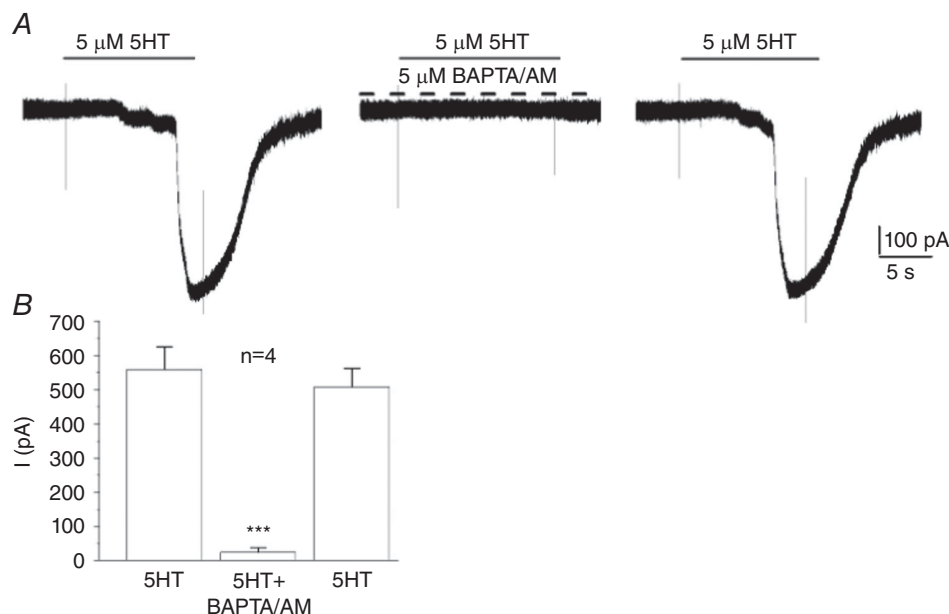


Figure 10. Effects of the Ca^{2+} chelator BAPTA-AM on the 5-HT-induced inward current in type II cells. A, chelating intracellular Ca^{2+} with BAPTA-AM ($5 \mu\text{M}$) reversibly abolished the 5-HT-induced inward current in a type II cell; summary data for a group of four cells are shown in (B) (***) $P < 0.001$.

(10 μM) (Thomas *et al.* 2006; Goodfellow *et al.* 2012) failed to affect the 5-HT-induced intracellular Ca^{2+} responses in type II cells. We attribute the apparent blockade of the 5-HT-induced inward current by SB 699551 to a non-specific blockade of the channels because similar blockade occurred when ATP was used as the ligand. Additional studies are required to determine whether other 5-HT receptors in addition to 5-HT_{2A}R are present on type II cells. Previous immunofluorescence studies demonstrated the expression of 5-HT_{2A}R in association with type I cell clusters in the rat CB *in situ* (Zhang *et al.* 2003; Liu *et al.* 2011) and, although the evidence was consistent with type I cell staining, the procedures used could not unambiguously exclude type II cell staining as well. Given that rat type I cells *in situ* have been shown to express not only the 5-HT biosynthetic enzyme tryptophan hydroxylase and the plasma membrane 5-HT transporter (Yokoyama *et al.* 2013), but also endogenous levels of 5-HT (Liu *et al.* 2011), the present study adds further support for a paracrine role of 5-HT possibly also involving 5-HT_{2A} receptors on type II cells.

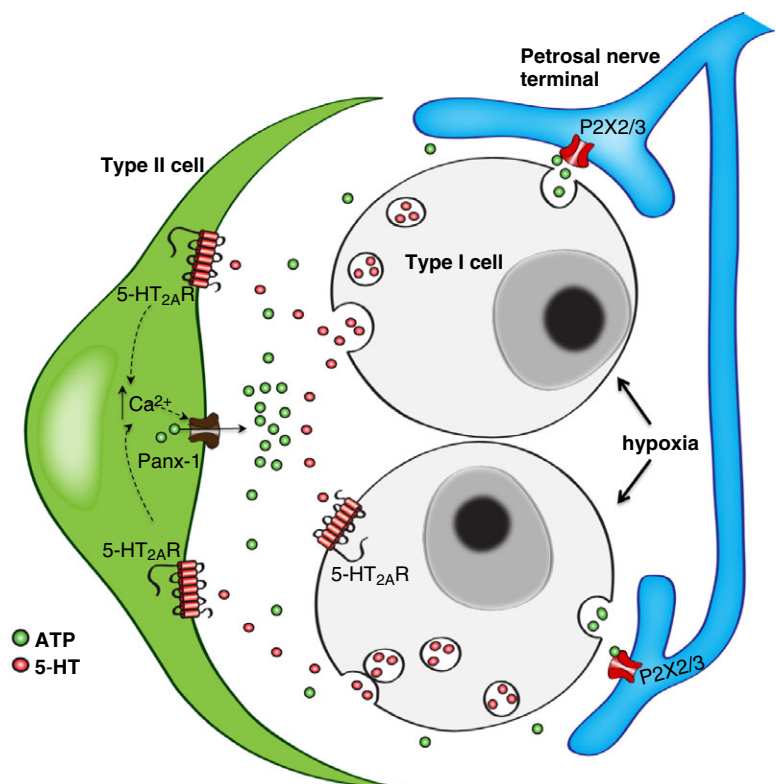
Contribution of 5-HT signalling to carotid body physiology

The present study adds to the complexity of 5-HT signalling in the CB by proposing an additional paracrine signalling pathway via glial-like type II cells. Although

there is strong evidence that chemoreceptor type I cells possess the machinery for synthesis, storage and release of 5-HT (Zhang & Nurse, 2000; Peng *et al.* 2009; Liu *et al.* 2011; Yokoyama *et al.* 2013), the role of 5-HT during CB chemotransduction is still unclear. For example, in one study using cultured CB cells from juvenile rats, exogenous 5-HT caused a protein kinase C-dependent depolarization in a subpopulation (~40%) of type I cells via ketanserin-sensitive 5-HT_{2A}R and, moreover, the hypoxia-induced depolarization in type I cells was partially inhibited by ketanserin (Zhang *et al.* 2003). This result is consistent with a positive feedback role for 5-HT acting via 5-HT_{2A}R on type I cells. Whether or not acute hypoxia releases detectable 5-HT from CB cells under normal conditions may depend on the method of detection, the sensitivity of the assay, the type of preparation (e.g. whole organ *vs.* isolated cells) and animal age, or perhaps the level of oxidative stress present (Zhang *et al.* 2003; Jacono *et al.* 2005; Peng *et al.* 2009; Ramirez *et al.* 2012). In a more recent study, exogenous 5-HT failed to elicit intracellular Ca^{2+} responses in adult rat type I cells in normoxia, although it did enhance hypoxia-induced Ca^{2+} responses via ketanserin-sensitive 5-HT₂ receptors (Yokoyama *et al.* 2015). These data contrast with those of the present study on juvenile rats where a small subpopulation of type I cells did respond to exogenous 5-HT and may reflect differences in culture conditions, animal age or sampling procedures. In excised intact CB-nerve preparations from

Figure 11. Schematic representation of the proposed autocrine–paracrine role of 5-HT at the carotid body sensory synapse

Chemostimuli such as hypoxia cause depolarization of type I cells and the release of several neurotransmitters of which only ATP (green circles) and 5-HT (red circles) are shown for clarity. 5-HT may act on autocrine–paracrine 5-HT_{2A} receptors located on a subpopulation of receptor type I cells and on the majority of adjacent glial-like type II cells. Stimulation of 5-HT_{2A} receptors on type I cells may facilitate membrane depolarization and the release of ATP, an excitatory neurotransmitter that acts on P2X2/3 receptors on petrosal afferent terminals. Stimulation of 5-HT_{2A} receptors on type II cells may cause a rise in intracellular Ca^{2+} , leading to the opening of Panx-1 channels, which act as conduits for the further release of ATP. Enhanced 5-HT signalling via type II cells, leading to ATP release, may contribute, in part, to the increased sensory discharge during exposure to chronic intermittent hypoxia. [Colour figure can be viewed at wileyonlinelibrary.com]



adult rats, exogenous 5-HT had no effect on the onset or magnitude of the hypoxia-evoked sinus nerve discharge; however, it did prolong the hypoxic sensory response via ketanserin-sensitive 5-HT₂ receptors, prompting the suggestion that 5-HT modulated the dynamics of the sensory discharge (Jacono *et al.* 2005). It should be noted that the effects of exogenous 5-HT on sensory discharge are complicated by the fact that 5-HT can have both postsynaptic and presynaptic effects, and the latter can lead to the release of both excitatory and inhibitory neurotransmitters (Nurse, 2014). A clear role has been demonstrated for 5-HT in the long-term facilitation of CB sensory discharge in adult rats exposed to CIH (Peng *et al.* 2006; Peng *et al.* 2009). This facilitation is dependent on release of 5-HT coupled to the 5-HT₂R-protein kinase C signalling pathway, leading to NADPH oxidase activation and reactive oxygen species generation in type I cells (Peng *et al.* 2009). Whether or not paracrine 5-HT-mediated type II cell signalling, as proposed in the present study, contributes to CB sensory physiology in adult rats, and especially in conditions associated with CIH, remains to be determined. In summary, the ability of 5-HT to activate Panx-1 channels in type II cells provides a potential paracrine signalling pathway for enhancing CB excitation via release of the excitatory transmitter ATP (Zhang *et al.* 2012), combined with the generation of adenosine from the breakdown of extracellular ATP by ectonucleotidases (Conde *et al.* 2012; Nurse, 2014; Murali & Nurse, 2016). A proposed model of the potential autocrine–paracrine interactions involving 5-HT at the CB chemosensory synapse is summarized in Fig. 11.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

SM helped to prepare the cultures, carried out all the Ca^{2+} imaging experiments and data analysis, and wrote the first draft of the manuscript. MZ performed all of the electrophysiological experiments, analysed the data, and helped prepare the figures. CAN was involved in planning and designing of all the experiments, helped to interpret the data, and revised the manuscript. All authors approved the final version of the manuscript submitted for publication. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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