

## ORIGINAL ARTICLE

# Transient receptor potential channels mediate the tussive response to prostaglandin E<sub>2</sub> and bradykinin

Megan Grace,<sup>1,2</sup> Mark A Birrell,<sup>1,2</sup> Eric Dubuis,<sup>1</sup> Sarah A Maher,<sup>1,2</sup> Maria G Belvisi<sup>1,2,3</sup>

► Additional materials are published online only. To view these files please visit the journal online (<http://dx.doi.org/10.1136/thoraxjnl-2011-201443>).

<sup>1</sup>Respiratory Pharmacology Group, Pharmacology and Toxicology Section, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, UK

<sup>2</sup>Centre for Integrative Physiology and Pharmacology, Imperial College London, London, UK

<sup>3</sup>Respiratory Research Group, University of Manchester, Wythenshawe Hospital, Manchester, UK

## Correspondence to

Professor Mara Belvisi, Imperial College London, Exhibition Road, London SW7 2AZ, UK; [m.belvisi@imperial.ac.uk](mailto:m.belvisi@imperial.ac.uk)

Received 29 November 2011  
Accepted 9 May 2012

Published Online First  
12 June 2012

## ABSTRACT

**Background** Cough is the most frequent reason for consultation with a family doctor, or with a general or respiratory physician. Treatment options are limited and a recent meta-analysis concluded that over-the-counter remedies are ineffective and there is increasing concern about their use in children. Endogenous inflammatory mediators such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and bradykinin (BK), which are often elevated in respiratory disease states, are also known to cause cough by stimulating airway sensory nerves. However, how this occurs is not understood.

**Methods** We hypothesised that the transient receptor potential (TRP) channels, TRPA1 and TRPV1, may have a role as 'common effectors' of tussive responses to these agents. We have employed a range of in vitro imaging and isolated tissue assays in human, murine and guinea pig tissue and an in vivo cough model to support this hypothesis.

**Results** Using calcium imaging we demonstrated that PGE<sub>2</sub> and BK activated isolated guinea pig sensory ganglia and evoked depolarisation (activation) of vagal sensory nerves, which was inhibited by TRPA1 and TRPV1 blockers (JNJ17203212 and HC-030031). These data were confirmed in vagal sensory nerves from TRPA1 and TRPV1 gene deleted mice. TRPV1 and TRPA1 blockers partially inhibited the tussive response to PGE<sub>2</sub> and BK with a complete inhibition obtained in the presence of both antagonists together in a guinea pig conscious cough model.

**Conclusion** This study identifies TRPA1 and TRPV1 channels as key regulators of tussive responses elicited by endogenous and exogenous agents, making them the most promising targets currently identified in the development of anti-tussive drugs.

## INTRODUCTION

Cough is the most frequent reason for consultation with a family doctor,<sup>1</sup> or with a general or respiratory physician. Patients with chronic cough probably account for 10–38% of respiratory outpatient practice in the USA.<sup>2</sup> Chronic cough of various aetiologies is a common presentation to specialist respiratory clinics, and is reported as a troublesome symptom by 7% of the population.<sup>3</sup> Treatment options are limited. A recent meta-analysis concluded that over-the-counter (OTC) cough remedies are ineffective<sup>4</sup> and there is increasing concern about the use of OTC therapies in children. Despite its importance, our understanding of the mechanisms which provoke cough is poor.

## Key messages

### What is the key question?

► Endogenous mediators which are often elevated in respiratory disease states, such as PGE<sub>2</sub> and bradykinin, are also known to cause cough. However, how this occurs is not known.

### What is the bottom line?

► Here we have elucidated the signaling mechanisms involved in this tussive response and identified a role for TRPA1 and TRPV1 channels.

### Why read on?

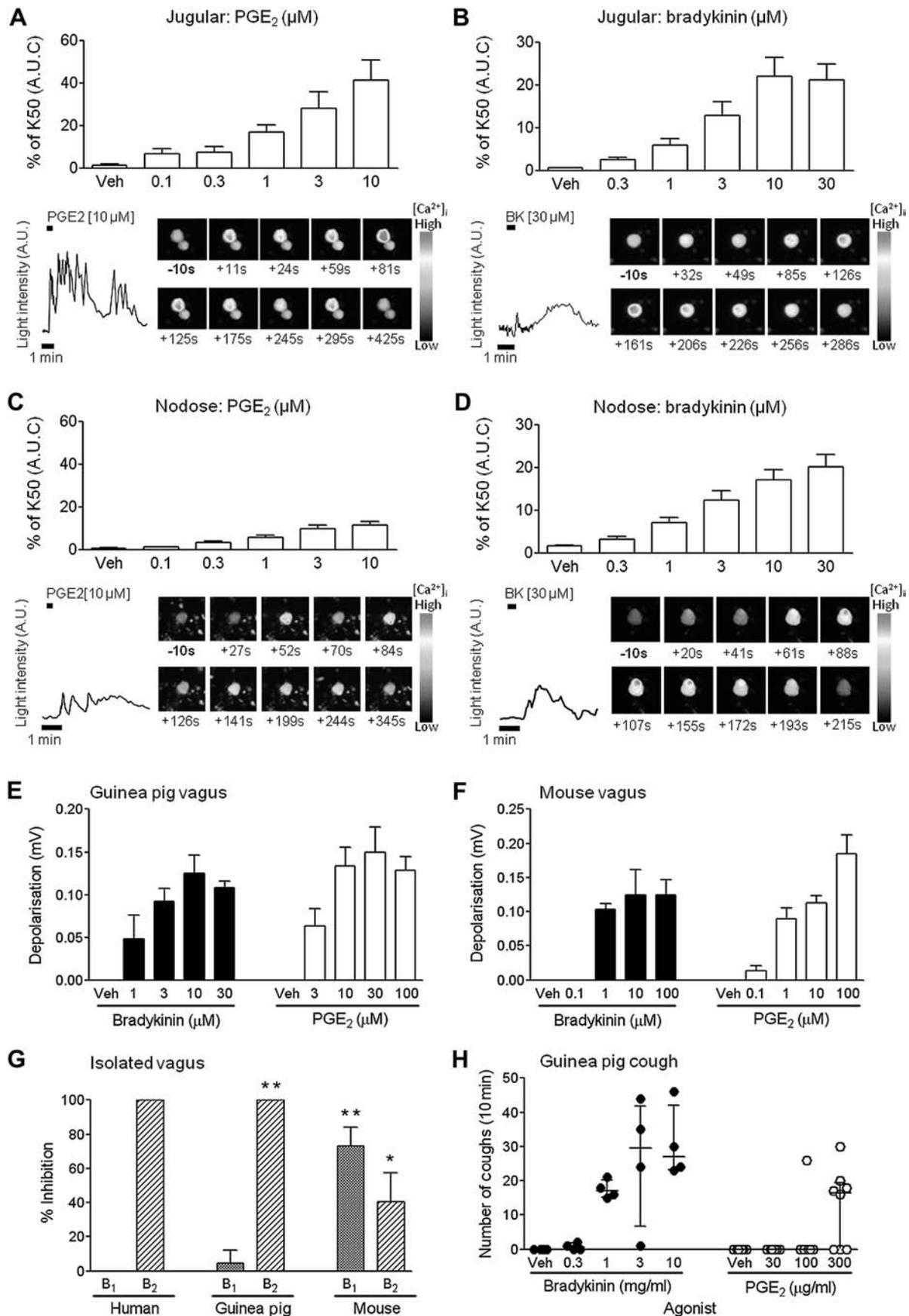
► These findings could have major implications for the treatment of cough which currently presents a significant unmet medical need.

The respiratory tract is innervated by sensory afferent nerves which are activated by mechanical and chemical stimuli.<sup>5</sup> Activation of capsaicin-sensitive C-fibres and acid-sensitive, capsaicin-insensitive mechanoreceptors innervating the larynx, trachea, and large bronchi regulate the cough reflex.<sup>5–6</sup> Endogenous inflammatory mediators are often elevated in respiratory disease states. For example, higher concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>7</sup> and bradykinin (BK)<sup>8</sup> have been found in the airways of patients with asthma and chronic obstructive pulmonary disease. PGE<sub>2</sub> and BK are also known to cause cough by stimulating airway sensory nerves.<sup>9–10</sup> Furthermore, increased PGE<sub>2</sub> levels have been found in idiopathic cough and cough associated with post-nasal drip, gastro-oesophageal reflux disease, cough variant asthma and eosinophilic bronchitis.<sup>11</sup> It has previously been demonstrated that PGE<sub>2</sub> activates guinea pig, mouse and human airway sensory nerves and causes cough via EP<sub>3</sub> receptor activation.<sup>10</sup> BK activates guinea pig airway sensory nerves and elicits cough via activation of the B<sub>2</sub> receptor, but it is not known if the same process occurs in other species.<sup>9</sup> Although we do have some information regarding which G-protein-coupled receptors (GPCRs) are activated by these endogenous tussive agents, it is still unclear what post-receptor signalling pathways are involved.

Recently, ion channels of the transient receptor potential (TRP) class such as TRPV1 have been implicated in the afferent sensory loop of the cough reflex<sup>12–13</sup> and in the heightened cough sensitivity seen in disease.<sup>14</sup> TRPA1 is a Ca<sup>2+</sup>-permeant



This paper is freely available online under the BMJ Journals unlocked scheme, see <http://thorax.bmj.com/site/about/unlocked.xhtml>



**Figure 1** Establishing concentration responses for prostaglandin (PGE<sub>2</sub>) and bradykinin (BK) in the in vitro preparations and in vivo cough model. (A–D) Concentration responses showing increases in intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) for PGE<sub>2</sub> and BK in primary neurons isolated from guinea pig jugular (A, B) and nodose (C, D) ganglia. In each panel, histograms show an increase in [Ca<sup>2+</sup>]<sub>i</sub> for increasing concentrations of tussive agent. To take into account multiphasic shapes of some responses and their lengths, the calcium flux (area under curve (AUC)) generated by applications of tussive

non-selective channel with 14 ankyrin repeats in its amino terminus which also belongs to the larger TRP family. TRPA1 channels are activated by a range of natural products such as allyl isothiocyanate, allicin and cannabidiol, found in mustard oil, garlic and cannabis<sup>15–17</sup> and by environmental irritants (eg, acrolein, present in air pollution, vehicle exhaust and cigarette smoke),<sup>18–20</sup> and is primarily expressed in small diameter, nociceptive neurons where its activation contributes to the perception of noxious stimuli such as itch.<sup>18 20 21</sup> It has been demonstrated that stimulating TRPA1 channels activates vagal broncho-pulmonary C-fibres in rodent lung,<sup>22–24</sup> inducing a late asthmatic response in sensitised rodents following allergen challenge<sup>25</sup> and causing cough in guinea pig models and in normal human volunteers.<sup>26</sup> Although many exogenous stimuli are known to activate TRPA1 and TRPV1, it is still unknown how cough and other reflexes are elicited in health and disease by endogenous agents, and whether these ion channels are involved. We hypothesised that the TRPA1 and TRPV1 ion channels may have a role as common effectors for such tussive agents.

## METHODS

### Isolated vagal ganglia

Intracellular free calcium ( $[Ca^{2+}]_i$ ) measurements were performed in dissociated jugular and nodose neurons. These studies were performed on all isolated vagal neurons (not airway specific), with the concentration–response data representing an overview of responding and non-responding cells. For subsequent antagonist studies only responding cells were analysed, with the criteria for a ‘responsive cell’ judged as an increase in  $[Ca^{2+}]_i$  of  $\geq 10\%$  of the  $K_{50}$  response. In each case,  $N$  = number of animals and  $n$  = number of cells tested. Comprehensive methods are detailed in online supplementary text.

### Isolated vagus nerve preparation

Guinea pigs or mice (*C57BL/6*, *Trpa1*<sup>-/-</sup> and *Trpv1*<sup>-/-</sup>) were sacrificed by injection of sodium pentobarbitone (200 mg/kg intraperitoneal injection). The vagus nerves were removed and experiments conducted in our fully characterised isolated vagus preparation, as described in previous publications.<sup>10 26</sup> Human vagal tissue ( $n=6$ , two men, 27–72-year-old donors with no respiratory disease) was obtained from two sources—transplant tissue surplus to requirements (Harefield Hospital, UK); and purchased from the International Institute for the Advancement of Medicine (Edison, New Jersey, USA). In all cases, the tissue was consented for use in scientific research and ethics approval obtained from the Royal Brompton & Harfield Trust. See online supplementary text for full methods.

## Conscious guinea pig cough model

Conscious unrestrained guinea pigs were placed in individual plastic transparent whole-body plethysmograph chambers (Buxco, Wilmington, North Carolina, USA) and cough detected as previously described.<sup>10 26</sup>

## Data analysis and statistics

For imaging,  $R_M$  is the maximum response observed expressed as a percentage of the  $K_{50}$  response.  $EC_{50}$  values quoted in the imaging studies are the concentrations of drug that produced 50% of the maximum response obtained. Inhibition of agonist responses in the isolated vagus nerve preparation was analysed by two-tailed paired *t* test, comparing responses to the agonist in the absence and presence of an antagonist in the same piece of nerve.

Inhibition of cough by TRPA1 and TRPV1 antagonists *in vivo* was analysed by Kruskal–Wallis test for multiple comparisons with Dunn’s post hoc test, comparing responses from each group of antagonist/vehicle combination to the vehicle-only control. Data are presented as median  $\pm$  IQR, with statistical significance set at  $p < 0.05$ .

## RESULTS

### Characterising agonist responses in isolated vagal ganglia

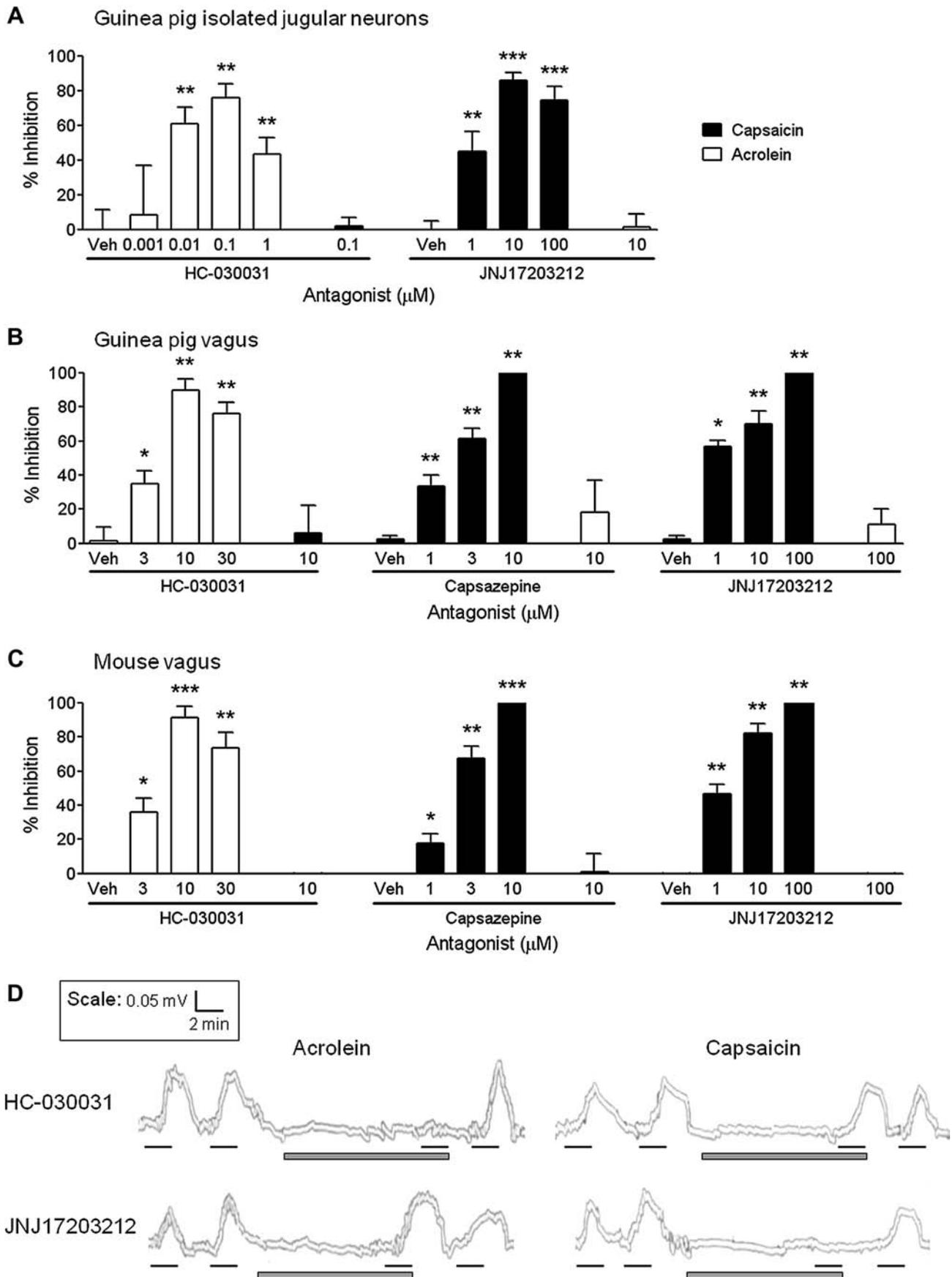
Capsaicin and acrolein produced concentration-related increases in  $[Ca^{2+}]_i$  in sensory neurons (supplementary figure 1A–D).  $PGE_2$  stimulation was multiphasic in both ganglia, of which 56.3% of jugular and 40% of nodose neurons responded. Overall,  $PGE_2$  increased  $[Ca^{2+}]_i$  in jugular neurons with an  $R_M$  of  $41 \pm 9\%$  at 10  $\mu M$  and an  $EC_{50}$  of  $5.07 \pm 1.0 \mu M$  ( $N=5$ ,  $n=16$ ); whereas, in nodose neurons  $R_M$  was only  $11 \pm 2\%$  at 10  $\mu M$  with an  $EC_{50}$  of  $3.11 \pm 0.4 \mu M$  ( $N=4$ ,  $n=15$ ). 52.9% of jugular neurons and 37.5% of nodose neurons responded to BK stimulation. Overall, BK induced  $22 \pm 4\%$   $R_M$  at 10  $\mu M$ , with an  $EC_{50}$  of  $2.32 \pm 0.36 \mu M$  in jugular neurons ( $N=5$ ,  $n=17$ ); and  $17 \pm 3\%$   $R_M$  at 30  $\mu M$  with an  $EC_{50}$  of  $2.2 \pm 0.2 \mu M$  in nodose neurons ( $N=5$ ,  $n=24$ ) (figure 1A–D).

### Characterising agonist responses *in vitro* and *in vivo*

Capsaicin and acrolein produced concentration-related increases in depolarisation of guinea pig, mouse and human vagus nerve (online supplementary figure 1E–G). BK and  $PGE_2$  concentration dependently activated both guinea pig and mouse isolated vagus nerves, whereas the corresponding vehicles did not induce depolarisation (figure 1E,F). BK (3  $\mu M$ ) and  $PGE_2$  (10  $\mu M$ ) also activated human afferent sensory nerves ( $n=5–6$ , data not shown). The GPCR mediating the tussive effects of  $PGE_2$  has already been established as the  $EP_3$  receptor.<sup>10</sup> Here, we show that BK activates only the  $B_2$  receptor in human and guinea pig, but  $B_1$  and  $B_2$  receptors in

[Continued]

agents is normalised, and expressed as percentage of response to  $K_{50}$ . The tussive agent used is indicated above each set of histograms and the concentration below each bar in  $\mu M$  ( $N=4–5$ ,  $n=15–24$ ). The trace in the lower left shows a typical recording of the light intensity over time following exposure to the agonist. Time and duration of drug application are indicated by a black bar above the trace. Time scale is given by the 1 min length-equivalent black bar shown below the trace. On the bottom right are display images taken during the recording. Time of the snapshot is indicated below each picture with zero being the start of tussive agent application. The pseudo colour code used for light intensity in the pictures is represented on the right of each set of images. (E, F) Perfusion for 2 min of BK (black bars) or  $PGE_2$  (white bars) activated (E) guinea pig and (F) mouse isolated vagus nerves in a concentration-dependent manner, measured as depolarisation of the nerve in mV ( $n=6$ ). (G) The G-protein coupled receptor mediating BK-induced depolarisation (3  $\mu M$  in guinea pig and 1  $\mu M$  in mouse tissue) was identified as the  $B_2$  receptor in human ( $n=1–2$ ) and guinea pig ( $n=6$ ), and a combination of  $B_1$  and  $B_2$  receptors in the mouse ( $n=6$ ) by incubating the nerve with either  $B_1$  (R715, 1  $\mu M$ ; checked bars) or  $B_2$  (WIN 64338, 10  $\mu M$ ; striped bars) selective antagonists for 10 min, measured as % inhibition of agonist responses. (E) BK (filled circles) and  $PGE_2$  (open circles) also induced concentration-related coughing in the conscious guinea pig, measured as the total number of coughs counted during 10 min of aerosol stimulation ( $n=4–8$ ). Data are expressed as mean  $\pm$  SEM of  $n$  observations (A–G) or median  $\pm$  IQR (H). Statistical significance is indicated by \* $p < 0.05$  and \*\* $p < 0.01$ , calculated as a paired *t*-test comparing responses in the same piece of nerve (human data were not analysed due to low numbers). Veh, vehicle. This figure is produced in colour in the online journal—please visit the website to view the colour figure.



**Figure 2** Characterisation of transient receptor potential channel A1 (TRPA1)-selective and TRPV1-selective antagonists in the in vitro primary ganglia and isolated vagus nerve preparations. The TRPA1 antagonist HC-030031 (HC) or TRPV1 antagonists JNJ17203212 (JNJ) or capsazepine (CAPZ) were assessed for their ability to inhibit capsaicin (black bars) and acrolein (white bars) responses in isolated guinea pig jugular neurons and guinea pig, mouse or human isolated vagus nerves. (A) HC concentration-dependently inhibited acrolein-induced (10 μM) increases in  $[Ca^{2+}]_i$  in guinea pig isolated jugular neurons, but

the mouse isolated vagus (figure 1G). It is possible that BK is inducing airway sensory afferent activation and cough via production of prostanoids.<sup>27, 28</sup> However, incubation of the vagus nerve with indomethacin did not alter BK-induced activation of either the guinea pig ( $20 \pm 11\%$  inhibition,  $p > 0.05$ ) or wild-type mouse sensory nerves ( $13 \pm 10\%$  inhibition;  $n = 6$ ,  $p > 0.05$ ; data not shown). The magnitude of BK-induced sensory nerve depolarisation was also similar in wild-type compared with  $EP_3^{-/-}$  mouse vagus ( $n = 6$ ,  $p > 0.05$ ; data not shown), which is the GPCR through which  $PGE_2$  causes cough. Depolarisations to BK,  $PGE_2$ , acrolein and capsaicin were abolished with the sodium channel blocker tetrodotoxin ( $n = 3$ ; 100% inhibition, data not shown). Further evidence for the observed depolarisation being mediated via sensory nerve activation comes from the in vivo experiments, demonstrating that BK and  $PGE_2$  successfully induce concentration-related coughing in conscious guinea pigs (figure 1H).

### Characterising antagonist responses in isolated vagal ganglia

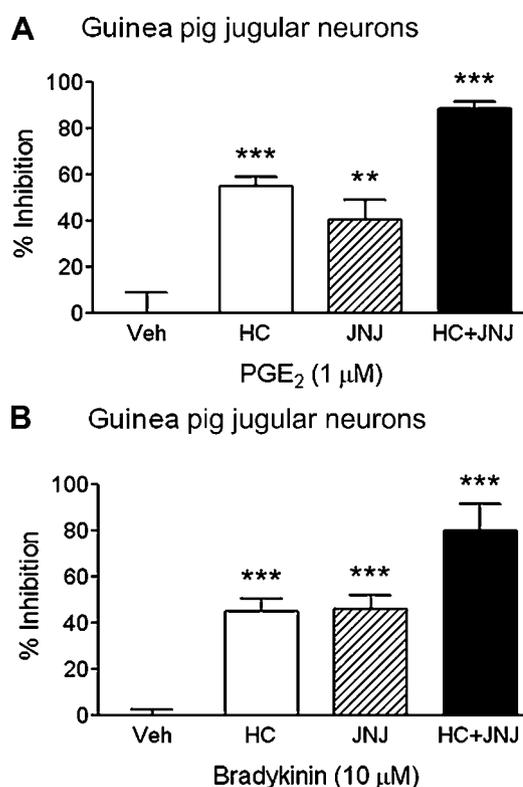
Concentration responses for the TRPV1-selective antagonist JNJ17203212 (JNJ) and TRPA1-selective antagonist HC-030031 (HC) were established in primary jugular cells for their ability to inhibit agonist-induced increases in  $[Ca^{2+}]_i$  (figure 2A). JNJ concentration-dependently inhibited increases in  $[Ca^{2+}]_i$  caused by the TRPV1-selective agonist capsaicin, with a maximal effect of  $86 \pm 2\%$  at  $10 \mu M$ . Alternatively, HC concentration-dependently inhibited increases in  $[Ca^{2+}]_i$  induced by the TRPA1-selective agonist acrolein, with a maximal effect of  $76 \pm 8\%$  at  $0.1 \mu M$ . At the concentration which caused maximal inhibition of its own receptor,  $10 \mu M$  JNJ did not inhibit acrolein, and  $0.1 \mu M$  HC did not inhibit capsaicin stimulation of jugular cells (figure 2A).

### Characterising TRP-selective antagonists in vitro

Depolarisation of guinea pig and mouse vagus nerve by acrolein was concentration-dependently inhibited with the TRPA1-selective antagonist HC. Similarly, capsaicin responses were concentration-dependently inhibited by the TRPV1-selective antagonists capsazepine (CAPZ) and JNJ (figure 2B,C). At the concentration which maximally inhibited acrolein, HC ( $10 \mu M$ ) did not inhibit capsaicin-induced nerve depolarisation; and equally CAPZ ( $10 \mu M$ ) and JNJ ( $100 \mu M$ ) did not inhibit acrolein-induced nerve depolarisation (figure 2B,C). This suggests that these compounds are not exhibiting off-target actions at these concentrations. Subsequently, the effects of HC ( $10 \mu M$ ) and JNJ ( $100 \mu M$ ) were investigated in human isolated vagus. In these experiments ( $n = 2-3$ ), acrolein responses were abolished by HC but not affected by JNJ; whereas, capsaicin responses were abolished by JNJ but not affected by HC (example traces shown in figure 2D). Vehicle control (0.1% dimethyl sulfoxide (DMSO) vol/vol) did not inhibit agonist responses (data not shown).

### Determining the role of TRPA1 and TRPV1 in $PGE_2$ and BK induced vagal ganglia and sensory nerve activation in vitro

Having characterised the available tools, and confirmed selectivity of the antagonists, the role of TRPA1 and TRPV1 in



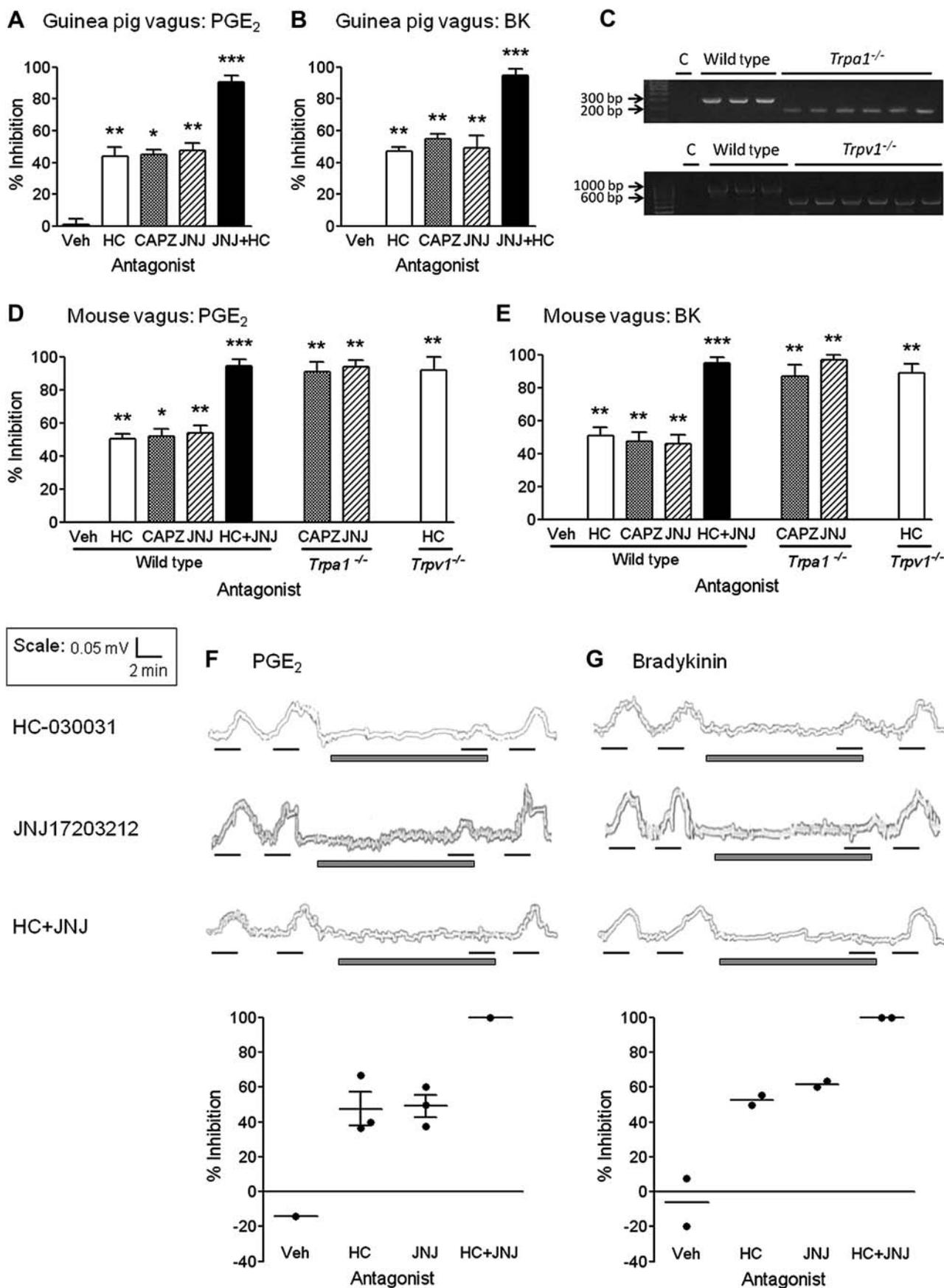
**Figure 3** Determining the role of transient receptor potential channel A1 (TRPA1) and TRPV1 in prostaglandin  $E_2$  ( $PGE_2$ ) and bradykinin (BK) induced isolated primary jugular neurons. The TRPA1 antagonist HC-030031 (HC,  $0.1 \mu M$ ; white bars); TRPV1 antagonist JNJ17203212 (JNJ,  $10 \mu M$ ; striped bars); and a combination of HC+JNJ (black bars) were assessed for their ability to inhibit (A)  $1 \mu M$   $PGE_2$  and (B)  $10 \mu M$  BK responses in isolated guinea pig jugular neurons. HC or JNJ partially inhibited  $PGE_2$  and BK responses, whereas HC+JNJ almost completely abolished increases in  $[Ca^{2+}]_i$ . Data are presented as mean  $\pm$  SEM of  $N = 3-5$ ,  $n = 10-19$  observations, calculated as % inhibition of agonist responses. \*\*( $p < 0.01$ ) and \*\*\*( $p < 0.0001$ ) indicate statistical significance, paired t-test comparing responses in the same neuron. Veh, vehicle for the antagonist (0.1% dimethyl sulfoxide).

$PGE_2$ -induced and BK-induced vagal ganglia and nerve stimulation was established. HC ( $0.1 \mu M$ ) or JNJ ( $10 \mu M$ ) partially inhibited  $PGE_2$ -induced ( $55 \pm 4\%$  and  $40 \pm 9\%$ , respectively) or BK-induced ( $45 \pm 5\%$  and  $46 \pm 7\%$ , respectively) increases in  $[Ca^{2+}]_i$  in guinea pig primary cells isolated from jugular vagal ganglia ( $p < 0.01$ ). Furthermore, when used in combination, HC and JNJ inhibited  $PGE_2$ -induced  $[Ca^{2+}]_i$  elevation by  $88 \pm 3\%$  and BK by  $80 \pm 12\%$  ( $p < 0.0001$ ). In contrast, vehicle incubation had no effect on  $[Ca^{2+}]_i$  ( $-1 \pm 11\%$  for  $PGE_2$  and  $-4 \pm 8\%$  for BK;  $p > 0.05$ ) (figure 3A,B).

TRPA1 antagonism with HC partially inhibited  $PGE_2$  and BK responses in the guinea pig isolated vagus nerve ( $44 \pm 5\%$  and  $47 \pm 3\%$ , respectively). Additionally, TRPV1 antagonism with CAPZ or JNJ also partially inhibited  $PGE_2$  ( $45 \pm 3\%$  and  $48 \pm 4\%$ ,

[Continued]

had no effect on capsaicin ( $1 \mu M$ ) at the concentration which maximally inhibited its own receptor ( $0.1 \mu M$ ). Similarly, JNJ concentration-dependently inhibited capsaicin-induced responses, but had no effect on acrolein at  $10 \mu M$  ( $N = 3-4$ ,  $n = 5-19$ ). (B, C) HC concentration-dependently inhibited acrolein-induced ( $300 \mu M$ ) depolarisation of the guinea pig and wild-type mouse isolated vagus nerves, but had no effect on capsaicin ( $1 \mu M$ ) stimulation. Conversely, TRPV1 antagonism with capsazepine or JNJ17203212 concentration-dependently inhibited capsaicin-induced depolarisation in guinea-pig and mouse isolated vagus nerves, but had no effect on acrolein stimulation at  $10 \mu M$  or  $100 \mu M$ , respectively ( $n = 6$ ). (D) Representative traces showing inhibition of human vagus nerve depolarisation with  $10 \mu M$  HC when stimulated with acrolein ( $300 \mu M$ ) but not capsaicin ( $1 \mu M$ ). Conversely,  $100 \mu M$  JNJ inhibited capsaicin but not acrolein responses ( $n = 2-3$ ). Black lines represent agonist incubation (2 min) and grey bars antagonist incubation (10 min). Data are presented as mean  $\pm$  SEM of  $n$  observations, calculated as % inhibition of agonist responses. \*( $p < 0.05$ ), \*\*( $p < 0.01$ ) and \*\*\*( $p < 0.0001$ ) indicate statistical significance, paired t-test comparing responses in the same piece of nerve. Veh, vehicle for the antagonist (0.1% dimethyl sulfoxide).



**Figure 4** Determining the role of transient receptor potential channel A1 (TRPA1) and TRPV1 in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and bradykinin (BK) induced sensory nerve activation. The TRPA1 antagonist HC-030031 (HC 10  $\mu$ M; white bars), TRPV1 antagonists capsazepine (CAPZ 10  $\mu$ M; grey bars) and JNJ17203212 (JNJ 100  $\mu$ M; striped bars), and a combination of HC+JNJ (black bars) were assessed for their ability to inhibit PGE<sub>2</sub> (10  $\mu$ M) and BK (3  $\mu$ M in guinea pig and human, and 1  $\mu$ M in mouse tissue) isolated vagus nerve responses. (A, B) HC, CAPZ or JNJ partially inhibited PGE<sub>2</sub> and BK

respectively) and BK responses ( $55\pm 3\%$  and  $49\pm 8\%$ , respectively) ( $p<0.05$ ). When used in combination, HC+JNJ abolished vagus nerve responses to PGE<sub>2</sub> ( $90\pm 4\%$ ) and BK ( $95\pm 4\%$ ) ( $p<0.0001$ ; figure 4A,B). To further confirm these results the experiments were repeated in a second species. The mouse was chosen because we have access to *Trpa1*<sup>-/-</sup> and *Trpv1*<sup>-/-</sup> mice, and have previously shown that mouse vagus responds in a similar fashion to human isolated vagus.<sup>10 26</sup> Knockdown of the TRPA1 or TRPV1 gene was confirmed by genotyping (figure 4C). Vagal nerve activation induced by acrolein and capsaicin were initially assessed to ensure phenotypical loss of TRPA1 and TRPV1 responses (data not shown). The results obtained in guinea pigs were then confirmed by comparing the magnitude of stimulation of the endogenous tussive agents in wild-type mice to that of *Trpa1*<sup>-/-</sup> and *Trpv1*<sup>-/-</sup> animals. The *Trpa1*<sup>-/-</sup> and *Trpv1*<sup>-/-</sup> responses to PGE<sub>2</sub> and BK stimulation were approximately half those seen in wild-type mouse vagal tissue ( $p<0.01$ ; data not shown).

In agreement with the results obtained from guinea pig vagus, wild-type mouse nerve responses to PGE<sub>2</sub> and BK were partially inhibited by HC ( $47\pm 2\%$  and  $51\pm 5\%$ , respectively), CAPZ ( $49\pm 4\%$  and  $48\pm 6\%$ , respectively) and JNJ ( $54\pm 5\%$  and  $46\pm 5\%$ , respectively) ( $p<0.05$ ). When used in combination, HC+JNJ abolished vagus nerve responses to PGE<sub>2</sub> ( $94\pm 4\%$ ) and BK ( $95\pm 3\%$ ) ( $p<0.0001$ ; figure 4D,E). And when tissue taken from genetically modified animals was tested in combination with the alternative antagonist, responses were again virtually abolished. In *Trpa1*<sup>-/-</sup> tissue PGE<sub>2</sub> was inhibited  $91\pm 6\%$  and  $94\pm 4\%$ ; and BK  $87\pm 7\%$  and  $97\pm 3\%$  by CAPZ and JNJ, respectively ( $p<0.01$ ; figure 4D,E). In *Trpv1*<sup>-/-</sup> tissue, PGE<sub>2</sub> was inhibited  $92\pm 8\%$  and BK  $89\pm 6\%$  by HC ( $p<0.01$ ; figure 4D,E).

We were able to confirm the above results in human vagal tissue ( $n=2-3$ ). In these experiments, both PGE<sub>2</sub> and BK were partially inhibited by either HC or JNJ antagonism with complete inhibition when the antagonists were used in combination. Vehicle control (0.1% DMSO vol/vol) did not inhibit agonist responses (figure 4E,G).

### Determining a role for TRPA1 and TRPV1 in PGE<sub>2</sub> and BK induced guinea pig cough

Concentration responses for the selective agonists capsaicin (TRPV1; figure 5A) and acrolein (TRPA1; figure 5B) were initially established to determine a submaximal concentration for which to test the antagonists (60  $\mu$ M capsaicin and 100 mM acrolein). Subsequently, the maximally effective doses of the selective antagonists HC (TRPA1) and JNJ (TRPV1) which did not display off-target effects on the alternative receptor were determined. HC dose-dependently inhibited acrolein-induced cough in vivo, but at 300 mg/kg had no effect on capsaicin; and JNJ concentration-dependently inhibited capsaicin-induced cough but at 100 mg/kg had no effect on acrolein in conscious guinea pigs (figure 5C,D).

The selective antagonists were then tested against PGE<sub>2</sub>-induced and BK-induced cough, appropriate concentrations of which had been determined earlier (300  $\mu$ g/ml PGE<sub>2</sub> and 3 mg/ml BK) (figure 1H). The in vivo guinea pig cough results agree with the in vitro findings. When pretreated with vehicle control, PGE<sub>2</sub> induced coughs in response to 10 min aerosol stimulation. This was reduced with either HC or JNJ pretreatment, respectively. Similarly, pretreatment with HC or JNJ antagonists reduced BK-induced coughing compared with vehicle control (figure 5E,F). When pretreated with a combination of HC+JNJ, the cough responses to PGE<sub>2</sub> and BK were completely abolished (figure 5E,F).

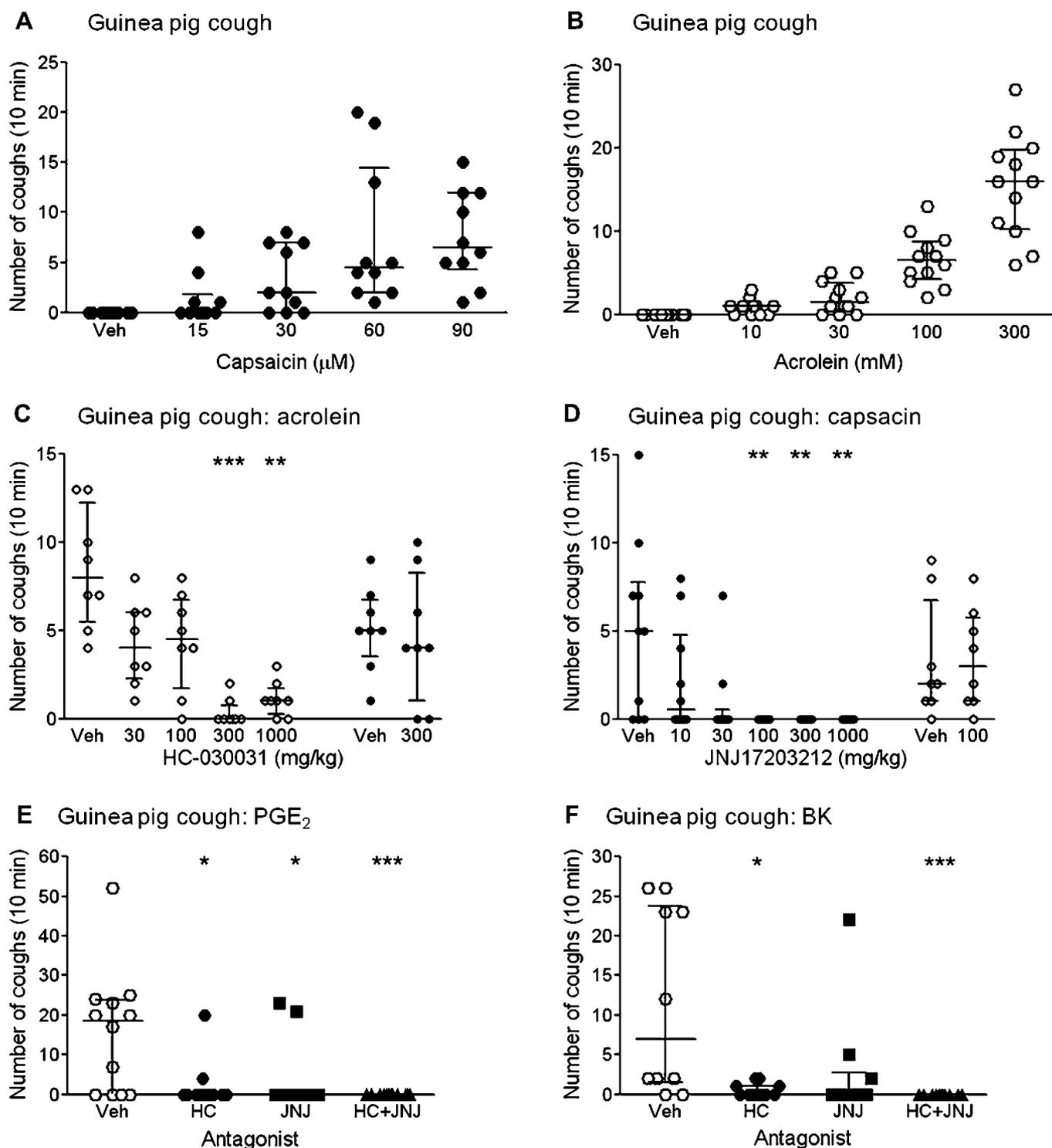
### DISCUSSION

Despite its importance, our understanding of the mechanisms which provoke cough and the endogenous tussive agents involved in health and disease is poor. Chronic cough is often associated with an underlying inflammatory condition, as in asthma and chronic obstructive pulmonary disease, but the endogenous mediators and signal transduction pathways which initiate cough are not known. Inflammatory diseases are associated with enhanced release of inflammatory mediators in the airways.<sup>7 8</sup> Two such mediators are PGE<sub>2</sub> and BK, which have been shown to induce coughing in humans<sup>29 30</sup> and animals.<sup>9 10</sup> Interestingly, cough associated with patients who take angiotensin-converting enzyme (ACE) inhibitors has also been suggested to be due to the increased levels of bradykinin.<sup>9</sup> Furthermore, PGE<sub>2</sub> levels have been found to be elevated in induced sputum of patients with chronic cough.<sup>11</sup> It has previously been demonstrated that PGE<sub>2</sub> activates guinea pig, mouse and human airway sensory nerves and evokes cough in guinea pigs via the EP<sub>3</sub> receptor.<sup>10</sup> However, BK stimulates guinea pig sensory nerves and elicits cough via activation of the B<sub>2</sub> receptor.<sup>9 31</sup> In these studies we demonstrate that BK and PGE<sub>2</sub> are able to activate sensory jugular ganglia; depolarise guinea pig, mouse and human vagal afferents; and evoke cough in a guinea pig model in a concentration-related fashion. Interestingly, the bradykinin B<sub>2</sub> receptor mediated sensory nerve activation in the isolated guinea pig and human vagal nerve assays but the B<sub>1</sub> receptor also played a role in the BK-induced activation of the mouse vagus, highlighting a species difference.

To induce coughing, post-receptor signalling pathways downstream of GPCR coupling are likely to cause the opening of membrane-bound ion channels leading to activation of airway sensory nerves and subsequent coughing. Previously other groups have presented data implicating the TRP family of ion channels in sensory nerve activation and the cough reflex elicited by BK.<sup>32</sup> In these studies we have confirmed these data and extended these findings by confirming a partial inhibition of BK-induced sensory nerve activation by TRPV1 antagonists. Similar results were obtained with PGE<sub>2</sub>, with the TRPV1 antagonists

#### [Continued]

responses in isolated guinea pig vagus tissue, whereas, HC+JNJ almost completely abolished nerve activation. (C) Knockdown of the TRPA1 or TRPV1 gene was verified by genotyping. Bands were expected at 317 bp for wild-type and 184 bp for *Trpa1*<sup>-/-</sup>; and 984 bp for wild-type and 600 bp for *Trpv1*<sup>-/-</sup> mice. C, water (negative control); bp, base pair. (D, E) HC, CAPZ or JNJ partially inhibited PGE<sub>2</sub> and BK responses in isolated wild-type mouse vagus tissue, whereas, HC+JNJ almost completely abolished nerve activation. In agreement with this, sensory nerves taken from genetically modified mice *Trpa1*<sup>-/-</sup> or *Trpv1*<sup>-/-</sup> tested in combination with the alternative TRPV1 or TRPA1 antagonist also largely eliminated sensory nerve responses to PGE<sub>2</sub> and BK. (F, G) HC and JNJ partially inhibited PGE<sub>2</sub> and BK responses in human isolated vagal tissue, whereas, HC+JNJ abolished nerve depolarisation. Example traces are shown above, where black lines represent agonist incubation (2 min), and grey bars represent antagonist incubation (10 min). Scatter plots of % inhibition are shown below and time and magnitude scales for the traces are shown in the top left hand corner. Data are presented as mean  $\pm$  SEM of  $n=6$  observations for guinea pig and mouse experiments, and  $n=2-3$  for human experiments, calculated as % inhibition of agonist responses. \* ( $p<0.05$ ), \*\* ( $p<0.01$ ) and \*\*\* ( $p<0.0001$ ) indicate statistical significance, paired t-test comparing responses in the same piece of nerve. Veh, vehicle for the antagonist (0.1% dimethyl sulfoxide).



**Figure 5** Determining the role of transient receptor potential channel A1 (TRPA1) and TRPV1 in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and bradykinin (BK)-induced cough in conscious guinea pigs. (A) Capsaicin and (B) acrolein concentration-dependently induced coughing in conscious, unrestrained guinea pigs. Tussive agents were aerosolised for 5 min, the number of coughs was counted during this time and for a further 5 min post stimulation (10 min total). Data are presented as mean  $\pm$  SEM of  $n=10-12$  observations. (C, D) Animals received intraperitoneal injections with a concentration of TRPA1 antagonist HC-030031 (HC), TRPV1 antagonist JNJ17203212 (JNJ) or vehicle (Veh) 1 h prior to 5 min aerosol stimulation with a tussive agonist. The number of coughs was counted during the 5 min stimulation plus a further 5 min (10 min total). (C) HC concentration-dependently inhibited acrolein-induced coughing (100 mM; open circles), but had no effect on capsaicin cough (60  $\mu\text{M}$ ; filled circles) at 300 mg/kg. (D) Conversely, JNJ concentration-dependently inhibited capsaicin-induced cough, with no effect on acrolein at 100 mg/kg. Data are presented as mean  $\pm$  SEM of  $n=8-10$  observations. (E, F) Animals received intraperitoneal injection with HC (300 mg/kg; filled circles), JNJ (100 mg/kg; filled squares), a combination of both antagonists (HC+JNJ; filled triangles), or appropriate Veh (open circles) 1 h prior to stimulation with a tussive agonist. (E) PGE<sub>2</sub> (300  $\mu\text{g}/\text{ml}$ ) or (F) BK (3 mg/ml) were aerosolised for 10 min, during which time the number of coughs was counted. Compared with vehicle control, pretreatment with either HC or JNJ significantly inhibited PGE<sub>2</sub>-induced or BK-induced coughing; and pre-treatment with HC+JNJ abolished cough altogether. Data are presented as median  $\pm$  IQR of  $n=10-12$  observations. \* ( $p<0.05$ ), \*\* ( $p<0.01$ ) and \*\*\* ( $p<0.0001$ ) indicate statistical significance, Kruskal–Wallis one-way analysis of variance with Dunn’s multiple comparison post-test.

producing partial inhibition of vagal sensory nerve activation in all species, guinea pig jugular neurons, and in a guinea pig cough model. At concentrations which were selective for inhibition of TRPA1 ligands (and not TRPV1 ligands) we also found that the TRPA1 antagonist, HC-030031 inhibited BK-induced and PGE<sub>2</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> in jugular neurons, vagal sensory nerve activation and cough. Furthermore, both antagonists together (JNJ17203212 and HC-030031) completely inhibited PGE<sub>2</sub> and BK in vitro and in vivo responses. In vitro pharmacological sensory nerve studies were confirmed in tissue from *Trpv1*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> gene deleted mice.

The mechanisms downstream of GPCR coupling that lead to either sensitisation or activation of ion channels are not yet fully understood, but phospholipase C (PLC) and protein kinase A (PKA) pathways are thought to be important in the signalling for a number of TRP channels.<sup>32–34</sup> GPCR binding to G<sub>q</sub>-coupled receptors can lead to activation of PLC, hydrolysis of phosphatidylinositol-(4,5)-biphosphate (PIP<sub>2</sub>) to yield inositol-(1,4,5)-triphosphate (IP<sub>3</sub>), production of diacylglycerol (DAG) and activation of phosphokinase C (PKC). PKC and DAG have been found to directly bind the TRPV1 receptor; and IP<sub>3</sub>-induced release of intracellular calcium stores may be involved in activation of TRPA1. Moreover, PIP<sub>2</sub> is thought to constitutively inhibit TRP receptors. Therefore, its hydrolysis by PLC may disinhibit these ion channels, sensitising them to subsequent stimulation.<sup>34</sup> Alternatively, PKA-dependent phosphorylation can occur through activation of G<sub>s</sub>-coupled receptors, thereby enhancing ion channel excitability.<sup>34</sup>

A number of the functional responses elicited by BK are caused via indirect effects, including the release of other endogenous mediators downstream of arachadonic acid. We established here that the stimulatory effects of BK on isolated vagus nerves were not due to subsequent release of prostanoids by using the general cyclo-oxygenase inhibitor indomethacin. However, a number of studies have also implicated downstream release of lipoxigenase products. For example, BK evokes the release of 15-HETE from airway epithelial cells.<sup>27</sup> Furthermore, 12-lipoxygenase and 5-lipoxygenase products have been implicated in BK-induced stimulation of airway afferent nerve terminals via TRPV1 channel activation.<sup>35</sup> It is therefore plausible that BK may be causing cough via the release of lipoxigenase products downstream of arachadonic acid.

In this paper, we have used in vitro cellular and tissue preparations, and an in vivo animal model to investigate the cough reflex. The data generated with these models are useful in attempting to understand cough; however, each model has its limitations. In the calcium-imaging preparation, we cannot determine if there are phenotypical changes induced in the primary ganglia cells during the isolation process. One of the benefits of the isolated vagus nerve preparation is that we can parallel our animal experiments in human tissue. However, the agents being tested are applied to the axon of the vagus nerve (not the nerve endings), meaning that the extracellular depolarisation signal recorded represents a summation of the change in membrane potential of all the nerve fibres being carried by the vagus. In addition, receptor expression and signal transduction mechanisms may differ from those at the peripheral endings. Finally, though the conscious guinea pig cough model is generally considered to be a valid tool for studying the cough reflex, there are a number of reported differences between the actions of certain drugs in guinea pig and man. These differences could be due to strong tachykinin-driven responses via sensory nerves in the guinea pig airways or because potential anti-tussives have been trialled in clinical studies in which cough was not the

primary endpoint and where there was no objective cough monitoring and so any efficacy may have been hard to capture. Furthermore, in guinea pig studies compounds are often not dose limited as they are in the clinic due to safety concerns.

The findings presented here are important for our understanding of the cough reflex (and in particular inflammatory and ACE inhibitor induced cough) and strongly support a role for TRPA1 and TRPV1 as common effectors of the tussive response to endogenous tussive agents. These studies were conducted in tissues and in vivo models under 'normal' physiological conditions and so a role for these TRP channels has not been established under pathophysiological conditions. However, it has been shown that patients suffering from chronic cough exhibit an increased TRPV1 expression within the lungs, which was correlated with an increase in cough sensitivity to capsaicin challenge,<sup>14</sup> indicating that TRP channels could be common effectors of tussive responses in disease and that these channels could be associated with long-term potentiation of the cough reflex. Studies have not yet been conducted to show if TRPA1 is overexpressed in pathological cough in man because suitable antibodies are not available but these will be important studies to perform when appropriate tools are developed. Current research investigating the pathogenesis of cough supports the development of TRP channel inhibitors as novel and selective treatment modalities.

**Contributors** MGB and MAB conceived and designed the studies; MG, ED, SAM, performed all the experiments and provided intellectual input; MGB wrote the manuscript.

**Funding** MAB, SAM and MG were funded by project grants from the Medical Research Council (MRC, UK) (MAB, G0800196; SAM, MG, G0800195). The human tissue experiments in this study were undertaken with the support of the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London. ED was funded by a Wellcome Trust project grant (089301/Z/09/Z).

**Competing interests** None.

**Ethics approval** Ethics approval was provided by Royal Brompton & Harefield Trust.

**Provenance and peer review** Not commissioned; externally peer reviewed.

## REFERENCES

1. **McCormick A**, Fleming DM, Charlton J; Office of Population Censuses and Surveys. *Morbidity Statistics from General Practice, Fourth National Study 1991–1992 (Series MB5 No 3)*. London: HMSO, 1995.
2. **Irwin RS**, Corrao WM, Pratter MR. Chronic persistent cough in the adult: the spectrum and frequency of causes and successful outcome of specific therapy. *Am Rev Respir Dis* 1981;**123**:413–17.
3. **Ford AC**, Forman D, Moayyedi P, et al. Cough in the community: a cross sectional survey and the relationship to gastrointestinal symptoms. *Thorax* 2006;**61**:975–9.
4. **Schroeder K**, Fahey T. Systematic review of randomised controlled trials of over the counter cough medicines for acute cough in adults. *BMJ* 2002;**324**:329–31.
5. **Canning BJ**, Chou Y-L. Cough sensors. I. Physiological and pharmacological properties of the afferent nerves regulating cough. *Handb Exp Pharmacol* 2009;**187**:23–47.
6. **Nasra J**, Belvisi MG. Modulation of sensory nerve function and the cough reflex: understanding disease pathogenesis. *Pharmacol Ther* 2009;**124**:354–75.
7. **Profita M**, Sala A, Bonanno A, et al. Increased prostaglandin E<sub>2</sub> concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol* 2003;**112**:709–16.
8. **Baumgarten CR**, Lehmkuhl B, Henning R, et al. Bradykinin and other inflammatory mediators in BAL-fluid from patients with active pulmonary inflammation. *Agents Actions Suppl* 1992;**38**:475–81.
9. **Fox AJ**, Laloo UG, Belvisi MG, et al. Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough. *Nat Med* 1996;**2**:814–17.
10. **Maher SA**, Birrell MA, Belvisi MG. Prostaglandin E<sub>2</sub> mediates cough via the EP<sub>3</sub> receptor: implications for future disease therapy. *Am J Respir Crit Care Med* 2009;**180**:293–8.
11. **Birring SS**, Parker D, Brightling CE, et al. Induced sputum inflammatory mediator concentrations in chronic cough. *Am J Respir Crit Care Med* 2004;**169**:15–19.
12. **Laude EA**, Higgins KS, Morice AH. A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulm Pharmacol* 1993;**6**:171–5.

13. **Laloo UG**, Fox AJ, Belvisi MG, *et al.* Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *J Appl Physiol* 1995;**79**:1082–7.
14. **Groneberg DA**, Niimi A, Dinh QT, *et al.* Increased expression of transient receptor potential vanilloid-1 in airway nerves of chronic cough. *Am J Respir Crit Care Med* 2004;**170**:1276–80.
15. **Story GM**, Peier AM, Reeve AJ, *et al.* ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003;**112**:819–29.
16. **Jordt SE**, Bautista DM, Chuang HH, *et al.* Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* 2004;**427**:260–5.
17. **Bandell M**, Story GM, Hwang SW, *et al.* Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* 2004;**41**:849–57.
18. **Bautista DM**, Jordt SE, Nikai T, *et al.* TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 2006;**124**:1269–82.
19. **Trevisani M**, Siemens J, Materazz S, *et al.* 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proc Natl Acad Sci U S A* 2007;**104**:13519–24.
20. **André E**, Campi B, Materazzi S, *et al.* Cigarette smoke-induced neurogenic inflammation is mediated by alpha, beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *J Clin Invest* 2008;**118**:2574–82.
21. **Wilson SR**, Gerhold KA, Bifulco-Fisher A, *et al.* TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. *Nat Neurosci* 2011;**14**:595–602.
22. **Bessac BF**, Sivula M, von Hehn CA, *et al.* TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest* 2008;**118**:1899–910.
23. **Nassenstein C**, Kwong K, Taylor-Clark T, *et al.* Expression and function of the ion channel TRPA1 in vagal afferent nerves innervating mouse lungs. *J Physiol* 2008;**586**:1595–604.
24. **Taylor-Clark TE**, McAlexander MA, Nassenstein C, *et al.* Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autotoxin 4-oxononenal. *J Physiol* 2008;**586**:3447–59.
25. **Raemdonck K**, de Alba J, Birrell MA, *et al.* A role for sensory nerves in the late asthmatic response. *Thorax* 2012;**67**:19–25.
26. **Birrell MA**, Belvisi MG, Grace M, *et al.* TRPA1 agonists evoke coughing in guinea pig and human volunteers. *Am J Respir Crit Care Med* 2009;**180**:1042–7.
27. **Salari H**, Chan-Yeung M. Release of 15-hydroxyicosatetraenoic acid (15-HETE) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by cultured human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1989;**1**:245–50.
28. **Ellis K**, Fozard J. Species differences in bradykinin receptor-mediated responses of the airways. *Auton Autacoid Pharmacol* 2002;**22**:3–16.
29. **Fuller RW**, Dixon CM, Cuss FM, *et al.* Bradykinin-induced bronchoconstriction in humans. Mode of action. *Am Rev Respir Dis* 1987;**135**:176–80.
30. **Costello JF**, Dunlop LS, Gardiner PJ. Characteristics of prostaglandin induced cough in man. *Br J Clin Pharmacol* 1985;**20**:355–9.
31. **Fox AJ**, Barnes PJ, Urban L, *et al.* An in vitro study of the properties of single vagal afferents innervating guinea-pig airways. *J Physiol* 1993;**469**:21–35.
32. **Chuang HH**, Prescott ED, Kong H, *et al.* Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P<sub>2</sub>-mediated inhibition. *Nature* 2001;**411**:957–62.
33. **Wang S**, Dai Y, Fukuoka T, *et al.* Phospholipase C and protein kinase A mediate bradykinin sensitisation of TRPA1: a molecular mechanism of inflammatory pain. *Brain* 2008;**131**:1241–51.
34. **Bessac BF**, Jordt SE. Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda)* 2008;**23**:360–70.
35. **Carr MJ**, Kollarik M, Meeker SN, *et al.* A role for TRPV1 in bradykinin-induced excitation of vagal airway afferent nerve terminals. *J Pharmacol Exp Ther* 2003;**304**:1275–9.

**On line Supplement**

**TRP channels mediate the tussive response to prostaglandin E<sub>2</sub> and bradykinin**

Megan Grace, Mark A. Birrell, Eric Dubuis, Sarah A. Maher, Maria G. Belvisi

## **METHODS**

### **Animals**

Male Dunkin-Hartley guinea-pigs (300-500g) and C57BL/6 mice (18-20g) were purchased from Harlan (Bicester, Oxon, U.K.), and housed in temperature-controlled (21°C) rooms with food and water freely available for at least 1 week before commencing experimentation. Breeding pairs of mice devoid of the TRPA1 (*Trpa1*<sup>-/-</sup>) or TRPV1 (*Trpv1*<sup>-/-</sup>) gene that had been backcrossed on to the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbour, ME). Breeding colonies were maintained at Imperial College, London. Experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986.

### **Isolated vagal ganglia**

#### *Cell dissociation*

Guinea-pigs were sacrificed by injection of pentobarbitone (20 mg/kg, i.p.). Nodose and jugular ganglia were dissected free of adhering connective tissue and isolated by enzymatic digestion. Ganglia were incubated with activated papain (Sigma, Papain type 200U/ml in Ca<sup>2+</sup>-free, Mg<sup>2+</sup>-free Hanks' balanced salt solution) for 30 minutes at 37°C followed by incubation for 40 minutes with type 4 collagenase (Worthington, 2mg/ml) and Dispase II (Roche, 2.4mg/ml), with gentle agitation every 5 minutes. The neurons were dissociated by titration with fire-polished glass Pasteur pipettes of decreasing tip pore size and washed by centrifugation for 1 minute (1400 rpm, 380g) at 37°C. The supernatant was decanted and the pellet of tissues and cells carefully homogenised in HBSS at room temperature. Cells were separated from the remaining tissue by two centrifugations at 22°C: 8 min (1400 rpm, 380g) in L15 medium containing 20% Percoll (v/v) and 2 min (2300 rpm, 1030g) in L15 medium. The cells were resuspended in complete F-12 medium and plated in poly-d-lysine/Laminin

(22.5 ug/ml) coated fluorodishes. Neurons were allowed to adhere for 2h in a 37°C, 5% CO<sub>2</sub> environment, then gently flooded with 2ml of complete F12 medium (10% FBS, 1% penicillin 10,000U/ml – streptomycin 10mg/ml). Plates were used for experimentation within 24h.

### *Calcium imaging*

Intracellular free calcium ( $[Ca^{2+}]_i$ ) measurements were performed in dissociated jugular and nodose neurons. Fluorodishes were loaded with Fluo-4 AM (6  $\mu$ M, Invitrogen) for agonist experiments, or Fluo-2 AM (3  $\mu$ M, Invitrogen) for antagonist experiments for 40 min in the dark at 25°C and allowed to rest for 30 min in the dark at 25°C. After washing, a fluoro dish was placed in a full incubation chamber mounted on the stage of a widefield inverted microscope Zeiss Axiovert 200 (Carl Zeiss Inc., NY, USA) and held at 37°C. Signals were recorded using an Hamamatsu EM-CCD C9100-02 camera run by Simple PCI software. CA Xenon gas Arc lamp Cairn ARC Optosource Illuminator, a Quad filter set Ex 485-20 Bs 475-495 Em 510-53120x and a LD Plan-Neofluar AIR Korr objective were used to generate excitatory signal and record emission.

Neurons were constantly superfused with 37°C ECS buffer using an in house designed pressurized solution-changing perfusion system allowing complete bath (600 $\mu$ l volume) replacement in 3s. Prior to experiments, the cells were superfused for 10 min with ECS-only. 50 mM potassium chloride solution (K50) was applied at the start and end of each experiment for 10s to assess cell viability and normalise responses. Stock solutions of agonists and antagonists were diluted 1/1000 in ECS to make a working solution. Capsaicin or acrolein was applied for 10-20s, PGE<sub>2</sub> for 20-30s and BK for 40-60s (N=4-6 animals, n=15-26 cells per drug tested). After each application of drug, cells were washed with ECS until complete recovery of baseline  $[Ca^{2+}]_i$ . Images were acquired with a frequency of 1Hz from 30s prior to

drug application and for 2min afterward, and at 0.2Hz otherwise. To take into account the multiphasic responses obtained in some cells the area under curve of calcium signal (total elevation of calcium above resting level over time or calcium flux) was used to measure responses, which were normalised to calcium flux generated by application of K50. Only neurons producing a fast response to K50 which was washable within 5min, and that had diameter of over 20  $\mu\text{m}$  were analysed.

The ability of TRPA1-selective (HC-030031) and TRPV1-selective (JNJ17203212) antagonists to inhibit submaximal acrolein (10  $\mu\text{M}$ ) and capsaicin (1  $\mu\text{M}$ )-induced changes in  $[\text{Ca}^{2+}]_i$  was investigated in jugular cells. CR curves were established for HC-030031 (0.001, 0.01, 0.1, 1  $\mu\text{M}$ ) or vehicle (0.1% DMSO) against acrolein; and JNJ17203212 (1, 10, 100  $\mu\text{M}$ ) or vehicle (0.1% DMSO) against capsaicin.  $[\text{Ca}^{2+}]_i$  responses were recorded using Fura-2, with only one concentration of antagonist assessed per plate. Once appropriate concentrations of the antagonists had been determined, the effect of 0.1  $\mu\text{M}$  HC-030031 on capsaicin and 10  $\mu\text{M}$  JNJ17203212 on acrolein stimulated  $[\text{Ca}^{2+}]_i$  elevation was investigated to establish that there was no off-target effect at the concentration chosen. These antagonist concentrations were subsequently used to inhibit  $\text{PGE}_2$  (1  $\mu\text{M}$ ) and BK (10  $\mu\text{M}$ ) responses in jugular neurons (N=3-4, n=10-19).

#### *Isolated vagus nerve preparation*

Initially, concentration-response curves were established for the agonists. Concentrations of vehicle (distilled water or ethanol, 0.1% vol/vol),  $\text{PGE}_2$  or BK were applied to guinea-pig ( $\text{PGE}_2$  3-100  $\mu\text{M}$ ; BK 1-30  $\mu\text{M}$ ) or mouse ( $\text{PGE}_2$  and BK 0.1-100  $\mu\text{M}$ ) nerves in a random order for 2 minutes each, including a wash period to retain baseline membrane potential between stimulations. No more than five stimulations were generated per section of nerve. From this, a submaximal dose of each agonist was chosen for future experiments: 10  $\mu\text{M}$

PGE<sub>2</sub>; and 1 μM BK in the mouse and 3 μM BK in the guinea-pig. The ability of PGE<sub>2</sub> (10μM) and BK (3μM) to stimulate human vagus nerves was also determined. Human vagal tissue (n = 6 patients, 2 male, 27-72 year old donors with no respiratory disease) was obtained from two sources – transplant tissue surplus to requirements (Harefield Hospital, UK); and purchased from IIAM (International Institute for the Advancement of Medicine, Edison, NJ). In all cases, the tissue was consented for use in scientific research and ethics approval obtained.

The GPCR through which PGE<sub>2</sub> induces cough has been identified as the EP<sub>3</sub> receptor [S1]. However, the GPCR through which BK signals has not yet been comprehensively investigated using selective ligands. Therefore, the ability of B<sub>1</sub> (1μM R715) or B<sub>2</sub>-selective (10μM WIN 64338) antagonists to inhibit BK-induced human, guinea-pig and mouse sensory nerve depolarisation was examined. These were based on using concentrations 10-fold higher than the reported antagonist affinity for the mouse (B<sub>1</sub>; [S2]) or guinea-pig (B<sub>2</sub>; [S3]) receptor. There has been a suggestion that BK may activate sensory nerves indirectly by inducing production of prostaglandins. To investigate this, a general cyclooxygenase inhibitor (10μM indomethacin) was tested according to our standard antagonist protocol to see if BK responses were altered. In addition, depolarisation to BK was assessed in both wild type and genetically modified mice with the EP<sub>3</sub> gene deleted (*EP<sub>3</sub><sup>-/-</sup>*). Tetrodotoxin (TTX, 3μM) was also used to assess whether depolarisation to these tussive agents was sensitive to sodium channel blockade indicating a role for sensory nerve activation. The concentration of TTX was based on previous functional experiments in isolated airway tissue shown to inhibit neuronal responses [S4].

Concentration-response curves were established for TRPA1-selective (HC-030031) and TRPV1-selective (capsazepine and JNJ17203212) antagonists, or vehicle (dimethyl sulfoxide [DMSO], 0.1% vol/vol) using previously established submaximal doses of the selective

agonists acrolein (300  $\mu$ M) and capsaicin (1  $\mu$ M). From this, the dose of antagonist exhibiting maximal inhibition of its receptor was chosen for further experiments. To demonstrate that these antagonists were not exhibiting off-target effects, 10  $\mu$ M HC-030031 was also tested against the TRPV1 agonist capsaicin; and 10  $\mu$ M capsazepine and 100  $\mu$ M JNJ17203212 were tested against the TRPA1 agonist acrolein. The ability of TRP-selective antagonists to inhibit PGE<sub>2</sub> and BK-induced sensory nerve depolarisation was comprehensively profiled in wild type mouse and guinea-pig tissue (n=6), and was also assessed in human tissue when available (n=2-3). Concentrations of both agonists and antagonists used for human vagus nerve were the same as for guinea-pig experiments. To further confirm that the inhibition observed with selective antagonists was due to inhibition of the TRPA1 or TRPV1 ion channel, we performed parallel experiments using vagal tissue from *Trpa1*<sup>-/-</sup> and *Trpv1*<sup>-/-</sup> mice. Knockdown of the TRPA1 or TRPV1 gene was confirmed in the genetically modified mice using standard genotyping techniques.

#### *Conscious guinea-pig cough model*

Conscious unrestrained guinea-pigs were placed in individual plastic transparent whole-body plethysmograph chambers (Buxco, Wilmington, NC, USA), and cough detected as previously described [S1, S5]. Concentration-responses were established for capsaicin (15-90  $\mu$ M, n=10), acrolein (10-300  $\mu$ M, n=12), PGE<sub>2</sub> (30-300  $\mu$ g/ml; n=8), BK (0.3-10 mg/ml; n=4), or appropriate vehicle (1% ethanol, 1% Tween 80 in 0.9% sterile saline; 0.9% sterile saline; 0.1M phosphate buffer; or 0.9% sterile saline, respectively). Stimuli were aerosolised for 5 min (capsaicin and acrolein) or 10 min (PGE<sub>2</sub> and BK). Coughs were counted for 10 min, both with the Buxco cough analyser and by a trained observer. From these experiments, a submaximal dose of agonist was identified for further experiments.

To determine an appropriate dose of TRPA1 and TRPV1-selective antagonist, guinea-pigs were injected i.p. with HC-030031 (30-1000 mg/kg), JNJ17203212 (10-1000 mg/kg) or appropriate vehicle (0.5% methylcellulose in 0.9% saline or 15% solutol in 5% dextrose solution, respectively). One hour later the guinea-pigs were exposed to 5 minutes of a submaximal dose of TRP-selective agonist (100 mM acrolein or 60  $\mu$ M capsaicin). Coughs were counted during this period, and for a further 5 minutes post-stimulation (10 minutes total). Once a concentration had been established that maximally inhibited its own receptor, this concentration was tested against the alternate agonist to confirm receptor selectivity at the chosen dose. Guinea-pigs received two i.p. injections of either: (a) TRPA1 vehicle (0.5% methylcellulose in 0.9% saline) plus TRPV1 vehicle (15% solutol in 5% dextrose solution); (b) TRPA1 antagonist (300 mg/kg HC-030031) plus TRPV1 vehicle; (c) TRPA1 vehicle plus TRPV1 antagonist (100 mg/kg JNJ17203212); or (d) TRPA1 antagonist plus TRPV1 antagonist. One hour later the guinea-pigs were exposed to 10 minutes of aerosolised PGE<sub>2</sub> (300  $\mu$ g/ml, n=12) or BK (3 mg/ml, n=10-11), and the number of coughs counted during this period.

### **Compounds and Materials**

PGE<sub>2</sub> was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The TRPA1 inhibitor HC-030031 was purchased from ChemBridge (San Diego, USA). The TRPV1 inhibitor JNJ17203212 was a kind gift from Glaxo SmithKline, who also provided the excipient Solutol (BASF, Ludwigshafen, Germany). All other agents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.)

*Isolated vagal ganglia experiments:* Fluo-4 AM and Fura-2 AM were purchased from Molecular Probes/Invitrogen. L-15 and Hanks' balanced salt solution (HBSS) were purchased

from Gibco/Invitrogen (Carlsbad, CA, USA). The 50mM potassium solution (K50) contained (in mM): 50 KCl, 91.4 NaCl, 1 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 10 HEPES; pH adjusted to 7.4 at 37°C using KOH). Capsaicin was dissolved in 100% DMSO, PGE<sub>2</sub> in 100% ethanol, and BK in dH<sub>2</sub>O. Stock solutions were diluted 1/1000 in extracellular solution (ECS) buffer (in mM: 5.4 KCl, 136 NaCl, 1 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 10 HEPES; pH adjusted to 7.4 at 37°C using NaOH) to the desired final concentrations. Final vehicle concentration was 0.1% for the calcium measurements.

*In vitro vagus experiments:* All Krebs salts were obtained from BDH (Dorset, U.K.), and Krebs Hanseleit solution was made fresh on a daily basis (mM: NaCl 118; KCl 5.9; MgSO<sub>4</sub> 1.2; CaCl<sub>2</sub> 2.5; NaH<sub>2</sub>PO<sub>4</sub> 1.2; NaHCO<sub>3</sub> 25.5; glucose 5.6). TTX stock was dissolved in 20mM citric acid. All other agonists and antagonists were dissolved in 100% distilled water, DMSO or ethanol. Aliquots were diluted down 1/1000 in Krebs solution for testing.

*In vivo cough experiments:* Acrolein was supplied in liquid form at 15M. A 1M stock was made in saline (0.9%) and diluted in saline to 100mM. A 10mM stock of capsaicin was made in ethanol and diluted in vehicle to obtain 1% ethanol and 1% Tween 80 in 0.9% saline. Bradykinin was dissolved in 0.9% saline to working solution, accounting for the acetate conversion factor (1.06). PGE<sub>2</sub> was dissolved in 0.1M phosphate buffer to a working solution. HC-030031 (300 mg/ml) was suspended in vehicle (0.5% methyl cellulose in sterile saline); JNJ17203212 (100 mg/ml) was suspended in vehicle (15% solutol in 5% dextrose solution or 0.5% methyl cellulose in sterile saline), total dosing volume 10 ml/kg i.p.

## **RESULTS**

### **Characterising agonist responses in isolated vagal ganglia**

Capsaicin increased intracellular free calcium ( $[Ca^{2+}]_i$ ) in jugular neurons with an  $EC_{50}$  of  $1.43 \pm 0.13 \mu M$  and a maximum response ( $R_M$ ) of  $75 \pm 14\%$  at  $10 \mu M$  ( $N=5$ ,  $n=24$ ), but on average triggered a small response in nodose neurons ( $R_M$   $5 \pm 2\%$  at  $10 \mu M$ ,  $EC_{50}$   $1.62 \pm 0.14 \mu M$ ;  $N=5$ ,  $n=18$ ). It should be noted that robust responses to capsaicin are observed in a small population of nodose neurons [S6, S7]; and in the capsaicin-responsive nodose cells in this study we did see some significant increases in  $[Ca^{2+}]_i$ . However, our data represent an overview of all responding and non-responding cells, of which 62.5% of jugular neurons and 16.7% of nodose neurons were responsive to capsaicin stimulation (Supplementary Figure 1A & 1C). Different types of response were observed for acrolein in the jugular compared to nodose ganglia. Primary jugular cells responded in a multi-phasic pattern, showing periods of repetitive sharp  $[Ca^{2+}]_i$  elevations, with an  $EC_{50}$  of  $3.4 \pm 0.39 \mu M$  and  $126 \pm 18\% R_M$  at  $30 \mu M$  ( $N=6$ ,  $n=24$ ). Whereas, acrolein-induced  $[Ca^{2+}]_i$  elevations in the nodose ganglia were mono-phasic, with an  $EC_{50}$  of  $8.14 \pm 1.1 \mu M$  and  $163 \pm 31\% R_M$  at  $30 \mu M$  ( $N=6$ ,  $n=26$ ) (Supplementary Figure 1B & 1D). Interestingly, 54.2% of jugular neurons and 53.8% of nodose neurons responded to acrolein stimulation.

## Figure Legends

### **Figure S1. Characterisation of TRPA1- and TRPV1-selective agonists in the *in vitro* primary ganglia and isolated vagus nerve preparations.**

Panels A-D: Concentration responses showing increases in intracellular calcium ( $[Ca^{2+}]_i$ ) for TRPV1 selective (capsaicin) and TRPA1 selective (acrolein) agonists in primary neurons isolated from (A & B) guinea-pig jugular and (C & D) nodose ganglia. In each panel, histograms show an increase in  $[Ca^{2+}]_i$  for increasing concentrations of tussive agent. To take into account multiphasic shapes of some responses and their lengths, the calcium flux (area under curve) generated by applications of tussive agents is normalised, and expressed as

percentage of response to K50. The response obtained is expressed as percentage of response to K50. The tussive agent used is indicated above each set of histograms and the concentration below each bar in  $\mu\text{M}$  (N=5-6, n=18-26). The trace in the lower left shows a typical recording of the light intensity over time following exposure to the agonist. Time and duration of drug application are indicated by a black bar above the trace. Time scale is given by the 1 minute length-equivalent black bar shown below the trace. On the bottom right are display images taken during the recording. Time of the snapshot is indicated below each picture with zero being the start of tussive agent application. The pseudo colour code used for light intensity in the pictures is represented on the right of each set of images.

Panels E & F: Perfusion for 2 minutes of capsaicin (black bars) or acrolein (white bars) activated (E) guinea-pig and (F) mouse isolated vagus nerves in a concentration-dependent manner, measured as depolarisation of the nerve in mV (n=6).

Panel G: Perfusion for 2 minutes of capsaicin (1  $\mu\text{M}$ ) or acrolein (300  $\mu\text{M}$ ) but not vehicle (0.1% DMSO v/v) activated human isolated vagus nerves, measured as depolarisation of the nerve in mV (n=4-8). Data is expressed as mean  $\pm$  s.e.m of  $n$  observations. Veh = vehicle.

## References

S1. Maher, S.A., Birrell, M.A., & Belvisi, M.G. Prostaglandin  $E_2$  mediates cough via the  $EP_3$  receptor: Implications for future disease therapy. *Am J Respir Crit Care Med* 2009; **180**: 293-298.

S2. Regoli D, Allogho SN, Rizzi A, et al., Bradykinin receptors and their antagonists. *Eur J Pharmacol* 1998; **348**: 1-10.

S3. Scherrer D, Daeffler L, Trifilieff, A, et al. Effects of WIN 64338, a nonpeptide bradykinin B<sub>2</sub> receptor antagonist, on guinea-pig trachea. *Br J Pharmacol* 1995; **115**: 1127-1128.

S4. Ward JK, Barnes PJ, Tadjkarimi D, et al. Evidence for the involvement of cGMP in neural bronchodilator responses in human trachea. *J Physiol* 1995; **483**: 525-536.

S5. Birrell MA, Belvisi MG, Grace M, et al. TRPA1 agonists evoke coughing in guinea pig and human volunteers. *Am J Respir Crit Care Med* 2009; **180**: 1042-1047.

S6. McLeod RL, Jia Y, Fernandez X, et al. Antitussive profile of the NOP agonist Ro-64-6198 in the guinea pig. *Pharmacology* 2004; **71**(3): 143-9.

S7. Kollarik M, Carr MJ, Ru F, et al. Transgene expression and effective gene silencing in vagal afferent neurons in vivo using recombinant adeno-associated virus vectors. *J Physiol* 2010; **588**(Pt 21): 4303-15.

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Page 2
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 2
<b>Introduction</b>			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 4
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 4
<b>Methods</b>			
Study design	4	Present key elements of study design early in the paper	Page 4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Page 5
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Page 5
		(b) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Page 4-5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Page 5
Bias	9	Describe any efforts to address potential sources of bias	Page 12
Study size	10	Explain how the study size was arrived at	Page 5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Page 5-6
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Page 5-7
		(b) Describe any methods used to examine subgroups and interactions	Page 5-7
		(c) Explain how missing data were addressed	Page 6
Results		(d) If applicable, explain how loss to follow-up was addressed	N/A
		(e) Describe any sensitivity analyses	N/A

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	Page 8-9 N/A N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	Page 8-9 Page 6 N/A
Outcome data	15*	Report numbers of outcome events or summary measures over time	N/A
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Page 9-10 Page 5 N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Page 8-9
<b>Discussion</b>			
Key results	18	Summarise key results with reference to study objectives	Page 3
<b>Limitations</b>			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Page 11-13
Generalisability	21	Discuss the generalisability (external validity) of the study results	Page 11
<b>Other information</b>			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Page 13

\*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at [www.strobe-statement.org](http://www.strobe-statement.org).

