SUPPLEMENTARY INFORMATION

METHODS

Participants

The study involved 16 participants with cough hypersensitivity and 16 age and sex matched healthy controls. All participants provided written informed consent to participate in the study, which was approved by the Melbourne Health Human Research Ethics Committee (Australia). Before inclusion, all participants underwent screening interviews to ascertain their eligibility for the study. All participants were non-smokers with no history of neurological disease or a recent history (over 8 weeks) of acute respiratory infections. Cough hypersensitive participants were recruited if they suffered from chronic cough (coughing for over 8 weeks) at the time of scan and scored 14 or above on the Hull Airway Reflux Questionnaire.[1] Healthy control participants had no history of chronic respiratory disease and were individually age and sex matched to each cough hypersensitive participant.

Psychophysical session

All participants underwent a psychophysical testing session where their level of sensitivity to capsaicin was measured. Patients provided estimates of their urge-to-cough using an eleven point numerical rating scale (0, no urge-to-cough; 10, maximum urge-to-cough). The capsaicin solution was delivered to participants as a vapour via a facemask connected to an air pump through irrigation tubing as previously described.[2-4]. Doubling doses used throughout the study ranged from 0.06μM to 125 μM.

Thresholds were determined using the method of limits, and included the minimum concentration of capsaicin needed for the participant to perceive an urge-to-cough (Cu Threshold) and the concentration of capsaicin needed to elicit two or more coughs (C2 Threshold). Participants inhaled successive, increasing doses of capsaicin with a single breath at vital capacity. Inhalations were separated by approximately 90-second inter-stimulus intervals. A third threshold was determined that involved repeated inhalation of capsaicin. Participants continuously inhaled a single dose of capsaicin during 24-seconds with the objective of suppressing cough. Successive dose increases were used in trials when the preceding dose was inhaled for 24 seconds without coughing. The dose one increment below the first dose to elicit uncontrolled coughing was deemed the maximum suppressible threshold (S_{max}). The S_{max} dose was one of two doses used during the brain imaging sessions.

The relationship between capsaicin dose and the intensity of urge-to-cough and cough frequency was tested using multiple, single breath challenges at five different doses based on each participant's sensitivity. Each of the five doses was delivered twice in random order. The doses were dictated by the participant's C2 threshold, and consisted of a two dose increment below the C2 (i.e., C2-2), a single dose increment below C2 (i.e., C2-1), the C2 dose, a single dose increment above C2 (i.e., C2+1), and a two dose increment above C2 (i.e., C2+2). Participants rated the level of urge-to-cough after capsaicin challenges and an auditory cough count was also recorded following each inhalation of capsaicin.

Brain imaging session

Image acquisition

Structural and functional MRI data were collected on a Siemens Magnetom Trio 3 Tesla scanner (Siemens AG, Erlangen, Germany) with a 32-channel head coil located at the Murdoch Children's Research Institute, Melbourne, Australia. Anatomical T1-weighted images were acquired in the sagittal plane (192 slices, 0.90 mm slice thickness, 0.84 × 0.84 mm2 in-plane resolution, echo time (TE) = 2.59 ms, repetition time (TR) = 1900 ms, flip angle = 9°). Three functional MRI (fMRI) scans of 558 seconds duration was performed using the BOLD contrast. Echo planar images (EPI) were acquired in the transaxial plane (36 slices, 4 mm slice thickness, 3.28 × 3.28 mm2 in-plane resolution, TE = 32 ms, TR = 2000 ms, flip angle = 90°)

During image acquisition, participants lay comfortably on the scanner bed with their head stabilised with foam padding and hearing protection. Participants were also fitted with the facemask and apparatus used in the psychophysical testing session Medical air (flow rate = 0.7 ml/min) in the scanner room was used to drive the apparatus. Online respiratory monitors were fitted around their chest

throughout the experiment (AD Instruments). A periscope mirror attached to the scanner head coil enabled participants to view a projector screen upon which visual cues were presented throughout the experimental session. Visual cues during fMRI scanning were delivered using Neurobehavioural Systems Presentation® software (San Francisco, USA).

fMRI protocol
Functional MRI scans included 8 stimulation blocks of 24 second interspersed with 42 seconds of rest. Participants were administered either saline, a low or high dose of capsaicin in a randomized order. A visual "Ready" cue displayed three seconds prior to stimulus onset was used to prompt participants to prepare for the impending saline or capsaicin challenge, and participants' were instructed to coordinate initial tidal inhalations to coincide with the onset of each challenge upon the presentation of the "Go" cue. Participants were instructed to breathe through their mouth at tidal volume during each challenge and to suppress coughing if possible. Another visual cue appeared 18 seconds after stimulus offset, instructing participants to rate their urge-to-cough. Participants were told to rate the most intense urge-to-cough they experienced during the preceding stimulus using both their hands on a scale of 0 (no urge) to 10 (maximal urge to cough. The occurrence of cough bouts during each challenge was also recorded and later confirmed by reference to the online recordings of thoracic movement. Scanning runs were repeated when uncontrolled coughing occurred. In a small number of cases the high doses was decreased to ensure cough suppression was achievable (Cough Hypersensitive patients n=3, Healthy Controls n=4).

The individually tailored S_{max} dose was used as the high dose for all participants to allow between-group comparisons on the basis of a matched urge-to-cough sensation. The second dose (low dose) was determined in different ways for the two groups. Participants in the cough hypersensitive group received capsaicin doses that were two increments lower than their S_{max} as their low dose. The healthy controls received the S_{max} dose of their matched cough hypersensitive participant to allow matched capsaicin dose comparisons. Not all control participants had a higher S_{max} threshold than their matched cough hypersensitive participant. Healthy participant with lower S_{max} thresholds received at least one dose that was equivalent to that of their individually matched cough hypersensitive pair (i.e., the low dose of the paired cough hypersensitive patient) (n=2).

Data analysis
Demographic and Psychophysical analyses
Independent t- tests were used to test the effects of group on C2, Cu and S_{max} thresholds. A repeated-measures ANOVA was used to test the effects of group, dose and their interaction on urge-to-cough ratings in response to random capsaicin challenges. Independent t-tests were used to assess group effects on capsaicin doses and urge-to-cough ratings associated with the fMRI scanning.

Brain imaging analyses
Pre-processing and statistical analysis of functional brain images were undertaken with the fMRI Expert Analysis Tool (FEAT) from the FMRIB Software Library (FSL) (www.fmrib.ox.ac.uk/fsl). The fMRI data were motion corrected, spatially smoothed with a Gaussian kernel of 6 mm full width at half maximum, and high pass filtered using a filter with cut-off frequency of 0.01 Hz. Anatomical images and functional images were stripped of non-brain voxels using the Brain Extraction Tool (BET).[5] The brain extracted anatomical image was used as an intermediate step to generate matrices using linear transformations for the co-registration of each participant's functional images to the MNI template brain.[6] Statistical analysis of participants' fMRI time series involved general linear modelling of BOLD signal changes using separate explanatory variables (EVs) that represented experimental events including the ready cue, ratings, saline inhalation, low capsaicin inhalation and high capsaicin inhalation blocks. Regressors for the experimental events were convolved with a gamma function to take account of the hemodynamic response. Motion correction parameters were included as regressors of no interest. Signal changes associated with infrequent, controlled coughing events were explicitly modelled using a regressor without convolution as we have previously described.[2 3] Physiological noise was also modelled as additional confounding EVs as detailed in [3] and [2]. These nuisance regressors were extracted from each participants' fMRI data from three regions likely to include signal changes associated with physiological processes, and unlikely to represent neural activation (i.e., lateral ventricles, white matter and a single voxel with the highest level of standard deviation across the time series of motion corrected images, typically located in the sagittal sinus.[7 8] An additional confound variable included in the model was based on the mean signal of non-activated

voxels using an iterative processes first advocated for analysis of positron emission tomography data and previously employed by our group to investigate BOLD signal changes associated with cough.[3 9] Our group has previously used saline blocks as a contrast against capsaicin challenges to cancel out shared attributes such as physiological noise created by breathing control and brief breath-holds, processing of visual cues, moisture content of inhaled vapour and sound of nebuliser. However, the limitation of contrasting saline inhalation challenges is that it could have differential effects on the groups as the patients and controls have significantly different airway sensitivity. This was indeed the case with cough hypersensitive patients where saline inhalation caused irritation and an urge-to-cough in some patients. It is possible that a saline contrast could attenuate capsaicin-related effects exclusively in the cough hypersensitive group, thus potentially driving a spurious group difference. For this reason, saline inhalation could not be counted as an accurate baseline for BOLD signal increase associated with a brief breath-hold and stimulus inhalation during healthy control and cough hypersensitive comparison analyses in this study. Furthermore, comparable amounts of activation caused by aerosol inhalation would be present in the control group and therefore, we would expect no difference in activation caused by aerosol inhalation during between-group comparisons. Comparisons of EVs were performed to identify regions showing increased BOLD signal activity during the two doses of capsaicin inhalation challenges and innocuous saline stimulation.

Higher-level analyses were carried out for inter-group averaging and between-group comparisons. Z (Gaussianised *T*/*F*) statistic images were thresholded to define clusters of contiguous voxels activated at a significance level of $Z > 2.3$. To correct for multiple comparisons, a corrected cluster probability threshold (based on Gaussian Random Field Theory[10] of $p < 0.05$ was applied. Between-group contrasts were made using two different comparisons: i) during the inhalation of S_{max} for all individuals in both groups (matched urge-to-cough sensation) and ii) during the inhalation of a matched capsaicin dose between each age and sex matched pair (matched capsaicin dose). Activation levels associated with Smax doses were tested for relationships with the Cu threshold and the frequency of coughing during the psychophysical sessions.

Activated voxels in homogenous anatomical regions identified by between-group comparisons during matched urge-to-cough sensation and matched capsaicin dose were used to define regions of interest (ROI) for further analysis. Estimates of percentage BOLD signal change in ROI were calculated using FEATQUERY. Subsequently, group effects on BOLD signal changes were tested with independent t-tests. Percentage BOLD signal changes of these ROIs were also correlated with Cu thresholds and cough frequency measures to investigate the relationship between capsaicin-inhalation activation and clinically related symptoms in cough hypersensitive participants.

References

1. Morice AH, Faruqi S, Wright CE, Thompson R, Bland JM. Cough hypersensitivity syndrome: a distinct clinical entity. Lung 2011;**189**(1):73-9 doi: 10.1007/s00408-010-9272-1.
2. Farrell MJ, Cole LJ, Chiapoco D, Egan GF, Mazzone SB. Neural correlates coding stimulus level and perception of capsaicin-evoked urge-to-cough in humans. NeuroImage 2012;**61**(4):1324-35 doi: 10.1016/j.neuroimage.2012.03.030.
3. Mazzone SB, Cole LJ, Ando A, Egan GF, Farrell MJ. Investigation of the neural control of cough and cough suppression in humans using functional brain imaging. The Journal of neuroscience : the official journal of the Society for Neuroscience 2011;**31**(8):2948-58 doi: 10.1523/JNEUROSCI.4597-10.2011.
4. Mazzone SB, McLennan L, McGovern AE, Egan GF, Farrell MJ. Representation of capsaicin-evoked urge-to-cough in the human brain using functional magnetic resonance imaging. American journal of respiratory and critical care medicine 2007;**176**(4):327-32 doi: 10.1164/rccm.200612-1856OC.
5. Smith SM. Fast robust automated brain extraction. Human brain mapping 2002;**17**(3):143-55 doi: 10.1002/hbm.10062.
6. Jenkinson M, Bannister P, Brady M, Smith S. Improved optimization for the robust and accurate linear registration and motion correction of brain images. NeuroImage 2002;**17**(2):825-41
7. Birn RM, Murphy K, Handwerker DA, Bandettini PA. fMRI in the presence of task-correlated breathing variations. NeuroImage 2009;**47**(3):1092-104 doi: 10.1016/j.neuroimage.2009.05.030.
8. Birn RM, Smith MA, Jones TB, Bandettini PA. The respiration response function: the temporal dynamics of fMRI signal fluctuations related to changes in respiration. NeuroImage 2008;**40**(2):644-54 doi: 10.1016/j.neuroimage.2007.11.059.
9. Andersson JL. How to estimate global activity independent of changes in local activity. NeuroImage 1997;**6**(4):237-44 doi: 10.1006/nimg.1997.0302.
10. Worsley KJ, Evans AC, Marrett S, Neelin P. A three-dimensional statistical analysis for CBF activation studies in human brain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 1992;**12**(6):900-18 doi: 10.1038/jcbfm.1992.127.

SUPPLEMENTARY TABLES

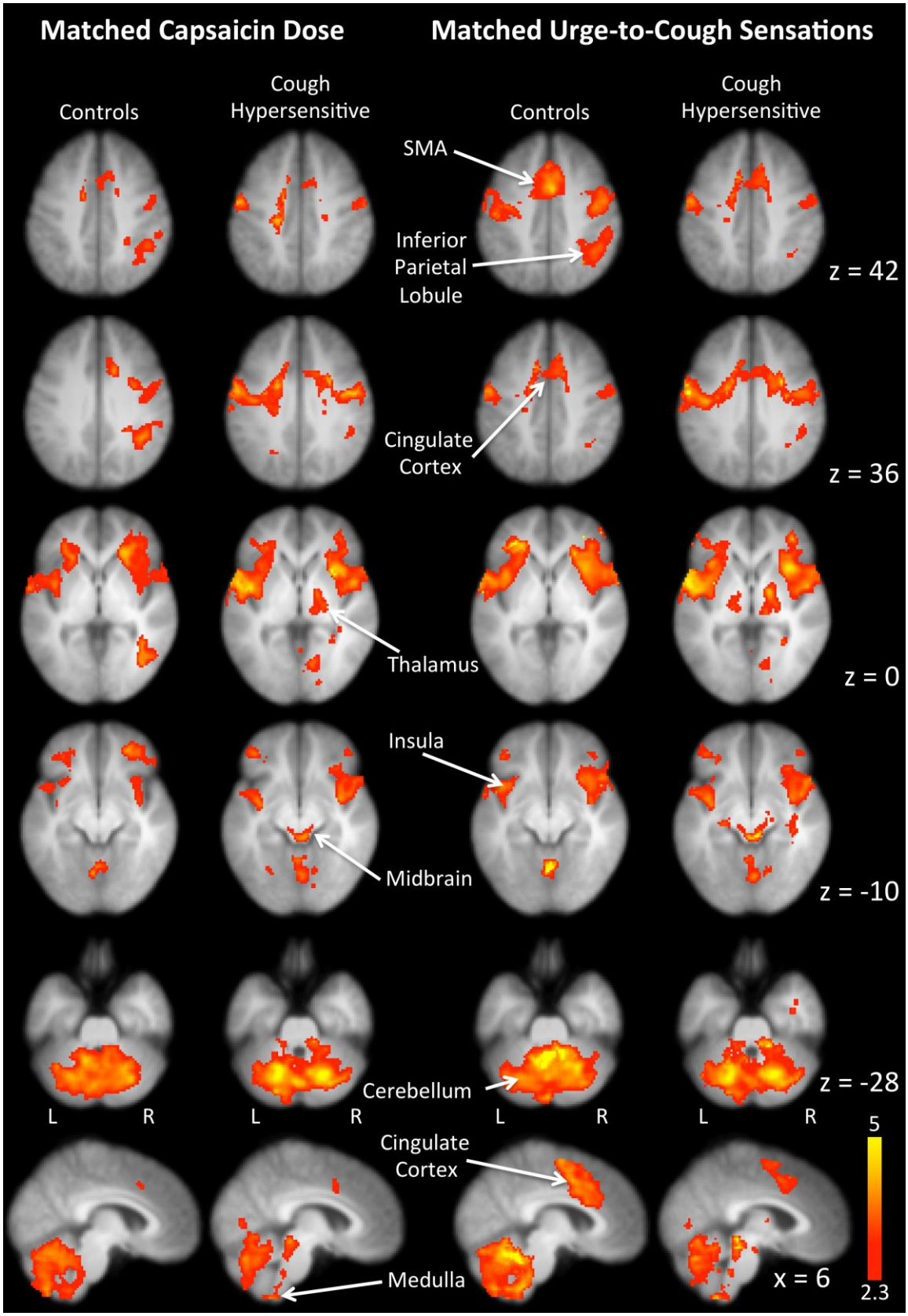
Supplementary Table 1.
Capsaicin-Inhalation Activations for Matched Capsaicin Doses in Control and Cough Hypersensitive Participants

	Controls				**Cough Hypersensitive**			
	Peak Voxel				Peak Voxel			
REGION	x	y	z	z stat	x	y	z	z stat
Cingulate cortex	14	12	36	3.72	-18	18	24	3.31
Middle frontal gyrus	24	32	-2	4.13	-42	44	-6	3.62
SMA								
S1/M1	32	-8	22	3.68	-64	-18	24	4.61
Superior parietal cortex	40	-48	34	4.14	44	-46	34	3.03
Lateral occipital cortex	38	-64	2	4.11				
Operculum	-68	-24	16	3.57	-50	-10	10	4.67
Orbitofrontal cortex	-48	14	-12	2.60	50	18	-10	3.25
Inferior frontal gyrus	-64	-2	0	3.62	-56	20	56	4.42
Insula	28	30	4	4.38	-50	-10	8	4.69
Thalamus					16	-4	-2	3.71
Midbrain					4	-32	-10	3.92
Pons	8	-38	-26	2.69	-2	-30	-16	3.73
Medulla	-8	-40	-50	3.50	4	-50	-60	4.11
Cerebellum	40	-64	-40	5.00	22	-64	-28	5.57

Supplementary Table 2.
Capsaicin-Inhalation Activations for Matched Urge-to-Cough Sensation Doses in Control and Cough Hypersensitive Participants

	Controls				**Cough Hypersensitive**			
	Peak Voxel				Peak Voxel			
REGION	x	y	z	z stat	x	y	z	z stat
Cingulate cortex	-14	14	28	5.41	-10	16	42	3.82
Middle frontal gyrus	32	46	0	5.59	30	30	-2	4.24
SMA	2	-8	62	4.04	-8	-6	58	3.91
S1/M1	-60	8	28	5.93	-56	-6	36	5.91
Superior parietal cortex	32	-46	34	3.64	44	-44	32	3.99
Lateral occipital cortex								
Operculum	62	-8	12	5.16	-48	-10	10	5.29
Orbitofrontal cortex	-44	14	-12	3.89	50	18	-10	3.74
Inferior frontal gyrus	-60	-4	0	5.87	54	16	8	6.43
Insula	-28	8	8	5.21	-48	-10	8	7.00
Thalamus					16	-2	-2	5.67
Midbrain					-2	-30	-14	5.27
Pons	8	-38	-26	3.46	-2	-32	-16	5.25
Medulla	2	-56	-52	5.17	14	-44	-60	4.37
Cerebellum	0	-50	-26	11.04	20	-64	-26	5.55

SUPPLEMENTARY FIGURE



Supplementary Figure Caption
Capsaicin-Inhalation Activations for Matched Capsaicin Dose and Matched Urge-to-Cough Sensation Doses in Control and Cough Hypersensitive Participants.

Group capsaicin-inhalation activations were generated for healthy controls and cough hypersensitive patients. The doses of capsaicin inhaled by each participant were tailored to their sensitivity. All participants inhaled a maximum suppressible dose (S_{max}) during repeated breaths over a 24 second time frame. Generally, the S_{max} dose was lower in cough hypersensitive patients than their age and sex matched healthy control pair. In order to compare the groups on the basis of equivalent doses (matched capsaicin dose), the cough hypersensitivity patients inhaled their personalised S_{max} dose and the healthy controls also inhaled the S_{max} dose of their individually matched cough hypersensitive pair. In almost all cases, the paired dose for the healthy controls was below there own S_{max} dose by 1 to 4 dose increments. Comparisons were also made between the groups using the personalised S_{max} dose for all participants. This comparison involved a systematic difference in mean doses because the patients were more sensitive, but constituted an equivalent sensory and motor event because the two groups reported similar levels of urge-to-cough and were both maximally attempting to suppress cough (matched urge-to-cough sensation).