Materials and methods

Participant recruitment and experimental protocol

All participants provided consent to be involved in the study in compliance with procedures approved by the Melbourne Health Human Research Ethics Committee (approval number 2013.262) and with the 1964 Helsinki declaration and its later amendments. 16 smokers without history of lung disease and 16 age and sex matched healthy non-smoking controls were recruited (10 males, 6 females, mean age 34.0 $\pm$ 12.2 years and 30.6 $\pm$ 12.3 years respectively). To avoid potential environmental and socio-demographic differences between groups, both groups of participants were recruited via advertisements displayed around the University of Melbourne campus; which included designated smoking areas that are dispersed around the University. Smokers were defined as having smoked at least five cigarettes per day for more than one year. All smokers supplied information on their smoking history (i.e., how many cigarettes they smoke per day on average and how many years they have been smoking) to calculate smoke exposure (i.e., pack-years).

Participants were excluded if they had recently experienced an acute respiratory infection 8 weeks preceding the experimental session, suffered from claustrophobia, or had a history of pathology likely to influence respiratory and brain function such as a history of brain injury and pulmonary, vascular or neurological diseases. Given the absence of prior studies, along with logistical issues associated with combining data from adults and children (e.g., problems in registering different sized brains), the study was restricted to adults (aged 18 years and over). Pregnant women were also excluded from participation due to the unknown effects of MRI on foetal development. People with intellectual or mental impairment and people taking psychotropic medication were also excluded from the study due to potential effects on brain regions and functions of interest.

Due to the potential effects of nicotine and carbon monoxide that could influence BOLD signals, smoker participants were asked not to smoke any cigarettes three hours prior to the scanning session. Studies have shown that blood nicotine levels return to baseline within three hours [1]. An additional confound that warrants discussion is the potential effect of elevated levels of carbon monoxide in smokers. A recent study reported the vulnerability of the fMRI (BOLD) contrast to low-level carbon monoxide exposure during hypercapnia, visual stimulation and finger-tapping tasks in participants with no history of smoking [2]. Carbon monoxide interactions with haemoglobin has a reported half-life of 4-6 hours, allowing for the persistence of caboxyhaemoglobin even 3 hours after smoking (the minimum time between

the last cigarette smoked and the time of scan in our study). To help control for the effects of carbon monoxide, we included saline inhalation challenges as a contrast against the capsaicin inhalation challenges. By doing so, the fMRI responses observed following capsaicin will reflect the difference in neuronal activation between capsaicin and saline, independent of the effects of carbon monoxide. Smoker participants completed the Wisconsin Smoking Withdrawal Scale (WSWS) to ensure that there were no significant withdrawal effects caused by the short period of smoking deprivation [3]. They also completed the Fagerström Test for Nicotine Dependence (FTND), which is a self-report measure of dependency on nicotine [4].

Psychophysical testing session

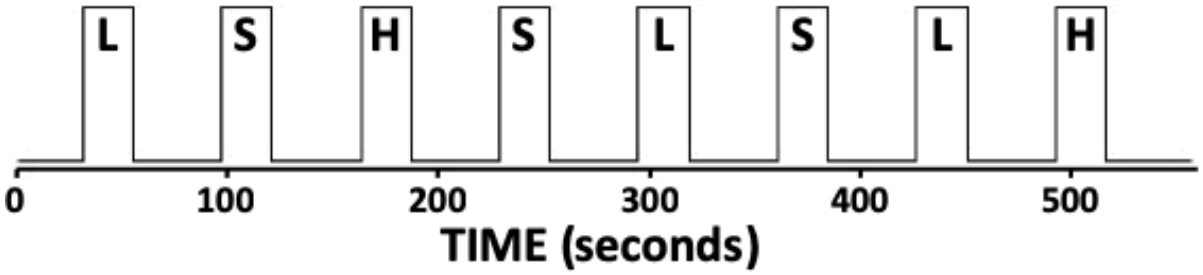
All recruited participants first underwent a psychophysical testing session where their level of sensitivity to capsaicin, the active ingredient of chilli peppers, was measured using method of limits. Participants inhaled a single vital capacity of nebulised capsaicin prepared in doubling concentrations (0.06 – 125µM) and rated their urge-to-cough sensation on a numerical rating scale (0, no urge-to-cough; to 10, maximum urge-to-cough). Capsaicin (Sigma, Sydney Australia, product #M2028) was delivered by an MRI compatible jet nubulizer (Allersearch, New South Wales, Australia) driven by medical air at flow rate of 0.7 mL/min) as previously described [5-7]. Cough frequency was noted after each challenge. Urge-to-cough threshold (C_u) was determined as the minimum concentration of capsaicin needed for the participant to perceive an urge-to-cough sensation (i.e., first instance of non-zero urge-to-cough rating). Cough motor threshold (C2) was determined as the concentration of capsaicin needed to elicit two or more coughs. The highest concentration that could be inhaled repetitively for 24 seconds without a coughing event was defined as the maximum suppressible (S_{max}) concentration and this concentration was used in the subsequent scanning session. This ensured that the concentration of capsaicin was tailored for each participant. After C_u, C2 and S_{max} concentrations were determined, a further 10 stimuli (2 x 5 concentrations) were delivered in randomised order to generate a stimulus response function as follows, i) capsaicin concentration at C2, ii) one concentration increment below and above the C2 (C2 ± 1) and iii) two concentration increments below and above the C2 (C2 ± 2). Participants were asked whether they experienced any other sensation during capsaicin inhalation (6 out of 32 participants (18.75%) reported ancillary effects; 3 participants had urge to sneeze and 3 participants had a runny nose, unrelated to smoking). Importantly, the general linear modelling of the task used for fmri analysis (see below) was tightly aligned to the onset and offset timing of capsaicin inhalation through the mouth, thereby excluding any persistent sensations, such as nasal burning, from confounding the resultant activation maps.

MRI testing session

The imaging protocol included 8 blocks of 24-second periods where the participants were administered either saline, a low or a high concentration of capsaicin in a randomised order interspersed by 42 seconds of no-stimulation periods. Participants were asked to rate the level of urge-to-cough after each capsaicin challenge by hand, as trained during the psychophysical session. During the scan, the nebulised capsaicin and saline were administered through a facemask and excess vapour exhausted via an outlet at the bottom of the mask to avoid irritation of the eyes, as described previously [5-7]. Participants were instructed to breath orally.

Two different concentrations of capsaicin (high and low concentration) were administered to participants during scanning. The higher concentration of capsaicin inhaled by participants was their individually tailored S_{max} concentration. This meant that concentrations varied among the participants and between the groups according to each participant's level of sensitivity. This stimulus level allowed for a group comparison that would identify differences in brain responses when all participants were having a comparable behavioural experience. The lower concentration of capsaicin inhaled was tailored to be equal (ie the same concentration) between matched pairs of smokers and controls, to allow for comparisons of brain activity between pairs of participants during inhalation of an identical stimulus intensity. In some instances, differences in sensitivity meant that a S_{max} concentration for a control was the low concentration for the paired smoker (i.e., two concentrations below the S_{max} concentration of smoker). In these cases, the control pair received their S_{max} concentration as the "high" concentration, equivalent to the "low concentration of their matched smoker, as well as their own "low" concentration, which was two concentrations lower than their own S_{max} concentration. Thus, a control in a pairing of this type received a similar stimulus format to other participants, where their S_{max} concentration was their high concentration and two concentrations below their S_{max} was their low concentration. Contrasts between the "like-stimulus" concentrations were treated as dependent comparisons, whereby the pairing of a smoker with a control of the same sex, same age, and inhaling the same concentration of capsaicin was modelled as a repeated measure. This paired approach involves variance between pairs in concentration levels but allows for a meaningful comparison that highlights differences between smokers and controls when challenged with the same level of stimulus. The pairing strategy stems from the impracticality of administering a single concentration to all participants. The wide range of sensitivity to capsaicin typically encountered in humans usually means that no single concentration can be chosen that wouldn't be imperceptible or cause uncontrolled coughing in some members of the sample.

The order of presentation of stimulus types (saline, low capsaicin, high capsaicin) during scanning was different for each of the three scans. Eight blocks of stimuli during a scan meant that one stimulus contingency occurred twice, whereas the other two occurred three times. The twice occurring stimulus was counterbalanced across the scans to ensure that each of the stimulus types was delivered on eight occasions across the three scans (see figure 1).



ORDERS

Scan 1	L	S	H	S	L	S	L	H
Scan 2	L	S	H	S	L	H	S	H
Scan 3	L	H	S	H	L	S	H	L

Figure 1. Experimental design schematic showing the timing and order of experimental challenges. Each participant underwent three scanning runs (scans 1-3). Each run involved eight blocks of nebulised challenges consisting of either saline (S), a low personally relevant concentration of capsaicin (L) or a high personally relevant concentration of capsaicin (H). The order of the challenges was varied across runs, although each participant received in total 8 challenges with each stimulus. Participants were informed visually immediately prior to the onset of a challenge but were blinded as to the identity of the challenge.

Image acquisition parameters

Scanning was performed at the Murdoch Children's Research Institute (Melbourne, Australia) using a Siemens Trio 3T scanner (Siemens) with a 32 channel head coil. Structural T1-weighted images were acquired in the sagittal plane (192 slices, 0.90 mm slice thickness, 0.84 × 0.84 mm^2 in-plane resolution, echo time (TE) = 2.59 ms, repetition time (TR) = 1900 ms, flip angle = 9°). Blood oxygen level dependent (BOLD) contrast echo-planar images (EPI) were acquired in the transaxial plane (36 slices, 4 mm slice thickness, 3.28 × 3.28 mm^2 in-plane resolution, TE = 32 ms, TR = 2000 ms, flip angle = 90°), producing a total of 279 sequential

volumes in 9:18 minutes of scanning time. Three EPI series were collected from all participants.

Imaging analysis

Statistical analyses of behavioural measures were performed with SPSS 21.0. Image analysis was performed with FMRIB Software Library (FSL) Expert Analysis Tool (FEAT) in FSL version 4.1 [8]. Regressors representing the timing for blocks of stimuli-inhalation challenges (saline, low and high concentration of capsaicin) and rating events were included in a general linear model that included motion parameters, as well as nuisance regressors as confound variables to take account of physiological noise. 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). To further dissociate vascular effects of respiration from its neuronal respiratory stimulant effect, the saline inhalation challenges were contrasted against capsaicin inhalation challenges (i.e., contrasts were made for low concentration > saline, high concentration > saline and low concentration + high concentration > saline). The saline contrast was used to take account of shared attributes such as BOLD signal variance associated with the generic aspects of chemical inhalation and respiratory control of brief breath holding prior to tidal volume inhalation of the stimulus [7]. Since all blocks of stimuli have a BOLD signal increase associated with respiratory fluctuation seen in the saline inhalation challenges, this event was used as the baseline. Brain regions activated due to task-related neural activations has a stronger relationship between the BOLD signal and the explanatory variables; and hence when contrasted with the baseline saline inhalation challenges, the BOLD response observed would represent the task-related neural activity above the baseline level [9]. An additional regressor calculated by averaging the time series of all non-activated voxels in preliminary analyses for each participant was also included to take further account of global signal variance associated with physiological noise, [6, 10]. Comparisons of explanatory variables were performed to identify regions showing increased BOLD signal activity during different concentrations of capsaicin inhalation challenges and innocuous saline stimulation.

Contrasts for high and low capsaicin concentrations were averaged across the three scans for each participant and used in the analysis of group and between-group effects. Significant activations for these primary group effects were determined using a single voxel inclusion threshold of $z > 3.09$ and a cluster-wise FWE-corrected threshold of $p_{corr}<0{\cdot}05$ corrected for

multiple comparisons [11, 12]. For between-group analyses, two separate contrasts were made. The first group contrast was performed for paired concentrations (matched capsaicin concentration; paired between control and smoker). For this matched capsaicin concentration group comparison, only 15 pairs were included (not the original 16 pairs) as one smoker-control pair did not inhale matching capsaicin concentrations due to the smoker having a higher "low" concentration than the matched control's S_{max} concentration. The other group contrast was performed during the inhalation of high concentrations for all participants (high urge-to-cough sensation for all participants; unpaired). As mixed-effects model, a conservative test, was used to test between-group differences, significant activations were determined using a single voxel inclusion threshold of $z > 2.3$ and a cluster-wise FWE-corrected threshold of $p_{corr}<0{\cdot}05$ corrected for multiple comparisons [11, 12].

Univariate correlation analyses were done with demeaned measures (i.e., mean value was subtracted from the original value) of smoke exposure measured by pack-years (number of packs per day smoked multiplied by the number of years smoked). This analysis allowed the identification of brain regions where variance in levels of capsaicin-inhalation activation among the group of smokers was explained by the severity of smoking behaviour. Significant activations were determined using a single voxel inclusion threshold of $z > 2{\cdot}3$ and a cluster-wise FWE-corrected threshold of $p_{corr}<0{\cdot}05$ corrected for multiple comparisons [11].

Region of interest analyses were performed to calculate the mean percentage BOLD signal changes in regions that showed significant group differences in capsaicin-inhalation activation, and in those regions where activation levels were associated to pack-years in the smokers.

Supplementary References

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