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Altered neural activity in brain cough suppression networks in cigarette smokers

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Abstract

Cough is important for airway defence and studies in healthy animals and humans have revealed multiple brain networks intimately involved in the perception of airway irritation, cough induction and cough suppression. Changes in cough sensitivity and/ or the ability to suppress cough accompany pulmonary pathologies, suggesting a level of plasticity is possible in these central neural circuits. However, little is known about how persistent inputs from the lung might modify the brain processes regulating cough. In the present study, we used human functional brain imaging to investigate the central neural responses that accompany an altered cough sensitivity in cigarette smokers. In non-smokers, inhalation of the airway irritant capsaicin induced a transient urge-to-cough associated with the activation of a distributed brain network that included sensory, prefrontal and motor cortical regions. Cigarette smokers demonstrated significantly higher thresholds for capsaicin-induced urge-to-cough, consistent with a reduced sensitivity to airway irritation. Intriguingly, this was accompanied by increased activation in brain regions known to be involved in both cough sensory processing (primary sensorimotor cortex) and cough suppression, (dorsolateral prefrontal cortex and the midbrain nucleus cuneiformis). Activations in the prefrontal cortex were highest among participants with the least severe smoking behaviour, whereas those in the midbrain correlated with more severe smoking behaviour. These outcomes suggest that smoking-induced sensitization of central cough neural circuits is offset by concurrently enhanced central suppression. Furthermore, central suppression mechanisms may evolve with the severity of smoke exposure, changing from initial prefrontal inhibition to more primitive midbrain processes as exposure increases.

Introduction

Cough is a neural process that plays an important role in airway defence and the maintenance of adequate ventilation. Functional brain imaging studies in humans and neuroanatomical investigations in animals provide insight into the central (brain and brainstem) sensory and motor networks involved in cough under nonpathological circumstances [1-5]. However, cough sensitivity can become altered, especially in disease states and from psychophysical (e.g., placebo or cognitive regulation of cough) or environmental (e.g., chronic exposure to airway irritants) factors [6-11]. Indeed, both enhanced and reduced cough sensitivity are possible, producing excessive or insufficient cough responses that prove challenging to control in the clinic [12].

The neural processes involved in altered cough sensitivity are poorly understood. Although altered airway peripheral sensory nerve activity and sensitivity is undoubtedly involved, aspects of the dynamic and chronic malleability of cough sensitivity is indicative that central mechanisms might also shape the sensorimotor processing of cough-related stimuli [7, 10, 13]. We recently reported alterations in the brain responses accompanying airway irritations in patients with chronic cough, demonstrating increased activity in cough sensory processing nuclei (evidence for sensitization) accompanied by concomitant reduced activity in brain regions involved in cough suppression (indicative of reduced inhibitory control) [7]. Consistent with this, Cho and colleagues [14] recently reported that patients with chronic refractory cough display significantly reduced capacity to voluntarily suppress capsaicin-evoked coughing, while also being hypersensitive to cough-evoking stimuli. Thus, the relative balance between facilitatory and inhibitory brain processing may be central to the development or maintenance of altered cough sensitivity in different pathologies.

In the present study, we reasoned that the brain processes involved in altered cough sensitivity could be further investigated using functional brain imaging in cigarette smokers because they typically demonstrate reduced cough reflex sensitivity to inhaled challenge

stimuli [13, 15, 16]. The previously reported central neural correlates of the urge-to-cough, cough induction and cough suppression mechanisms in healthy humans and in people who suffer from chronic cough provides a strong *a priori* prediction for how cigarette smoking could affect the central nervous system regions regulating cough [2-4, 7, 10]. Reductions in brain region responsivity associated with cough sensory processing, including in the sensorimotor or anterior insula cortices (important for urge-to-cough perception and stimulus intensity grading), posterior or parietal cortices (involved in spatial discrimination) or in medullary and pontine neural circuitry (needed for integrating airway primary afferent inputs for reflex coughing) might be associated with reduced evoked cough sensitivity in smokers. Alternatively, changes in cough reflex thresholds observed in smokers could be due to enhanced cough suppression network activity, perhaps because exposure of the airways to smoke enlists tonic central adaptive processes to countervail the unwanted perceptions or responses to irritation. Brain regions involved in the voluntary suppression (cingulate cortex, inferior frontal gyrus, anterior insula and supplementary motor area) or involuntary suppression (dorsolateral prefrontal cortex, midbrain) of cough and the urge-to-cough could display enhanced activity in smokers [4, 10, 17].

Materials and methods

Participant recruitment and experimental protocol

All participants consented 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 Helsinki declaration. 16 active smokers without history of lung disease and 16 age and sex matched non-smoking controls were recruited (10 males, 6 females, mean age 34.0 ± 12.2 years and 30.6 ± 12.3 years respectively). Smokers were defined as having smoked at least five cigarettes per day for more than one year.

Exclusion criteria included a respiratory infection during the 8 weeks preceding the experimental session, claustrophobia, a history of respiratory or brain pathology, pregnancy, intellectual or mental impairment and use of psychotropic medication. Smoker participants abstained from cigarettes three hours prior to the scanning session. Smokers completed the Wisconsin Smoking Withdrawal Scale (WSWS) to ensure that there were no significant withdrawal effects caused by the short period of smoking deprivation [18] and the Fagerström Test for Nicotine Dependence (FTND), which is a self-report measure of dependency on nicotine [19].

Psychophysical testing session

Sensitivity to capsaicin, the active ingredient of chilli peppers, was initially assessed using a modified fMRI adapted protocol. Participants inhaled a single vital capacity of capsaicin vapour delivered from an MRI compatible jet nebulizer, prepared in doubling concentrations (0.06 – 125µM) and rated their urge-to-cough sensation (0, no urge-to-cough; to 10, maximum urge-to-cough). 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. Cough threshold (C2) was determined as the concentration of capsaicin needed to elicit two or more coughs. The $\log_{10}$ values of the three thresholds were tested for associations with pack-years in the group of smokers by calculating Pearson r correlation coefficients between these continuous variables. 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). Guided by these measures, the highest concentration of capsaicin that could be inhaled for 24 seconds (the challenge time used in fMRI) without coughing was

defined as the maximum suppressible (S_{max}) concentration. Participants were asked whether they experienced any other sensation during capsaicin inhalation.

MRI testing session

The imaging protocol included 8 blocks of 24-second periods where the participants were administered saline, a low or a high concentration of capsaicin in a randomised order interspersed by 42 seconds of no-stimulation periods (Figure 1). Participants rated the level of urge-to-cough after each capsaicin challenge.

High and low concentrations of capsaicin were administered. The high concentration was the individual's S_{max} concentration while the low concentration was tailored to be equal between matched pairs of smokers and controls. Thus, concentrations either (a) varied among the participants allowing for brain responses to be tested when participants were having a comparable behavioural experience (Like-Behaviour), or (b) allowed for comparisons of brain activity between participants during an identical stimulus intensity (Like-Stimulus).

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 as previously described [2, 4, 7, 10] and is described in detail in the supplementary methods.

Contrasts for high and low capsaicin concentrations against saline challenges were used in the analysis of group and between-group effects. Primary group effects were determined using a single voxel inclusion threshold of $z > 3.09$ and a cluster-wise familywise error (FWE)-corrected threshold of $p_{corr}<0{\cdot}05$ based on the FSL expert analysis tool (FEAT) implementation of the random field theory [20, 21]. A group contrast was performed for paired concentration between controls and smokers for the Like-Stimulus 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. An unpaired contrast of high concentrations for all participants was also performed to test the Like-Behaviour effect. Univariate correlation analyses were performed with demeaned measures of smoke exposure measured by pack-years (number of packs per day smoked multiplied by the number of years smoked) to identify brain region activations 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 $p_{corr}<0{\cdot}05$ [20].

Results

Behaviour

The smokers' mean score for the Fagerstrom Test for Nicotine Dependence was 3.2±1.6 (range 1–6), which indicates a low to moderate level of dependence on nicotine. The mean total score for smokers for the Wisconsin Smoking Withdrawal Scale was 11.6±3.2 (range 6.7–16.9) indicating that the smokers were neutral to all seven subscales (anger, anxiety, concentration, craving, hunger, sadness and sleep). Mean pack-years for smokers was 6.6±6.0 (range 0.38–23).

Smokers had significantly higher thresholds for C_u, C2 and S_{max} measures ($t_{(30)}$=2.502, P<0.018; $t_{(30)}$=2.820, P<0.008; $t_{(30)}$=2.096, P<0.045 respectively; Figure 2A, B). Repeated-measures ANOVA revealed a main effect for capsaicin concentration (F(4,120)=127.534, P<0.001). The groups did not differ in their urge-to-cough ratings of the personally relevant concentrations (F(1,30)=0.286, p=0.597), nor was there an interaction between group and concentration (F(4,120)=0.556, p=0.695). The number of coughs recorded during the randomised capsaicin challenges did not differ between the groups ($t_{(30)=}$1.1, ns) (box insert in Figure 2B). Capsaicin concentrations required to elicit a perceptible urge-to-cough $\log_{10}(C_u)$ were positively correlated with pack-years (r=0.687, P<0.003, 95% CI [0.29, 0.88]) in smokers, whereas thresholds to elicit coughing ($\log_{10}$(C2); r=0.224, P=0.403, 95% CI [-0.31, 0.65]) and maximum suppressible concentrations ($\log_{10}(S_{max})$; r=-0.205, p=0.447, 95% CI [-0.32, 0.64]) were not associated with smoking behaviour. Six 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. No patients reported eye irritation.

Brain Imaging

Capsaicin-induced brain activation for non-smoking controls and smokers

The group mean BOLD signal increase, irrespective of matched-capsaicin concentration or urge-to-cough sensation comparisons, was widely distributed across brain regions in the two participant groups including the cingulate cortex, supplementary motor area, primary somatosensory and motor regions (S1/M1), superior parietal cortex, frontal regions, insula, cerebellum, regions of the basal ganglia and brainstem at a cluster-wise FWE-corrected threshold of $p_{corr} < 0.05$ (voxel inclusion of Z>3.09) (Tables 1 and 2, see Supplementary figure 2 for activation maps), This pattern of activation was consistent with previous capsaicin inhalation studies [2, 4, 5].

Paired comparison: matched capsaicin concentrations (Like-Stimulus)

Non-smoking participants were more often inhaling the S_{max} concentration for the comparison of equivalent concentrations in pairs of smokers and controls (X^2=6.788, p<0.009). Ratings of urge-to-cough during inhalation of equivalent concentrations were 4.3±0.7 for non-smoking participants and 3.3±0.5 for smokers, but this difference was not significant (t(14)=1.2, ns; Figure 3A). Increased BOLD signal during inhalation of equivalent concentrations of capsaicin between pairs was noted in the orbitofrontal cortex, bilateral ventral accumbens, cuneal cortex and cerebellum for controls greater than smokers at a cluster-wise FWE-corrected threshold of $p_{corr} < 0.05$ (voxel inclusion of Z>3.09) (Table 3; Figure 3B). The substantial BOLD signal change in the orbitofrontal cortex in controls was in contrast to that in smokers where the BOLD signal change in this region was negative/approaching zero ($t_{(14)}$=3.657, P<0.003) (Figure 3C). Smokers showed increased BOLD signal compared to the controls in the right sensorimotor ($t_{(14)}$=2.241, P<0.042) and left insula cortices ($t_{(14)}$=5.277, P<0.001) and regions of the basal ganglia including bilateral putamen ($t_{(14)}$=3.653, P<0.003) (Table 3; Figure 3D, E).

Between-group comparison: maximum suppressible concentration (Like-Behaviour)

Smokers tolerated significantly higher capsaicin challenge concentrations compared to controls ($t_{(30)}$=2.131, P<0.041), despite no significant differences in reported urge-to-cough between the two groups (Figure 4A) and similar regional distributions of brain responses to S_{max} inhalation. However, the spatial distribution of sensorimotor activation in the smokers appeared to be more extensive than in controls (Figure 4B). A between-group difference was seen in sensorimotor cortex, as well as the posterior parietal cortex in smokers compared to controls at a cluster-wise FWE-corrected threshold ($Z > 2.3$, $p_{corr} < 0.05$) (Figure 4C, Table 4). Notably, BOLD signals extracted from the left posterior parietal and extended region of the left sensorimotor cortices showed positive increases during inhalation of S_{max} concentrations in the smokers whereas controls showed negative or no mean signal change during the same stimulus (Figure 4D).

Correlations between regional capsaicin activations and pack years in smokers

Both negative and positive associations were identified between the severity of smoking behaviour and brain regional responses (Figure 5). Negative correlations between S_{max} activation levels and pack years were evident in the superior frontal gyri and middle frontal gyri bilaterally (Table 5, Figure 5A), as well as in the left precentral gyrus. Estimates of %BOLD signal change in the middle frontal gyri were predicted by pack years, with an R^2=0.330 in the right hemisphere, and an R^2=0.568 in the left.

Positive correlations between pack years and S_{max} activation were evident in bilateral peaks of activation in the lateral midbrain (Figure 5C, Table 5), two rostrocaudal levels of the pons (Figure 5D, E, Table 5), a central location of the rostral, dorsal medulla (Figure 5F, Table 5) and in symmetrical cerebellar regions incorporating the dentate nuclei (Table 5). Estimates of %BOLD signal change from clusters of activation in the brainstem were regressed against pack years and showed levels of shared variance ranging from R^2=0.203 to R^2=0.583.

DISCUSSION

Although cough can be a troubling symptom associated with cigarette smoking, smokers often display reduced sensitivity in evoked cough reflex testing indicative of complex peripheral or central sensory nervous system adaptation to chronic airway stimulation [13, 15, 16]. We similarly demonstrated reduced sensitivity to airway challenge with capsaicin in smokers compared to non-smokers and showed this to be reflected by differences in capsaicin-evoked brain responses. Although smokers showed increased activation in brain regions thought to encode airway sensations, there was a concomitant increase in putative central inhibitory network activity. Interestingly, brain responses to capsaicin inhalation among smokers were shown to vary according to individuals' smoking histories with a transition from prefrontal (dorsolateral prefrontal cortex) to midbrain (nucleus cuneiformis) activations as the severity of smoking behaviour increased. Both the DLPFC and nucleus cuneiformis have been implicated in the modulation of responses to tussive stimuli in other circumstances [7, 10], suggesting an evolving pattern of brain processing as smoking behaviour persists. Smoking was also associated with decreased capsaicin-evoked activation compared to controls in the orbitofrontal cortex and cerebellum, although these differences were only seen when participants inhaled comparable capsaicin concentrations and not when challenged with concentrations eliciting maximum levels of cough suppression. Collectively, these data further highlight the importance of central facilitatory and inhibitory neural network balance for shaping sensory and motor responses to airway irritation.

Evidence for altered airway sensory processing in the brain of smokers

Capsaicin inhalation challenge of both smokers and controls activated brain regions that are consistent with previous reports, and adds further support to the conclusion that airways irritation and associated processes are represented in a distributed brain network [1, 2, 7, 22-24] The constituent regions of the network include prefrontal, cingulate, sensorimotor, posterior parietal and insula cortices, as well as thalamus, cerebellum, basal ganglia and brainstem. Differences between smokers and controls did not occur uniformly throughout the brain regions activated, which implies differential effects of smoking on functional modules within the broader network.

Regional capsaicin activation levels in smokers showed both increases and decreases relative to non-smoking controls. Most notably, the primary somatosensory and posterior parietal cortices were more activated in the smokers. However, the regions of somatosensory cortex commonly activated in both groups did not show differences in BOLD signal levels. Instead, the differences occurred in neighbouring lateral cortical regions where activation occurred exclusively in the smokers. These patterns of BOLD signal changes suggest an expanding representation of the airways in the somatosensory cortices of the smokers. Changes in the somatosensory homunculus have been reported in response to alterations of sensory input, and representations have shown both expansions and contractions depending on whether afferent inputs from the periphery increased [25-28] or decreased [29-32].

The increased spatial extent of somatosensory responses in the smokers occurred in the context of decreased acuity for detecting capsaicin and a rightward shift of urge-to-cough ratings. It has been speculated that a loss of sensitivity to tussive stimuli in smokers could be due to functional loss of airway afferents [15]. However, other conditions involving deafferentation are usually associated with shrinking

somatosensory representations [30, 32], and consequently the expansion seen in the cortices of the smokers is not consistent with a loss of airway afferents. Studies demonstrating rapidly reversible changes in sensory sensitivity when smoking is ceased and resumed again also argue against any substantive loss of afferent nerve terminals themselves as a consequence of smoking [16]. It may be that other factors, including the influence of nicotine [33, 34], discrepant impacts of embryologically different sensory neural inputs [23, 24], or the recruitment of a countervailing central neural process could (present study) explain the concurrence of increased somatosensory activation with decreased subjective sensitivity to capsaicin inhalation in the smokers.

Capsaicin-inhalation activation in the controls exceeded that of the smokers in the orbitofrontal cortex and cerebellum, although this was confined to the contrast involving paired concentrations and not when concentrations were adjusted to produce similar levels of urge-to-cough ratings. The activations in the orbitofrontal cortex and cerebellum may therefore have reflected a more challenging situation for the control participants, a greater proportion of whom were inhaling the maximum suppressible concentration. The unpleasantness of the experience when inhaling a more challenging concentration could have been a factor in shaping the activation levels, most notably in the orbitofrontal cortex which has a role in emotional responses to sensory experiences [35, 36].

Evidence for altered central inhibitory network activity in smokers

Previous studies by our group have implicated prefrontal and midbrain regions in the modification of urge-to-cough. For instance, bilateral activation in the dorsolateral prefrontal cortex accompanies placebo-related decreases in urge-to-cough [10], and

the loci of these activations are similar to the regions that were increased to a greater degree among participants with the least severe smoking behaviour. Additionally, capsaicin-inhalation responses in midbrain regions encompassing nucleus cuneiformis distinguish patients with chronic cough, and activation levels in these regions correlate with airway sensitivity in hypersensitive cough patients [7]. The severity of smoking behaviour bore relationships with regional levels of capsaicin-inhalation activation. Relatively increased capsaicin-inhalation activations in the dorsolateral prefrontal cortex were seen among participants with the lowest pack years, whereas a more severe smoking behaviour was associated with increased activation in the midbrain and hindbrain.

The midbrain regions showing a positive association with smoking behaviour are very similar to that identified in chronic cough patients [7]. A parsimonious explanation for the respective positive and negative correlations between pack years and regional capsaicin-inhalation activation levels is that modulation of airway afferent input undergoes development as the severity of smoking behaviour increases. It could be that activity in prefrontal regions represents recruitment of cognitive processes that down-regulate responses to airway afferent inputs when smoking behaviour is less severe, possibly due to the motivational drive to continue smoking despite the frequent irritation of the airways. As smoking behaviour increases, prefrontal responses may be replaced by activity in midbrain and brainstem regions that directly regulate incoming airway afferent processing. It is salient to note that increasing pack years were associated with increases in thresholds to detect an urge-to-cough (i.e. less sensitivity), which supports speculation that midbrain and brainstem responses could contribute to decreased sensitivity to capsaicin challenge among smokers and may be an adaptive response in chronic coughers [7, 17, 37] in attempts to suppress persistent airway inputs.

Conclusion

This study demonstrates that cigarette smoking may impact on the central processing of airway sensations. Adaption to regular airway irritation in smokers may involve the development of responses with the potential to modulate airway sensory processing. One caveat of the study is that we assess pulmonary function in smokers, and although they universally reported no history of lung disease, we cannot exclude the possibility that the brain imaging results are impacted by an underlying pathology. Additionally, we can only speculate about the molecular mechanisms underlying the observed functional effects. Chronic exposure to nicotine is known to upregulate the expression of many nicotinic receptor subunits, both peripherally and in the central nervous system, and this is thought to contribute to the addictive properties of smoking [38]. Airway sensory neurons are directly activated by nicotine to induce coughing, notably via $\alpha 3,\beta 4$ nicotinic receptors [39]. Upregulation of these would be expected to enhance sensitivity to airway delivery of nicotine, perhaps consistent with the increased neural activation seen in the brain regions directly encoding these sensory inputs. Conceivably, the enhanced activity of central suppressive networks may also be nicotinic receptor dependent. Consistent with this, a single inhaled exposure to nicotine in non-smokers is sufficient to reduce cough and urge-to-cough evoked by inhaled capsaicin [33, 34]. Smoking may further enhance inhibitory processing by upregulating nicotinic receptor expression in these central suppressive pathways. In this regard, the outcomes of current early phase trials with centrally acting $\alpha 7$ nicotinic receptor agonists in chronic refractory cough will be interesting.

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Table 1 Capsaicin-inhalation activation for matched capsaicin concentration in control and smoker participants

Regions	Controls				Smokers			
	Peak coordinate		voxel		Peak coordinate		voxel	
	x	y	z	Z score	x	y	z	Z score
Precuneous cortex	2	-76	48	5.54				
Anterior midcingulate cortex	-6	22	32	5.03	-2	36	22	4.60
Posterior midcingulate cortex					-4	-32	26	4.53
SMA	10	10	56	5.01	-4	2	54	3.97
S1/M1	48	-10	34	4.20	58	2	40	5.09
Superior parietal cortex					-58	-38	36	3.88
Operculum	-56	-2	8	4.53	52	-6	8	4.90
Orbitofrontal cortex	50	22	-10	5.07	50	18	-10	3.97
Inferior frontal gyrus	48	16	-6	4.90	-56	8	0	5.29
Insula	40	22	-8	4.79	-38	-6	0	5.34
Putamen					-30	-8	6	5.43
Thalamus	2	-18	8	3.84	12	-14	6	5.13
Pons					8	-32	-26	4.79
Medulla	12	-34	-50	3.74	-8	-40	-40	3.90
Cerebellum	4	-62	-8	4.92	12	-56	-30	5.14

Table 2 Capsaicin-inhalation activation during S_{max} inhalation in control and smoker participants

Regions	Controls				Smokers			
	Peak coordinate		voxel		Peak coordinate		voxel	
	x	y	z	Z score	x	y	z	Z score
Precuneous cortex	10	-76	52	5.15	-12	-74	38	4.28
Anterior midcingulate cortex	-12	16	38	4.65	6	26	38	6.69
Posterior midcingulate cortex					4	-22	30	6.42
SMA	-8	-10	58	4.74	-10	-2	64	4.21
S1/M1	-34	-12	46	4.73	-58	-24	24	6.72
Superior parietal cortex	54	-30	40	4.51	56	-40	42	5.51
Operculum	58	-10	6	4.04	-52	-2	10	9.93
Orbitofrontal cortex	48	18	-8	3.79	-46	14	-12	5.72
Inferior frontal gyrus	-58	4	2	4.86	-52	-2	12	10.38
Insula	34	14	6	4.31	38	10	8	5.67
Globus Pallidus	18	2	0	3.12				
Putamen					-26	-8	6	7.01
Thalamus	-4	-14	4	4.81				
Pons	8	-38	-24	3.57	8	-36	-24	4.77
Medulla	10	-40	-48	6.01	16	-36	-42	3.56
Cerebellum	0	-84	-26	6.08	14	-56	-34	7.87

Table 3 Between-group differences during pair-matched capsaicin concentration inhalation

Regions	**Controls > Smokers**			
	Peak voxel coordinate			
	x	y	z	Z score
Cuneal cortex	2	-76	50	3.55
Orbitofrontal cortex	22	20	-20	3.46
Cerebellum	-18	-82	-26	4.62
Regions	**Smokers > Controls**			
	Peak voxel coordinate			
	x	y	z	Z score
Post-central gyrus	66	-18	26	3.91
	58	-16	34	3.74
Inferior parietal lobule	60	-22	32	4.28
Insula	-36	-8	10	3.95
Thalamus	-6	-16	14	3.37
Caudate	20	16	6	3.24
Putamen	-28	0	-6	3.17
	22	10	2	3.31

Table 4 Between-group differences during S_{max} sensation

Regions	Smokers > Controls			
	Peak voxel coordinate			
	x	y	z	Z score
Posterior parietal cortex	-40	-38	36	3.30
Pre-central gyrus	-60	-2	34	2.96
Post-central gyrus	-60	-12	38	3.29

Table 5 Activation during S_{max} inhalation correlated with pack years of smoking

Regions	Positive correlation			
	Peak voxel coordinate			
	x	y	z	Z score
Midbrain	12	-24	-12	3.69
	-8	-26	-16	3.62
Pons	4	-30	-36	4.06
	-4	-28	-34	4.25
	8	-32	-40	4.35
	-6	-30	-40	4.37
Medulla	2	-38	-48	3.07
Cerebellum	18	-64	-38	3.80
	-12	-62	-36	4.27
Regions	**Negative correlation**			
	Peak voxel coordinate			
	x	y	z	Z score
Superior Frontal Gyrus	38	44	28	3.96
	30	56	24	4.70
Middle Frontal Gyrus	-34	40	34	4.24
	44	46	18	4.10

FIGURE LEGENDS

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.

Figure 2 Behavioural data representing cough sensitivity measured during psychophysical testing session. (A) Participants inhaled doubling concentrations of nebulised capsaicin during single maximum inhalations. Urge-to-cough ratings (numerical rating scale of 0-10) and cough events were recorded. Thresholds were measured for detection of urge-to-cough (C_u), provocation of two coughs (C2), and maximum suppressible concentration during 24-seconds of repeated inhalations (S_{max}). All three thresholds were significantly increased in the smokers compared to the controls. (B) Relationships between capsaicin concentrations and urge-to-cough ratings were assessed using repeated inhalations of five capsaicin concentrations at concentration increments less than, greater than and corresponding to participants' C2 thresholds. Cough frequency was also recorded (inset). The smokers showed a rightward shift of the stimulus/response function and there were no differences in cough frequently during challenges. Histograms represent the group mean ± S.E.M. **p<0.05, **p<0.01*

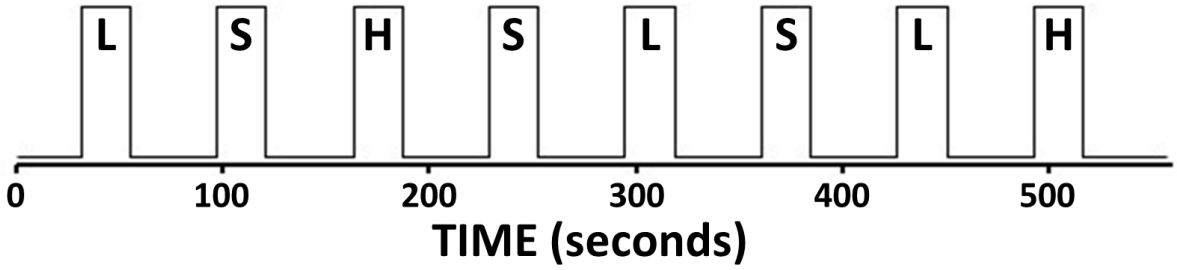
Figure 3 Brain responses during paired comparison of matched capsaicin concentration. Participants were challenged with low and high concentrations of capsaicin during fMRI scanning. "High" corresponded to participants' S_{max}, and smokers' "low" concentration was matched to controls. Not all smokers had a higher capsaicin threshold than their matched controls, in which case, at least one of the concentrations that the non-smoking controls inhaled was matched to the capsaicin concentration of their paired smoker for the matched capsaicin-concentration

between-group comparison. (A) The similar stimulus concentrations were associated with marginally, but not significantly, decreased urge-to-cough responses represented by their ratings in smokers (the difference in ratings did not reach significance (p<0.2). (B) Differential regional activation was seen where controls had increased BOLD signal in the orbitofrontal cortex (OFC) compared to smokers also evident in the line graph showing individual BOLD signal responses of the paired subjects (C). (D) Regions of the sensorimotor (S1/M1) and insula (Ins) cortices, as well as regions of the basal ganglia (in particular, putamen, Put) showed increased BOLD signal in smokers compared to controls (E). All axial brain images are presented in the neurological convention (left side of the page is the left hemisphere of the brain).

Activations have voxel inclusion of Z>2.3 and cluster-wise FWE-corrected threshold of p_{corr}<0.05. Histograms represent the group mean ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 4 Brain responses during between-group comparisons of maximum suppressible concentration comparison. Participants were stimulated with low and high concentrations of capsaicin during fMRI scanning. “High” corresponded to participants’ S_{max}. During matched urge-to-cough sensation comparison, all participants were inhaling their S_{max} concentration. (A) Despite a significantly lower mean stimulus in the controls compared to smokers, high concentrations were associated with similar mean urge-to-cough rating response between the two groups. (B) Logical map of group activation shows brain regions activated during match urge-to-cough sensation in controls (blue), smokers (red) and overlapping regions of activation (green)(Group activations have voxel inclusion of Z>3.09 and cluster-wise FWE-corrected thresholds of p_{corr}<0.05.). (C) Differential regional activation was seen where smokers had increased BOLD signal in the posterior parietal (PPC) and sensorimotor (S1/M1) cortices. (Activations for group differences have voxel inclusion of Z>2.3 and cluster-wise FWE-corrected thresholds of p_{corr}<0.05.) (D) Between-group differences are evident in the mean percentage BOLD signals extracted from both PPC and S1/M1 regions. All axial brain images are presented in the neurological convention (left side of the page is the left hemisphere of the brain). Histograms represent the group mean ± S.E.M. *$p<0.05$

Figure 5 Behavioural measure correlation analyses. (A) Levels of capsaicin-inhalation activation in the dorsolateral prefrontal cortices in both hemispheres were negatively correlated with pack years in the smokers group. (B) Positive correlations between pack years and levels of capsaicin-inhalation activation were seen in clusters distributed throughout the brainstem. Black lines through the expanded midline section indicate the position of axial slices in the remaining panels of the figure. (C) Symmetrical clusters in the lateral midbrain showed activation associated with pack years. (D) Capsaicin-inhalation activation in bilateral dorsal pons was positively correlated with pack years. (E) The rostral, ventral medulla contained symmetrical clusters of capsaicin-inhalation activation that correlated with pack years. (F) Pack years was positively correlated with capsaicin-inhalation activation in the dorsal medulla at a level approximately 10mm rostral of the obex. Left side of axial images is left side of the brain, and ventral is top of image. Activations have a voxel inclusion of $Z>2.3$ and a cluster-wise FWE-corrected threshold of $p_{corr}<0.05$.



ORDERS

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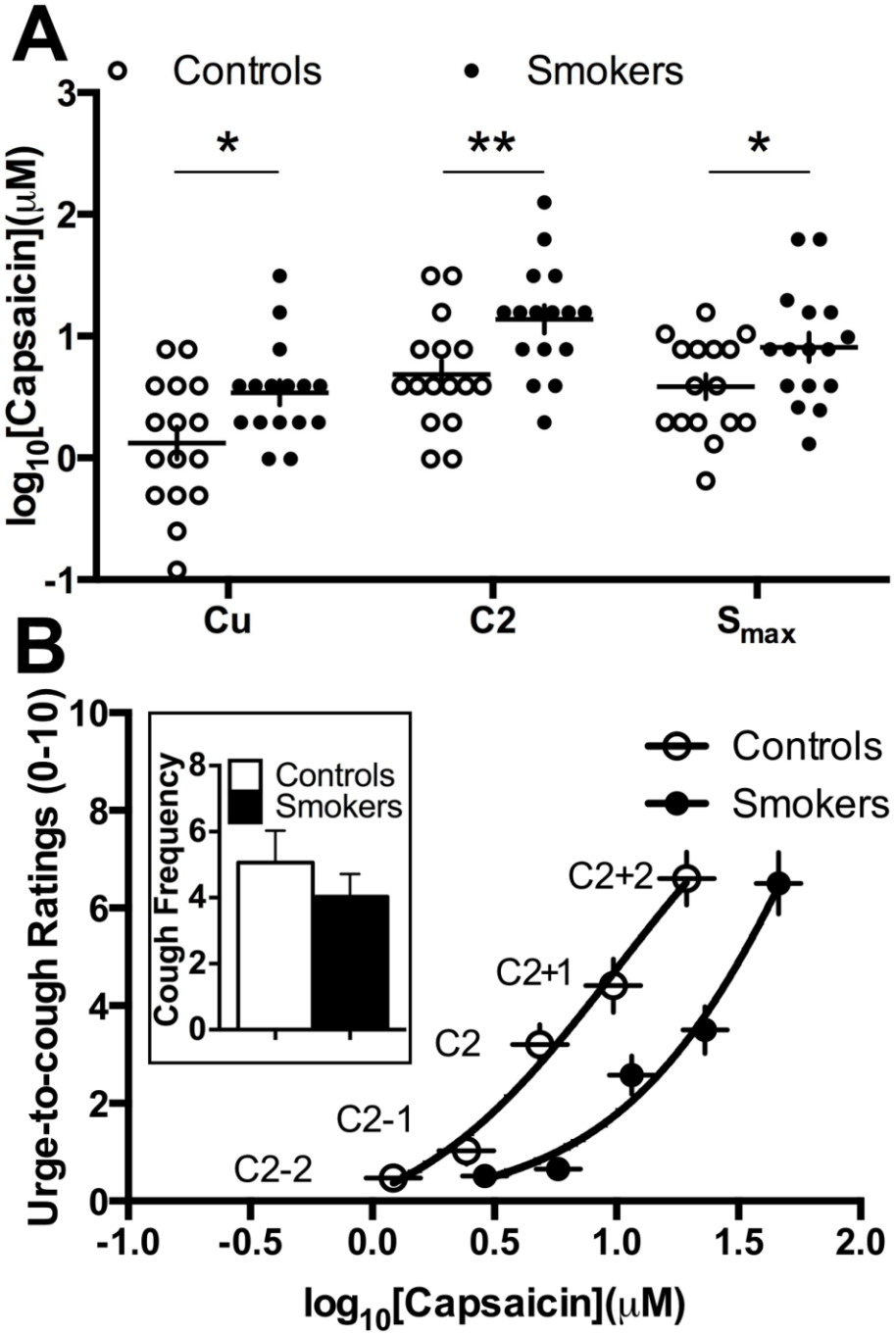
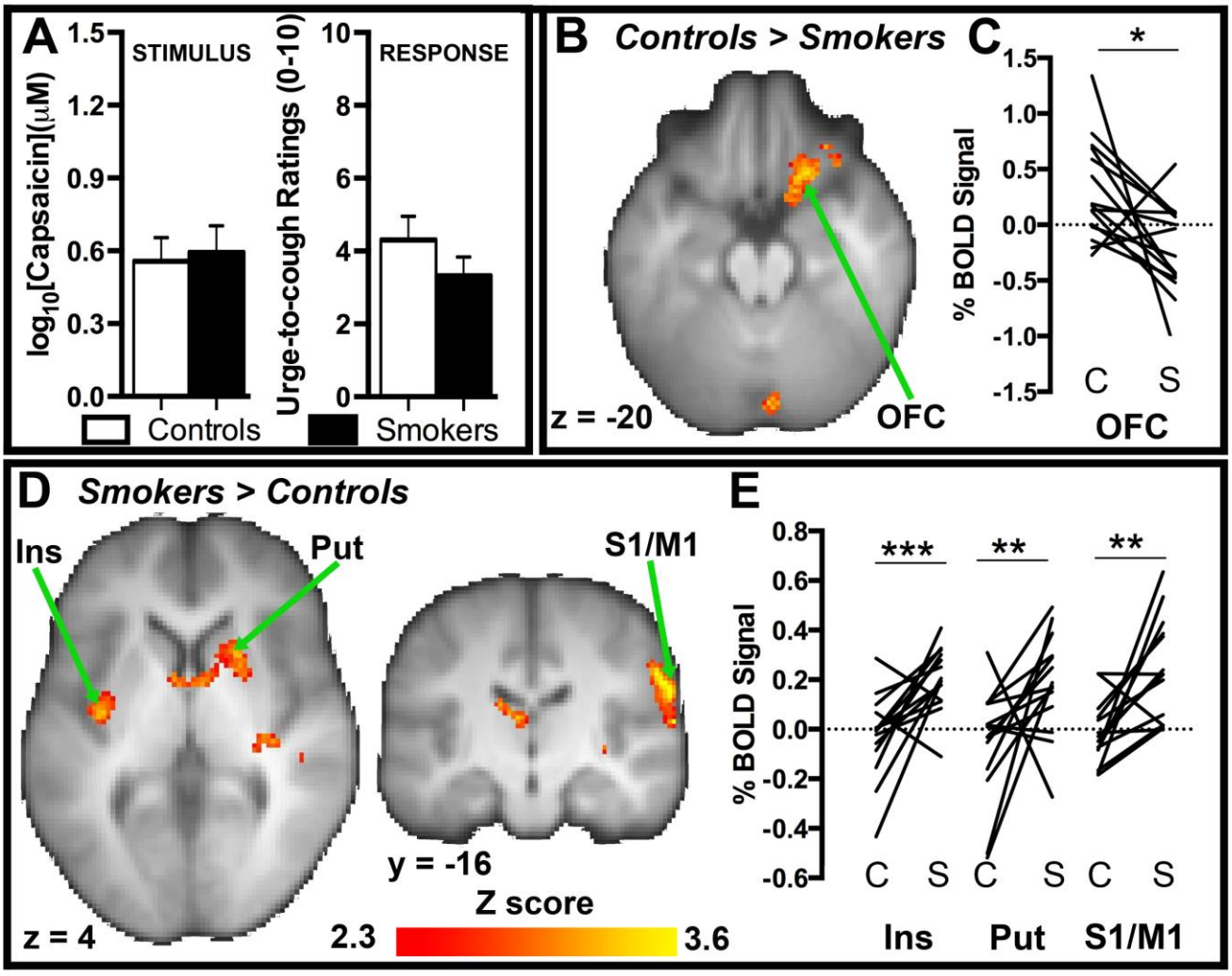
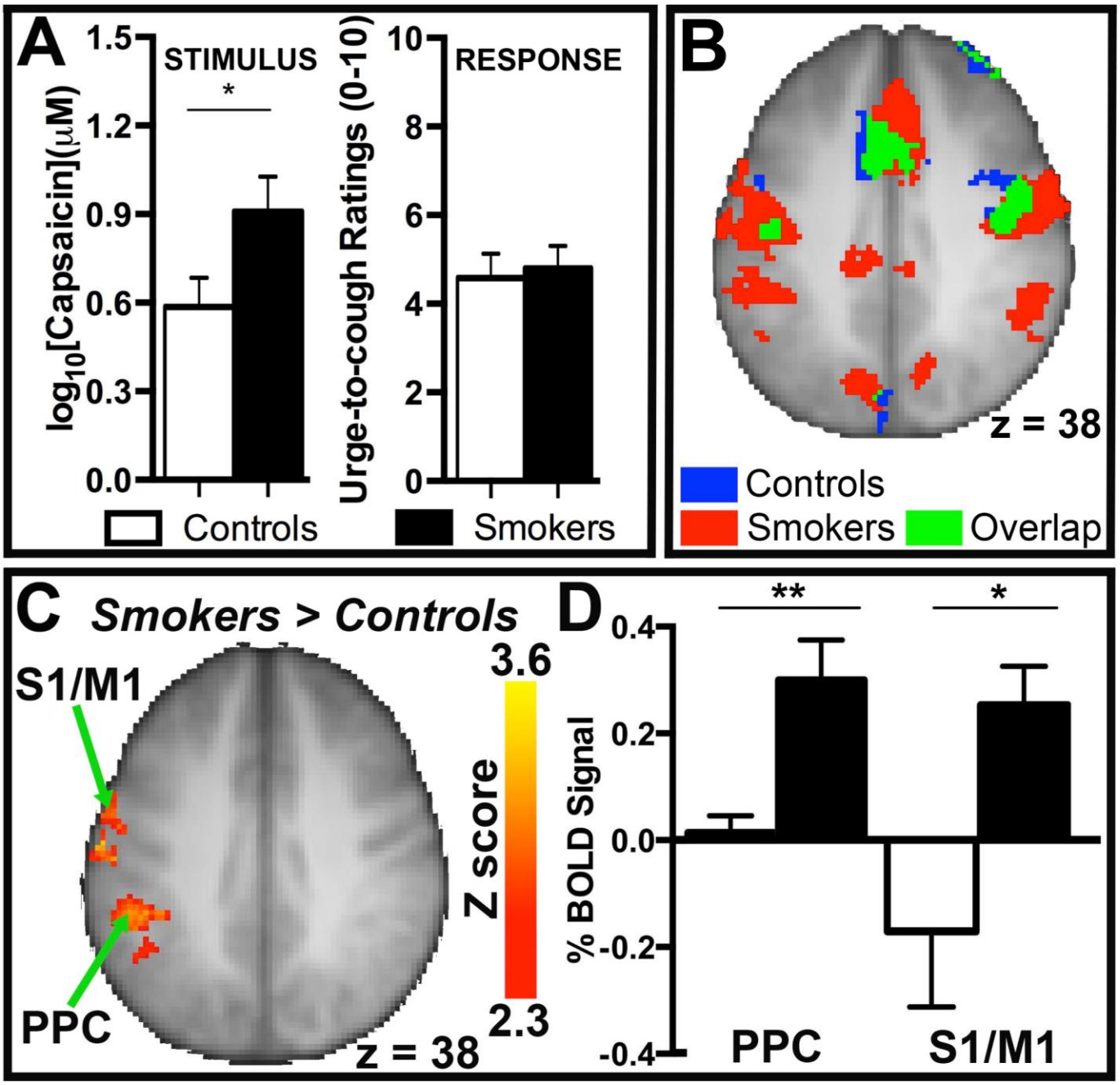
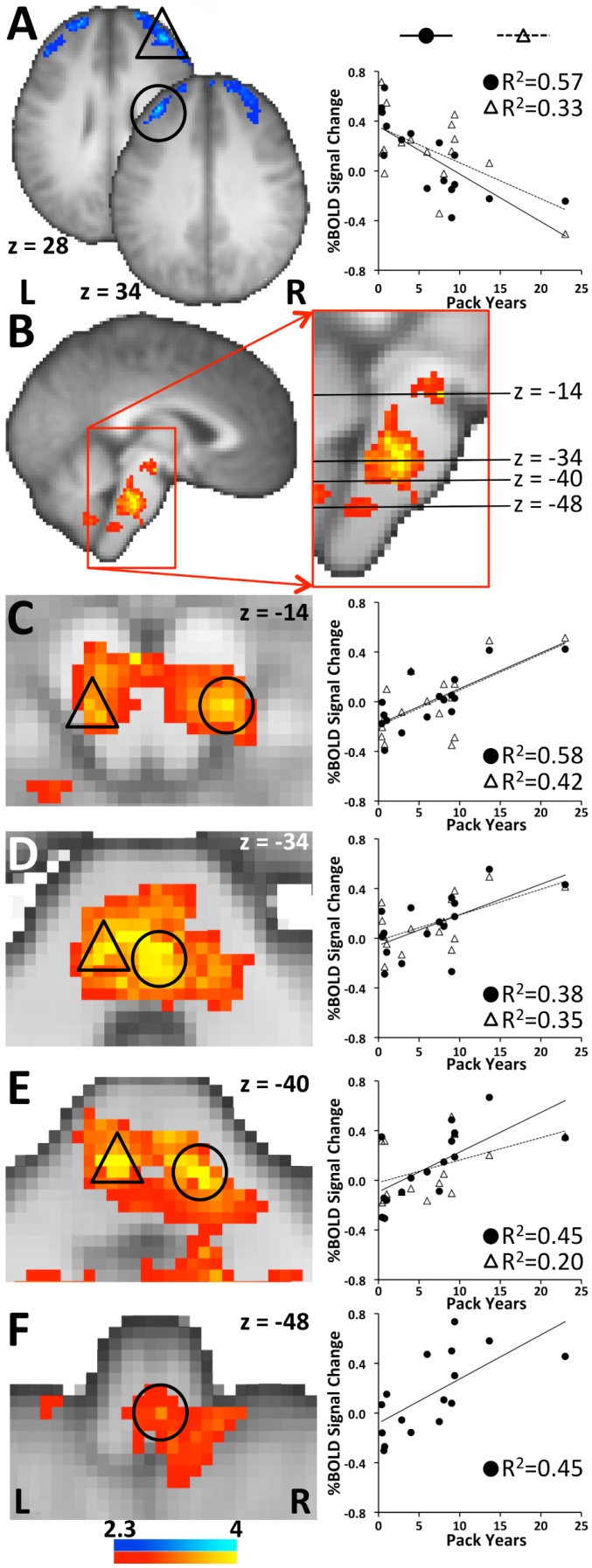
Scan 2	L	S	H	S	L	H	S	H
Scan 3	L	H	S	H	L	S	H	L


A
Controls
Smokers
*
**
*
log10[Capsaicin](μM)
3
2
1
0
-1
Cu
C2
Smax
B
Urge-to-cough Ratings (0-10)
10
8
6
4
2
0
Controls
Smokers
C2+2
C2+1
C2
C2-1
C2-2
Cough Frequency
8
6
4
2
0
Controls
Smokers
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
log10[Capsaicin](μM)


A
STIMULUS
RESPONSE
log10[Capsaicin](μM)
Urge-to-cough Ratings (0-10)
1.5
1.2
0.9
0.6
0.3
0.0
10
8
6
4
2
0
Controls
Smokers
B
Controls > Smokers
z = -20
OFC
C
*
% BOLD Signal
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
C
S
OFC
D
Smokers > Controls
Ins
Put
S1/M1
y = -16
z = 4
Z score
2.3
3.6
E
***
**
**
% BOLD Signal
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
C
S
C
S
C
S
Ins
Put
S1/M1


A
STIMULUS
RESPONSE
*
log10[Capsaicin](μM)
Urge-to-cough Ratings (0-10)
1.5
1.2
0.9
0.6
0.3
0.0
10
8
6
4
2
0
Controls
Smokers
B
z = 38
Controls
Smokers
Overlap
C
Smokers > Controls
S1/M1
PPC
z = 38
3.6
Z score
2.3
D
**
*
% BOLD Signal
0.4
0.2
0.0
-0.2
-0.4
PPC
S1/M1


A
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L
z = 34
R
B
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z = -34
z = -40
z = -48
C
z = -14
D
z = -34
E
z = -40
F
z = -48
L
R
2.3
4
%BOLD Signal Change
Pack Years
R2=0.57
R2=0.33
R2=0.58
R2=0.42
R2=0.38
R2=0.35
R2=0.45
R2=0.20
R2=0.45

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.

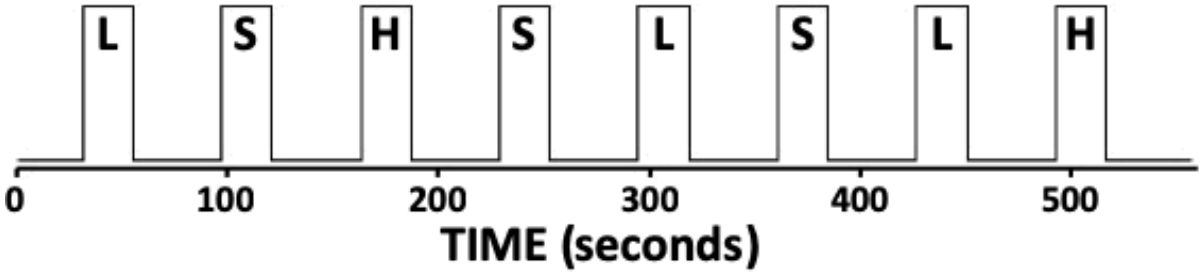
MRI testing session

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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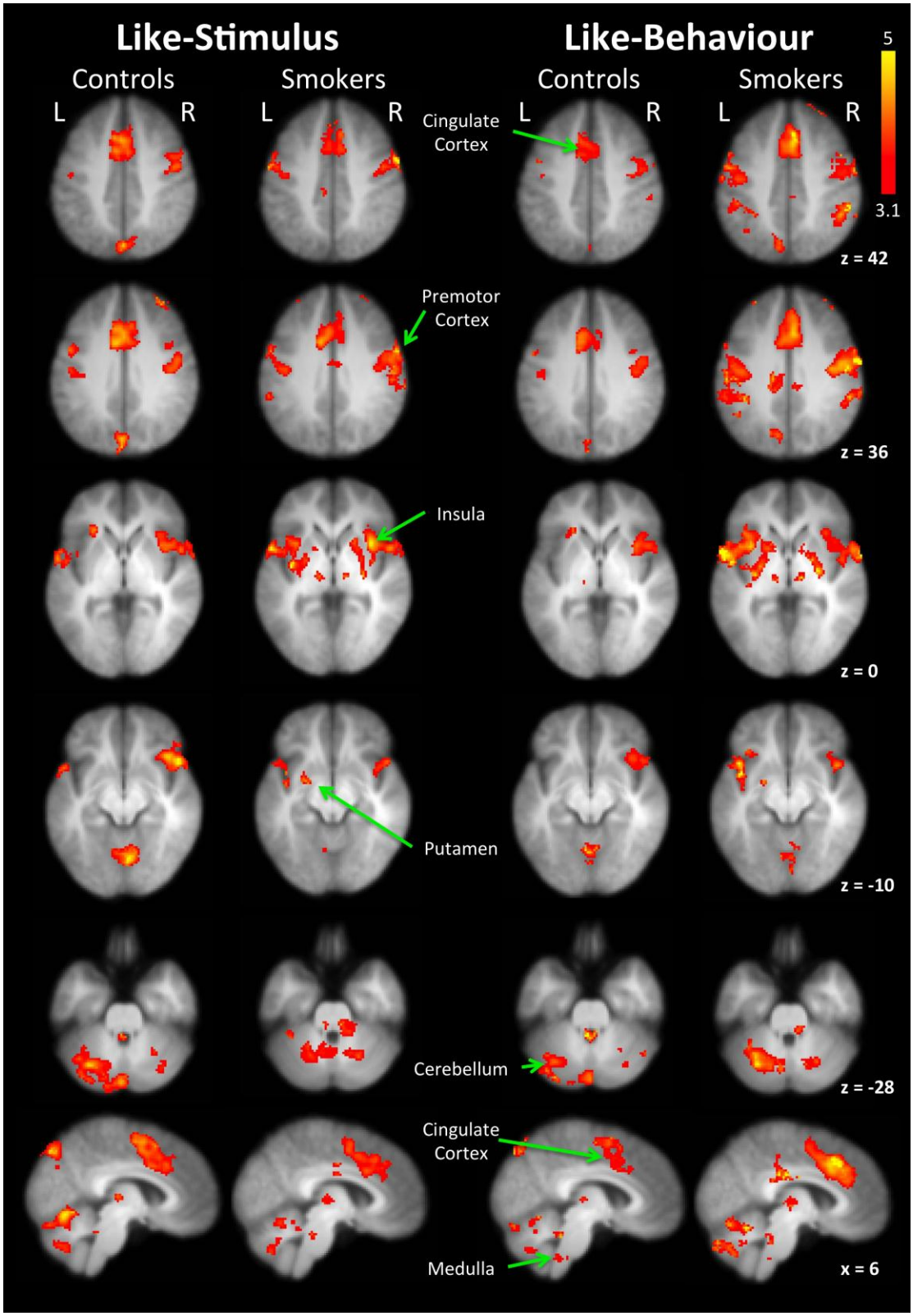

Supplementary Figure 1: Capsaicin-induced brain activation for non-smoker controls and smokers during matched capsaicin dose inhalation challenges (Like-stimulus) and S_{max} inhalation challenges (Like-behaviour). Group capsaicin-inhalation activations were generated for non-smoking controls and smokers. The concentrations of capsaicin inhaled by each participant was tailored to their sensitivity or matched to their pair. *Abbreviations*: Supplementary motor area (SMA); sensorimotor cortex (S1/M1). All axial brain images are presented in the neurological convention (left side of the page is the left hemisphere of the brain).