Low-level carbon monoxide exposure affects BOLD fMRI signal

Supplementary material

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1. Description of breathing system

The breathing system used for this study was derived from earlier systems used in the MRI unit and introduces some breathing resistance, although care was taken to ensure this was minimal. Standard anaesthetic tubing was used throughout to reduce resistance, and Hans Rudolf low-resistance one-way valves were used to direct flow as appropriate. The participant was attached to the breathing system via a mouthpiece using a plastic Y-piece connector. The inhalation arm of the system received room air from the operating room, and two reservoirs (anaesthetic bags) of pre-mixed gas (air+CO) was attached and sealed with valves at this part of the circuit. The exhalation arm of the system piped exhaled air into the air-conditioning vents in the MRI room.

Participants were breathing on the circuit for the entirety of the experimental protocol. Half-way through, the CO mixture (or air for control) was delivered. The CO mixture was introduced into the circuit by emptying the reservoir bags into the one-way circuit slowly over a 5-minute period, after which the reservoir valves were again closed. As the participant was breathing non-pressurised room air, they received the full amount of the gas, irrespective of breathing pattern. The participant was not able to see the experimenter adding the gas from their supine position in the scanner. For the air protocol, the participant breathed room air for the 5-minute period. None of the participants, when prompted following each experiment, were able to identify when they had received the mixture or if it had been air only or air mixed with CO. The structural scan required for registration was obtained during gas delivery, which may have aided in the concealment of the stimulus.

Due to the system using room air and requiring long tubing for gas delivery (6 m), some breathing resistance was associated with the circuit. This was minimised as much as possible through the use of wide-bore adult anaesthetic tubing, and the level of resistance was the same throughout both CO and air protocols. To further minimise any effect of the circuit on breathing, participants had been allowed to familiarise themselves with this during a preliminary session prior to the MRI protocols. No participant exhibited any problems breathing on the circuit, and only one participant mentioned noticing resistance. When prompted, this participant was revealed to be highly used to breathing systems using air under pressure in similar settings; they did not experience any breathing discomfort and were happy to commence with the study.

Some participants were unfamiliar with the mouthpiece, which may have altered their breathing patterns. This was counteracted by allowing all participants time to familiarise themselves with the circuit prior to the MRI sessions. All participants were told on each experimental day that they could stop the experiment and remove the mouthpiece at any point of their choosing, and that they were to inform the experimenters immediately if they experienced any discomfort. No participant interrupted the experiment on any of the experimental days.
2. Extended protocol

**Preliminary visit.** Participants were asked to attend a preliminary laboratory visit. During this visit, medical history and state and trait anxiety inventory (STAI) questionnaires were completed. A breathing test was conducted to let the participant familiarise themselves with the breathing system and the CO exposure. Participants were asked to breathe on a custom-made breathing system through a mouthpiece with their nose occluded, and were given time for their breathing to stabilise before commencing the experiment. After stable breathing had been recorded for five minutes, CO was added to the inspired air over five minutes, out of sight of the participant. Following CO administration, five more minutes of stable breathing was recorded. During the experiment, ECG, pulse pressure and saturation was continuously measured. Expired CO measurements were made before, immediately after, and 10 minutes after the breathing test (Micro+ Smokelyzer, Intermedical Ltd., Kent).

**Main visits.** MRI scans were conducted on two separate days. The experimental days were never less than 7 days apart and never more than 2 months apart for each participant. Scans were conducted at approximately the same time of day for each participant, and each participant was asked to adhere to the same routine prior to each scan. The participants were instructed to refrain from alcohol consumption on the day (and the evening before), keep to the same diet and level of exercise. On each experimental day, the participants were asked what they had eaten/drunk, if their general health was good and if they were on any medications, with large deviations in the above (i.e. getting a cold) being cause for the experiment to be rescheduled. No rescheduling was deemed necessary during the study.

The order of the experimental days was randomised and balanced. Participants were told that they would receive CO on one experimental day and air on the other, but that they would not be informed of which they were receiving. Participants were asked verbally after each protocol if they felt any change in their breathing, and which protocol they believed they had undertaken. The wording of these questions was standardised as follows: “Did you feel a change in your breathing during the experiment today?” and “Can you guess which gas you were breathing today?” No further debriefing was conducted.

On each experimental day, participants were asked to complete the state anxiety part of STAI on arrival and no more than 15 minutes after the end of the experiment. Training in all fMRI tasks were given by an experimenter prior to the first scan on each day, to ensure that the participant could reliably complete these on their own in the scanner. Expired CO measurements were made before the first scan, immediately after the second scan (~20 minutes after the breathing intervention) and 10 minutes after the second scan (~30 minutes after the breathing intervention).

Whilst in the scanner, participants were asked to undertake the following tasks: breath holds, a visual stimulation task, a motor task and a simple reaction time task. Breath holds were conducted end-expiration (to promote repeatability), signalled by visual cues (‘Get ready’ two seconds before breath hold, ‘Hold your breath’ during breath hold) and lasted 15 seconds, repeated 4 times with 15 second breaks in between. The visual stimulation was a flashing checkerboard (8Hz, lasting 10 seconds, separated by 10-second breaks, repeated thrice). The motor task was tapping of the right index finger, signalled by visual cues (‘Tap your right index finger’) and lasted 15 seconds, repeated 4 times with 15-second breaks in between. The reaction time task required participants to immediately press a button upon the appearance of a red dot on the screen (24 appearances, random intervals). These tasks were conducted twice, once before the breathing intervention (baseline) and once after (post-intervention).
3. Extended participant demographics

We recruited 12 (8F, age 25.3+/−4.3 years, range 19-34, median 24.5 years) healthy never-smokers to the study. Never smokers were chosen to ensure a uniform sample group as smokers typically have varying levels of COHb and may exhibit variation in e.g. craving. None of the participants reported being exposed regularly to passive smoking, and all but two lived apart from main roads. All participants were right-handed. Average alcohol consumption was 2.8+/−3.0 units per week across the sample. All participants had obtained a higher education degree or were currently undertaking higher education. None of the participants had any history of cardiovascular or respiratory illness, and none reported any allergies. At the time of study, none of the participants were ill or taking any medication. All female participants were on oral hormonal contraceptives, as exhaled CO level fluctuates within the normal menstrual cycle⁴, and were not pregnant at the time of study.

4. Extended physiological data

Figure S1 shows the exhaled CO data for all participants during both protocols (CO and air). Data from the preliminary visit showed an increase in exhaled CO from 3.0+/−0.8ppm to 6.4+/−0.7ppm measured immediately after the 5-min gas exposure (t(7)=−10.4, p<0.001).

![Figure S1: Exhaled CO (ppm) for CO and air control protocol. Baseline, post-scan (~20 min after end-inhalation) and 10 min post-scan (~30 min after end-intervention). Individual values plus average and standard deviation (bold line).](image)

Expired CO² and O² were sampled continuously throughout the MRI scans. End-tidal CO₂ (P_{ET}CO₂) and (P_{ET}O₂) were calculated across scans for all tasks (Table S1), and P_{ET}CO₂ was introduced in the general linear model during the image analysis to correct for potential fluctuations in the gas. There was no significant difference between the CO and Air protocols for any of the gases sampled. P_{ET}O₂ was lowered for both CO and Air protocols in the second scan compared to baseline, but this difference was not significantly different between protocols. Respiratory rate was calculated based on expired CO₂ data. No significant differences in respiratory rate were found for any of the tasks.

| Protocol | CO₂ (%) | Scan       | Breath hold task | Visual task | Motor task |
|----------|---------|------------|------------------|-------------|------------|
| CO       | Baseline| Air        | 5.3 (0.7)        | 5.3 (0.7)   | 5.3 (0.6)  |
|          |         | Air Post-scan| 5.4 (0.6)        | 5.4 (0.6)   | 5.4 (0.5)  |
|          |         | CO         | 5.5 (0.6)        | 5.5 (0.7)   | 5.5 (0.7)  |
|          |         | CO Post-scan| 5.4 (0.7)        | 5.5 (0.7)   | 5.5 (0.8)  |
|        | Air Baseline | Air Post-scan | CO Baseline | CO Post-scan |
|--------|--------------|---------------|-------------|--------------|
| O₂ (%) | 15.8 (0.1)   | 15.5 (0.7)    | 15.4 (0.1)  | 15.0 (0.7)   |
| RR (bpm) | 9.1 (1.8)    | 14.3 (3.8)    | 15.2 (3.7)  | 14.5 (2.8)   |

Table S2: $P_{ET}$CO₂ data (%) associated with breath hold task. Mean(SD). S1=scan 1 (pre-intervention), S2=scan 2 (post-intervention). Data is end tidal values collected immediately prior to (pre) and following (post) breath hold. Rise is calculated as post – pre. S2>S1 is calculated as S2(rise)-S1(rise).

|        | Air | CO |
|--------|-----|----|
| N      |     |    |
| S1     |     |    |
| S2     |     |    |
| S2>S1  |     |    |

End-tidal PCO₂ rise following breath holds for all scans:

For air, S1 rose from 5.04%+/-0.6 to 6.20+/−0.8 (t(11)=−9.6, p<0.001) and S2 from 5.10%+/-0.6 to 6.15+/−0.7 (t(11)=−13.4, p<0.001).

For CO, S1 rose from 5.27+/−0.6 to 6.33+/−0.6 (t(11)=−9.5, p<0.001) and S2 from 5.16+/−0.7 to 6.14+/−0.9 (t(11)=−11.8, p<0.001).

$P_{ET}$CO₂ rise in the air protocol was not significantly different between S1 and S2 (t(11)=1.3, p=0.22). $P_{ET}$CO₂ rise in the CO protocol was not significantly different between S1 and S2 (t(11)=0.6, p=0.57). Rise between protocols (Air (S2 vs S1) vs CO (S2 vs S1)) was not significantly different (t(11)=−0.5, p=0.64).
Individual expired CO$_2$ raw data, and end-tidal CO$_2$ traces are presented in Figures S2, S3 and S4 for the breath hold task, visual task and motor task, respectively.

**Figure S2.** Individual CO$_2$ traces for the breath hold task (dotted lines). Average end-tidal values presented (solid line). Blue=Air pre-intervention scan; Red=Air post-intervention scan; Green=CO pre-intervention scan; Black=CO post-intervention scan. Averages for all scans also presented separately.
**Figure S3.** Individual CO₂ traces for the visual task (dotted lines). Mean end-tidal values presented (solid line). Averages for all scans also presented separately.

**Figure S4.** Individual CO₂ traces for the motor task (dotted lines). Mean end-tidal values presented (solid line). Averages for all scans also presented separately.
5. CVR maps for breath hold task

CVR maps in units of %BOLD/mmHg CO₂ were calculated for the breath hold task. This was done by calculating %BOLD signal for each individual scan from the contrast of parameter estimates derived from the end-tidal CO₂ regressor, scaled by rise in P_{ET}CO₂ in units of mmHg, to yield %BOLD/mmHg. Mean %BOLD/mmHg values were compared across scans using paired Student’s T Tests. No difference in baseline (pre-intervention) scans were observed (air: 0.09+/−0.10, CO: 0.07+/−0.08, p=0.34). A significant difference was found between post-air and post-CO scans (air: 0.09+/−0.06, CO: 0.04+/−0.08, p=0.048).

Figure S5. Cerebrovascular reactivity maps for breath hold task. %BOLD/mmHg CO₂. Maps are average across subjects.
6. Additional slices: BOLD response change in the CO protocol, breath hold task

Figure S6. BOLD fMRI response associated with breath-by-breath end-tidal CO₂ during the breath hold task, pre versus post intervention difference maps for the CO protocol. Whole-brain analysis. Images are colour-rendered statistical maps (Z scores) superimposed on a standard (MNI) brain. Significant regions are displayed with a threshold of Z>3.1 with a cluster probability threshold of p<0.05 (corrected for multiple comparisons). Blue-lightblue indicates where BOLD response was reduced in the post-inhalation scan.

Figure S7. Change in BOLD fMRI response for the CO protocol (associated with breath-by-breath end-tidal CO₂ during the breath hold task) correlating with CO level change. Whole-brain analysis. Images are colour-rendered statistical maps (Z scores) superimposed on a standard (MNI) brain. Significant regions are displayed with a threshold of Z>3.1 with a cluster probability threshold of p<0.05 (corrected for multiple comparisons). Red-yellow indicates a positive correlation between BOLD response change and CO rise.
7. Activation maps for the tasks using a 2.3 cluster-forming threshold

Figure S8. BOLD fMRI response associated with breath-by-breath end-tidal CO$_2$ during the breath hold task. Whole-brain analysis. Images are colour-rendered statistical maps (Z scores) superimposed on a standard (MNI) brain. Significant regions are displayed with a threshold of $Z>2.3$ with a cluster probability threshold of $p<0.05$ (corrected for multiple comparisons). Maps are BOLD response associated with air and CO inhalation (pre- and post-intervention), pre versus post-intervention difference maps for each protocol ($\Delta$), and contrasts between protocols (contrast between the pre- versus post-intervention difference maps). For contrasts, blue-lightblue indicates where BOLD response following CO (i.e. CO(post>pre)) was lower than BOLD response following air – i.e. on the day the participants inhaled CO, the BOLD response was reduced in the post-inhalation scan, but this did not occur on the day the participants inhaled Air. This difference between protocols was significant. In no area was BOLD response following CO increased compared to BOLD response following Air.

Figure S9. BOLD fMRI response during visual stimulus. Whole-brain analysis. Images are colour-rendered statistical maps superimposed on a standard (MNI) brain. Significant regions are displayed with a threshold of $Z>2.3$. $p<0.05$ (corrected for multiple comparisons). Maps are BOLD response associated with air and CO inhalation (pre- and post-intervention), pre vs post-intervention difference maps for each protocol ($\Delta$), and contrasts between protocols. Blue-lightblue indicates where BOLD response following CO (i.e. CO(post>pre)) was lower than BOLD response following air. In no area was BOLD response following CO increased compared to BOLD response following Air.

Figure S10. BOLD fMRI response during motor stimulus. Whole-brain analysis. Images are colour-rendered statistical maps superimposed on a standard (MNI) brain. Significant regions are displayed with a threshold of $Z>2.3$. $p<0.05$ (corrected for multiple comparisons). Maps are BOLD response associated with air and CO inhalation (pre- and post-intervention), pre vs post-intervention difference maps for each protocol ($\Delta$), and contrasts between protocols. Blue-lightblue indicates where BOLD response following CO (i.e. CO(post>pre)) was lower than BOLD response following air, Red-Yellow indicates where response following CO was greater than response following Air for Contrasts on the right hand side, and mean BOLD response elsewhere.

8. %BOLD activation for visual and motor tasks

Featquery (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FEAT/UserGuide) was used to interrogate the results for both visual and motor tasks (block designs), but not for the breath hold task (fluctuations in CO$_2$ do not conform to a block design). Parameter estimates were extracted for each subject at the second level. Region of Interest (ROI) mask images were used (created from the high level thresh_zstat image files for each task, located in the visual cortex) without weighing. These ROIs were not part of the original study design or power calculation, and care should thus be taken in the interpretation of the findings below. Output was converted to percentage signal change values within featquery. Statistical comparisons of means were made using Students t-tests, correlation was done using a linear regression model in Matlab (Mathworks, Natick, USA) and the data is presented in Figure S11.

The visual task showed a significant difference between protocols for the post-intervention scans ($p=0.039$). The change in %BOLD activation between scans was significantly different between protocols (Air: 0.02+/-.79, CO: -0.94+/-.86, $p=0.005$). %BOLD activation change
for the visual task correlated with CO levels (R^2=0.264, R^2(adj)=0.231, p=0.010, Figure S10).

The motor task showed no significant difference between protocols for the post-intervention scans, but the change in BOLD signal between scans was significantly different between protocols (Air: 0.21+/-0.75, CO: -0.48+/-0.83, p=0.019). %BOLD activation change for the motor task for the ROI mask did not correlate with CO levels.

A second analysis of the motor task was done using a mask in the motor cortex (anatomical, derived from the Juelich Histological Atlas in FSL). This was also in part done to assess the positive activation observed in the group analysis using the lower cluster forming threshold of 2.3. This analysis showed a significant difference between protocols for the post-intervention scans (p=0.020). The change in BOLD signal between scans was significantly different between protocols (Air: -0.23+/-0.21, CO: 0.30+/-0.47, p=0.002). The decrease in the air protocol could suggest that the differences between protocols in the motor region (seen at the 2.3 zstat threshold, Figure S10) could be driven to some extent by the control condition. This might be due to habituation effects or could be artifactual. The reason why signal in the CO protocol does not decrease like the Air protocol, but rather increases, remains unanswered, and highlights further that the impact of CO on BOLD signal may be complex. %BOLD activation change for the motor task in the motor area correlated with CO levels (R^2=0.204, R^2(adj)=0.168, p=0.0266, Figure S10).

![Figure S10. Correlation between %BOLD and CO (exhaled) for visual and motor tasks.](image-url)
9. Individual activation maps for all tasks

**Figure S11. Breath hold. Individual zstat statistical images.** Maps are mean activation associated with end-tidal CO₂ fluctuations after air inhalation.

**Figure S12. Visual stimulation. Individual zstat statistical images.** Maps are mean activation associated with viewing a flashing checkerboard.
Figure S13. Fingertapping task. Individual zstat statistical images. Maps are mean activation associated with tapping the index finger on the right hand.

10. References

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