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CO₂, CO, and N₂ Euthanasia in Laboratory Mice: a multisystem welfare assessment

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25 **ABSTRACT**

26 Millions of laboratory mice are euthanized worldwide every year, most often with carbon dioxide (CO₂).
27 However, CO₂ raises major animal welfare concerns, because it is aversive and induces strong
28 respiratory distress, which may cause anxiety and pain.

29 Currently, insufficient data are available to assess and classify the impact of CO₂ and other gases
30 proposed as alternatives. In this context, it is crucial to accurately determine when animals lose
31 consciousness as the humaneness of an euthanasia method depends on the experience of pain or
32 distress before loss of consciousness (LOC).

33 Here, we analyzed and compared the effect of CO₂, N₂, and CO euthanasia in female and male
34 C57Bl/6N mice, one of the most widely used mouse strains. Mice were implanted with transmitters to
35 record neurological and cardiovascular signals while assessing also respiration, behavior by video-
36 tracking, and measuring biochemical stress parameters. This multi-modal assessment enabled us to
37 determine the time points of loss of posture (LOP) and LOC.

38 CO₂ induced gasping and muscle fasciculations before and after LOP whereas CO and N₂ caused severe
39 seizures and strong agitation, and delayed cessation of electrical brain activity. LOC, induced by the
40 anesthetic properties of CO₂, was the fastest with a high-volume displacement rate of CO₂. Multi-
41 modal assessment revealed that CO and N₂ led to a hypoxia-induced death that is less humane than
42 with CO₂ euthanasia. CO and N₂ cannot be recommended for a more humane euthanasia of laboratory
43 mice.

44

45 **Keywords:** Gas Euthanasia, Laboratory Mice, CO₂, Refinement, Distress

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48 INTRODUCTION

49 Mice are the most commonly used species in biomedical research¹. In 2022, approximately 4 million
50 mice were used and euthanized in Europe for research purposes^{2,3}. Animal welfare legislation requires
51 that euthanasia methods reduce suffering, pain and distress to an absolute minimum^{4,5}. Laboratory
52 rodents are most often euthanized with CO₂⁶. However, CO₂ is considered aversive and induces forced
53 breathing (“gasping”) and respiratory distress⁷, which may cause anxiety and pain^{6,8,9}. Exposure to CO₂
54 triggers panic-like escape behavior, increases locomotor activity, vertical jumps, freezing episodes, and
55 even epileptiform activity^{10,11}. In addition, nosebleeds, pulmonary edema and hemorrhage have been
56 reported after CO₂ exposure^{15–18}.

57 Inert gases have been proposed as promising alternatives to CO₂. They induce severe hypoxia by
58 lowering the concentration of atmospheric oxygen to levels below 10% leading to unconsciousness
59 and death within a few minutes (min)^{14,21,22}. However, hypoxia caused anxiety or panic attacks in
60 humans and anxiety-like behaviors in mice and rats^{23–26}. In addition, epileptiform activity has been
61 observed in mice exposed to nitrogen (N₂), helium (He), or argon (Ar)¹¹. N₂ also increased locomotor
62 activity, vertical jumps, and freezing episodes in mice, but to a lesser extent than CO₂¹⁰. Consequently,
63 a recent amendment of the European directive 2010/63/EU from 2024 bans the use of N₂ and member
64 states must implement this prohibition by December 2026 at the latest²⁷. According to the American
65 Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals, the use of N₂ is only
66 considered conditionally acceptable for pigs in combination with CO₂ but unacceptable for other
67 mammals¹⁴.

68 Carbon monoxide (CO) may represent a more humane alternative to CO₂. According to the AVMA
69 guidelines for euthanasia, CO can be highly effective for euthanasia, mainly because of the gradual
70 intracellular hypoxemia caused by carboxyhemoglobin formation¹⁴, which may lead to minimal distress
71 and pain. CO is commonly used to kill pest rodents and fur animals and is reported to be less aversive
72 than CO₂¹⁴. However, inhalation of CO induces rapid death in some species and agitation in others<sup>28–
73 31</sup>.

74 Currently, there is insufficient data to assess the full systemic impact of CO₂ euthanasia and its different
75 volume displacement rates (VDR) on the welfare of laboratory rodents. The available evidence on
76 potential benefits of alternatives, such as CO and N₂, remains highly controversial and inconclusive due
77 to narrow endpoints, complicating cross-study conclusions³². A decisive factor in determining the
78 humanity of any euthanasia method is, whether (and to what extent) an animal experiences pain or
79 distress before loss of consciousness (LOC)³³. However, we still lack objective and reproducible
80 parameters to evaluate the animal’s experience during the transition to unconsciousness. Additionally,
81 it is crucial to accurately determine when animals lose consciousness and the temporal
82 correspondence with the animals’ LOP commonly used as an approximation for LOC. Furthermore,
83 distress may manifest differently in different species, so that species- (or even strain-) specific markers
84 need to be established and validated³³.

85 For this study female and male C57Bl/6N mice were used as they represent one of the most widely
86 used mouse strain. We first analyzed the effects of two AVMA-referenced CO₂ VDRs (30 % and 70 % of
87 the chamber volume/min) to identify and validate mouse-specific neurological, cardiovascular,
88 respiratory, behavioral, biochemical, and histological parameters. In a second step, we used our
89 validated setup to assess the overall impact of N₂ and CO euthanasia on animal welfare. Our presented
90 data strongly support the validity of banning the use of N₂ for rodent euthanasia by the amended EU
91 directive²⁷ and the recommendations in the AVMA Guidelines for the Euthanasia of Animals¹⁴.

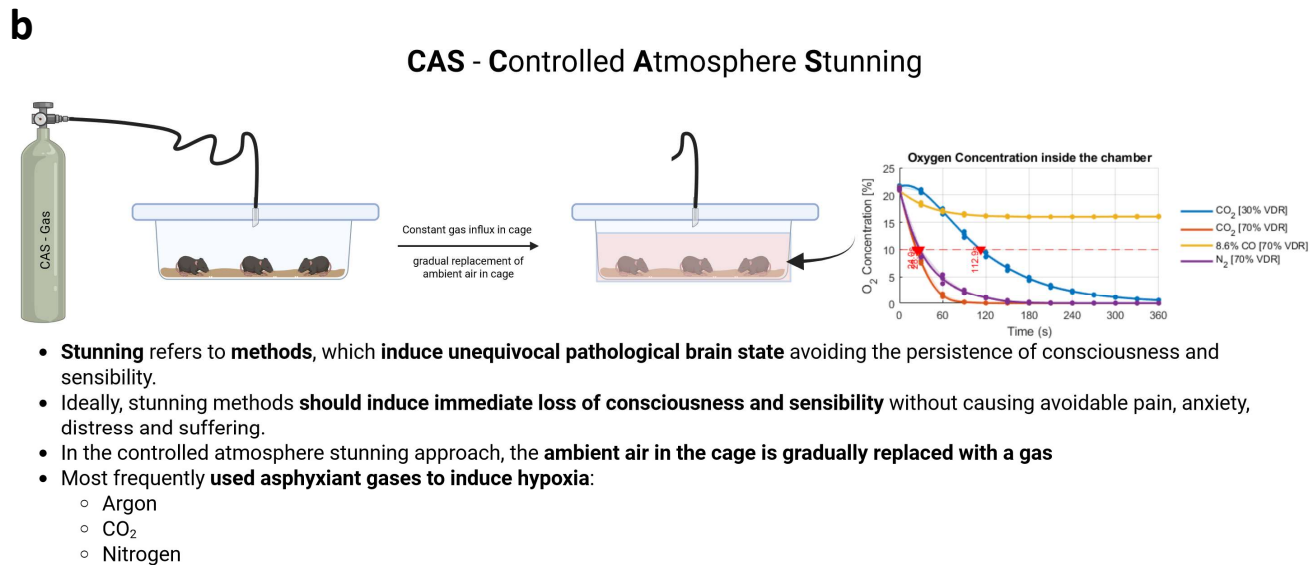
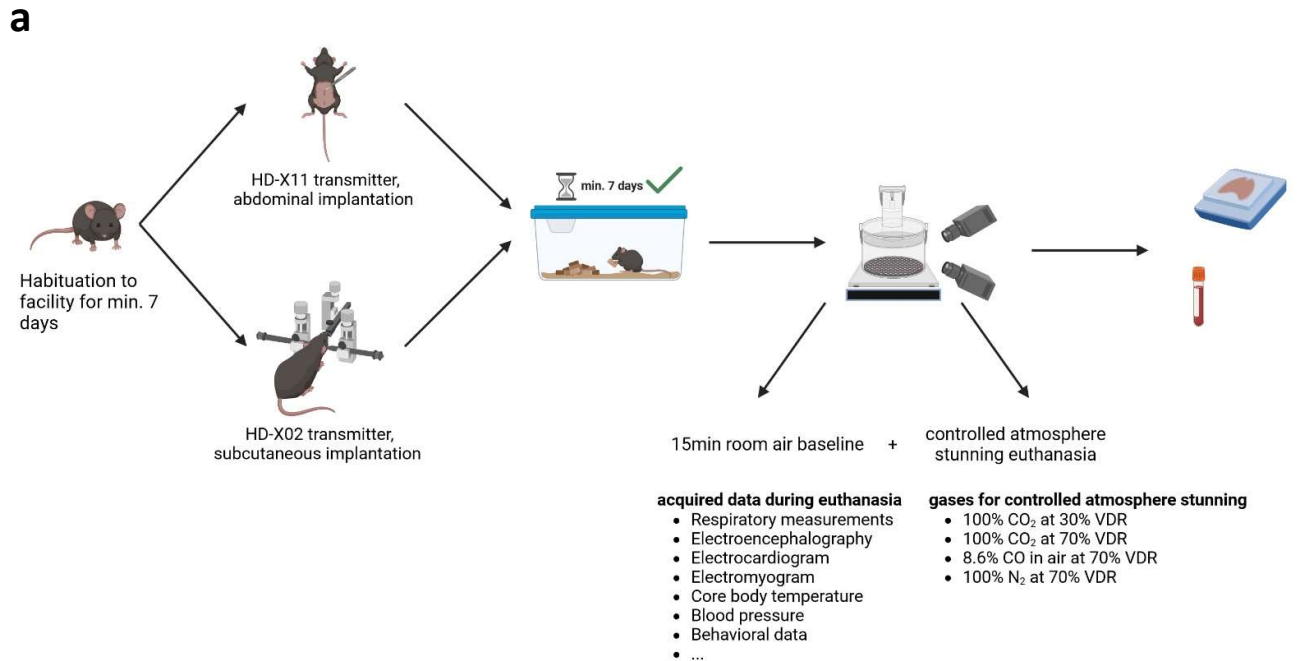


Figure 1: Schematic representation of the experimental process

a Overview of the experimental procedure, including transmitter implantation (HD-X11 blood pressure/ECG or HD-X02/ EEG and EMG) and euthanasia in a whole-body plethysmography chamber with different controlled atmosphere stunning protocols: 100% CO₂ at 30% VDR, 100% CO₂ at 70% VDR, 8.6% CO at 70% VDR or 100% N₂ at 70% VDR. **b** controlled atmosphere stunning (CAS) method, used in this study to euthanize laboratory mice. Further, the residual O₂ concentration, measured inside the chamber is shown for the different controlled atmosphere stunning protocols.

93 RESULTS

94 100 % CO₂ at 30 % and 70 % VDR (fraction of chamber air replaced per min with the introduced gas),
 95 8.6 % CO in air delivered at 70 % VDR, and 100 % N₂ at 70 % VDR were tested for euthanasia (**Fig. 1a**).
 96 The gases and VDRs differed greatly in the time course of oxygen replacement (**Fig. 1b**). CO₂ (70 % VDR)
 97 and N₂ rapidly created a severe hypoxic environment (< 10 % ambient oxygen) after 24 s and 28 s,
 98 respectively. For CO, ambient oxygen did not decrease notably since it was administered with 18.3 %
 99 O₂. For CO₂ (30 % VDR) it took 112 s to cause a severe hypoxic environment.

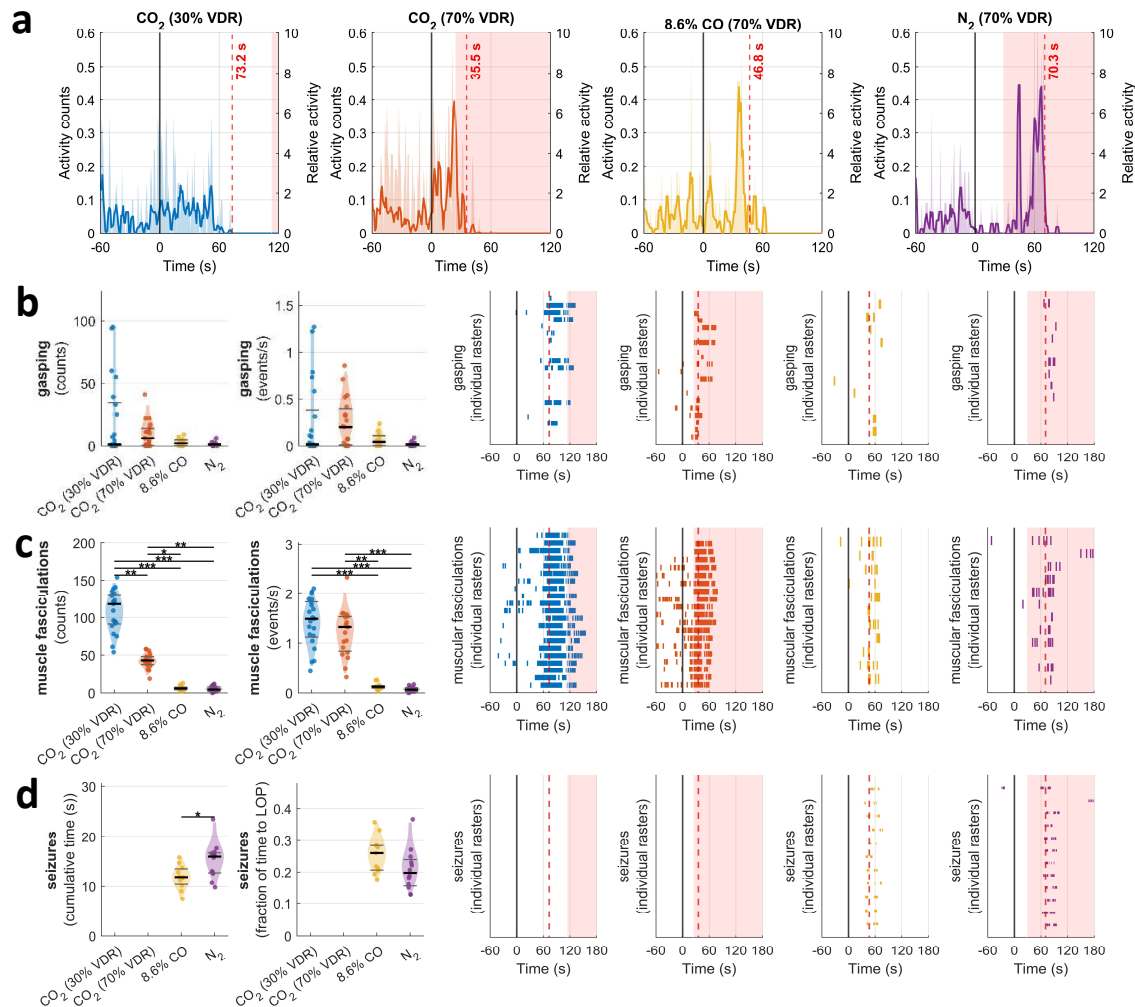


Figure 2: Behavioral responses to different gases during controlled atmosphere stunning euthanasia

Mice were euthanized in a whole-body plethysmography chamber with 100% CO₂ at a 30% volume displacement rate (VDR, n = 21), 100% CO₂ with a 70% VDR (n = 20), 8.6% CO with a 70% VDR (n = 11) or 100% N₂ with a 70% VDR (n = 12). During euthanasia, videos were acquired for behavioral analysis. **a** Activity counts inside the chamber: absolute counts (faded color, left axis) and baseline-normalized counts (solid color, right axis). **b-d** from left to right – cumulative events per group, frequency normalized to the latency to loss of posture (LOP), raster plots representing individual animals in each group, – showing **b** gasping, **c** muscular fasciculations, **d** video-scored seizures. Group comparisons for cumulative counts and LOP-normalized frequencies were performed using Kruskal–Wallis tests followed by Dunn–Sidak-corrected pairwise Wilcoxon rank-sum tests. Seizure durations between CO and N₂ were compared using one-way ANOVA. Gas onset is marked by a black line; LOP by a red dashed line; the shaded red area denotes severe hypoxia (<10% O₂).

Different activity patterns after gas influx

Locomotor activity was measured with telemetry-derived activity counts (**Fig. 2a**). Relative to each animal's baseline, peak activity increased 6 – 8-fold across CO₂ (70 % VDR), CO and N₂ after gas influx, whereas activity under CO₂ (30 % VDR) remained low and stable until LOP. For CO₂ (70 % VDR), and especially CO and N₂, activity peaked shortly before LOP (**Fig. 2a**). The timing of maximum activity (mean ± SD) differed significantly across gases (*Kruskal–Wallis* $H(3)=29.5$, $n=62$, $p<0.001$), occurring at 9.2 ± 9.6 s for CO₂ (70 % VDR), 31.7 ± 15.7 s during CO exposure, 55.5 ± 18.1 s during N₂, and 19.9 ± 15.7 s for CO₂ (30 % VDR). CO₂ (70 % VDR) peaked significantly earlier than CO and N₂ (*Dunn–Sidak post hoc*, $p=0.005$ and $p<0.001$, respectively). This rise occurred before severe hypoxia (shaded in red in **Fig. 2a**) for CO₂ (70 % VDR) and coincided with the onset of hypoxia under N₂. In addition, the timing of LOP also differed significantly across gases (*Kruskal–Wallis* $H(3)=48.9$, $n=62$, $p<0.001$). LOP times (mean ± SD) were 73.2 ± 8.1 s for CO₂ (30 % VDR), 35.5 ± 7.3 s for CO₂ (70 % VDR), 46.8 ± 5.5 s for CO, and 70.3 ± 4.4 s for N₂. *Dunn–Sidak post hoc* comparisons showed that CO₂ (70 % VDR) was faster than CO₂ (30 % VDR) ($p<0.001$), CO ($p=0.004$), and N₂ ($p<0.001$). CO₂ (30 % VDR) was slower than CO₂ (70 % VDR) and CO but did not differ from N₂ ($p=0.82$, **Fig. 2a**).

Muscular fasciculations and gasping in both CO₂ groups, seizures in the CO and N₂ groups

Mice exposed to either CO₂ (30 % VDR) or CO₂ (70 % VDR) showed more gasps (median gasping counts were 1 (Q1–Q3: 0–34.5) and 6 (0.25–13.75), respectively) than those euthanized with CO ($M = 2$ (0–4.75) under CO and 1 (0–1.5) under N₂). Normalizing to time until LOP, gasping showed a similar pattern, with medians of 0.20 (0–0.38) for CO₂ (30 % VDR) and 0.25 (0.01–0.39) for CO₂ (70 % VDR), versus 0.04 (0–0.11) under CO and 0.01 (0–0.02) under N₂. Despite this trend, group differences did not reach statistical significance for either absolute counts or normalized values (*Kruskal–Wallis, counts: H(3)=5.09*, $n=63$, $p>0.1$; *normalized: H(3)=6.25*, $n=63$, $p=0.1$; **Fig. 2b**). Muscular fasciculations were markedly more frequent under CO₂ exposure than under CO or N₂ (*Kruskal–Wallis, counts: H(3)=54.75*, $n=63$, $p<0.001$; *normalized: H(3)=44.72*, $n=63$, $p<0.001$; **Fig. 2c**). Median fasciculation counts were 118 (91.3–129.5) for CO₂ (30 % VDR) and 43 (37.5–48.0) for CO₂ (70 % VDR), compared with 6 (4.25–7.5) under CO and 4.5 (2.0–8.0) under N₂. **Fig. 2c**). The persistence of these fasciculations under CO₂ exposure indicates a CO₂-specific increase in involuntary somatic motor output, consistent with the irritant and aversive properties of CO₂. In relation to seizure-like activity, visually (blinded) scored observations were present exclusively in the CO and N₂ groups. Median cumulative seizure duration was longer with N₂ than with CO (15.9 s (12.6–16.7) vs 11.8 s (10.4–13.5); *one-way ANOVA, F(1,21)=6.30*, $n=23$, $p<0.05$; **Fig. 2d**). When fractioned to time to LOP, seizure-like activity did not differ significantly between CO and N₂ ($F(1,21)=3.16$, $p>0.05$), indicating that absolute duration rather than timing in relation to LOP distinguished CO from N₂ group.

Effects of gases on cardiovascular parameters

To characterize autonomic and cardiovascular burden during gas-induced euthanasia, heart rate (HR) and mean arterial pressure (MAP) were quantified. During baseline, HR and MAP were similar across groups (HR: CO₂ (30 % VDR) 664.8 ± 44.4 beats/min, CO₂ (70 % VDR) 704.2 ± 56.9 beats/min, CO 683.7 ± 41.2 beats/min, N₂ 702.5 ± 50.1 beats/min; MAP: CO₂ (30 % VDR) 113.8 ± 7.9 mmHg, CO₂ (70 % VDR) 114.7 ± 10.9 mmHg, CO 115.4 ± 6.6 mmHg, N₂ 109.5 ± 7.2 mmHg). After gas influx, CO₂ (30 % VDR)

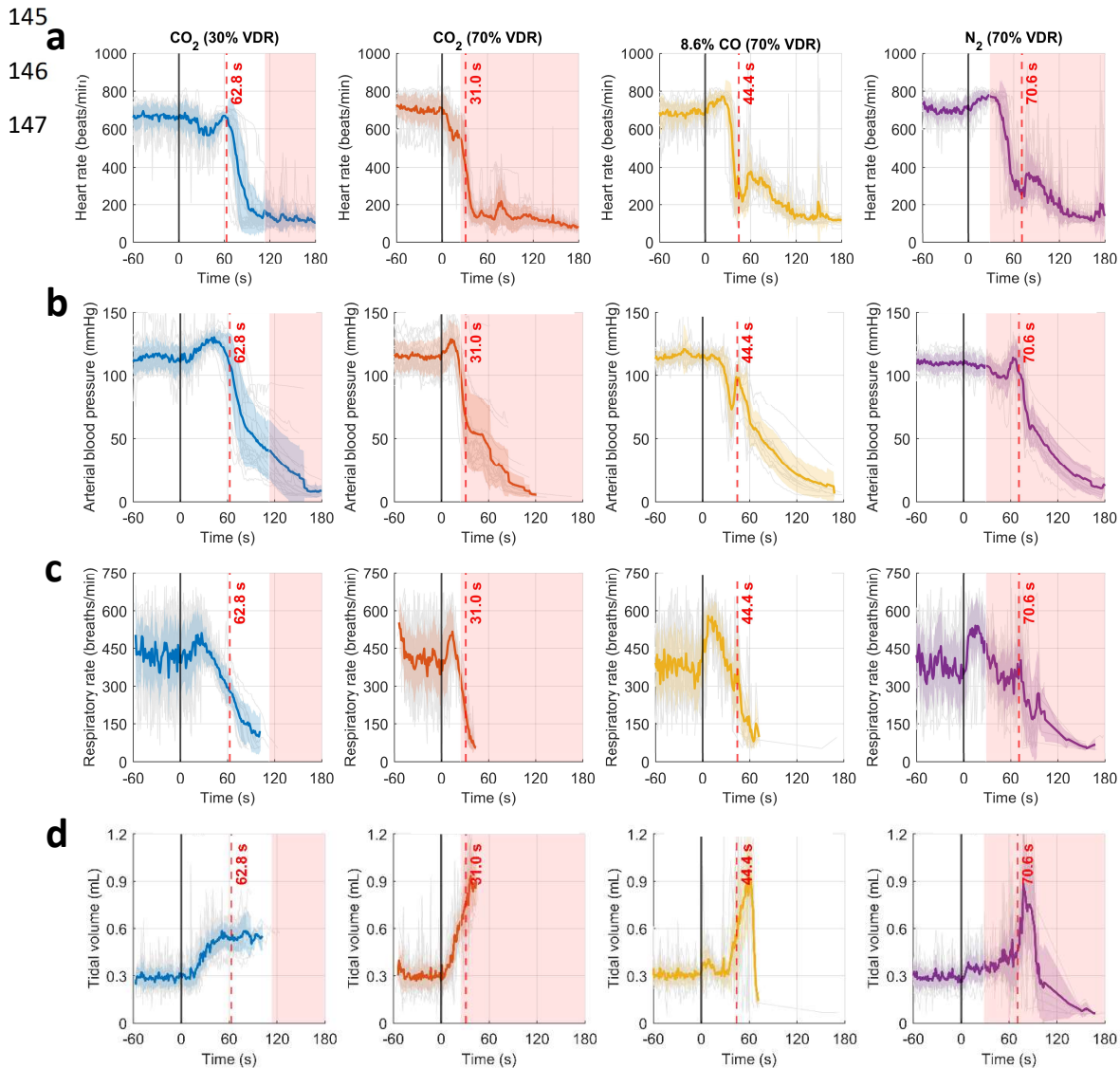


Figure 3: Cardiovascular and respiratory responses to different gases during controlled atmosphere stunning euthanasia

Male and female C57BL/6N mice implanted with a HD-X11 telemetry transmitter were euthanized in a whole-body plethysmography chamber using 100% CO₂ at a 30% volume displacement rate (VDR), 100% CO₂ at a 70% VDR, 8.6% CO at a 70% VDR or 100% N₂ at a 70% VDR. During euthanasia, the following physiological parameters were measured simultaneously: heart rate obtained from ECG (a), mean blood pressure (b), respiratory rate (c) and tidal volume (d). Animals with insufficient blood-pressure signal quality were excluded (final n: CO₂ 30% VDR = 21, CO₂ 70% VDR = 22, CO = 11, N₂ = 12). For all graphs, the data is displayed as mean ± SD; individual animals are plotted in grey. Statistical comparisons of group differences in peak values, values at loss of posture (LOP), and slopes were performed using Kruskal–Wallis tests with Dunn–Sidak-corrected pairwise Wilcoxon rank-sum tests. The black vertical line shows the gas influx. The red vertical line indicates the average LOP. The red zone shows from when mice were in the severe hypoxic zone (<10% ambient O₂).

exhibited a brief HR rise before LOP (maximum 740.1 ± 36.9 beats/min), with HR remaining close to baseline-level at LOP (668.5 ± 44.1 beats/min, **Fig. 3a**), while MAP increased early (peak 135.7 ± 11.8 mmHg) and then declined towards baseline-level at LOP (109.6 ± 17.8 mmHg, **Fig. 3b**). CO₂ (70 % VDR) showed a pronounced bradycardic response, with HR dropping from 704.2 ± 56.9 beats/min at baseline to 421.3 ± 151.1 beats/min at LOP (**Fig. 3a**), whereas MAP rose modestly (peak 138.5 ± 12.3 mmHg) and then declined markedly to 65.8 ± 29.5 mmHg at LOP (**Fig. 3b**). In contrast, CO and N₂ elicited an immediate tachycardia followed by a steady decline until animals lost motion. CO reached a maximum of 792.3 ± 18.0 beats/min and N₂ of 795.8 ± 21.4 beats/min shortly after gas onset, then decreased to 293.5 ± 189.4 beats/min (CO) and 265.0 ± 52.6 beats/min (N₂) at LOP (**Fig. 3a**). In these groups, MAP profiles were biphasic: MAP fell to low minima (CO 54.5 ± 17.6 mmHg; N₂ 82.8 ± 13.2 mmHg) and then rose again at LOP (CO 99.9 ± 13.1 mmHg; N₂ 105.1 ± 11.7 mmHg; **Fig. 3b**).

Changing respiratory patterns with all gases

Similarly to HR and MAP, respiration rate (RR) and tidal volume (TV) at baseline were also similar across gas groups (RR: CO₂ (30 % VDR) 424.3 ± 71.4 breaths/min, CO₂ (70 % VDR) 410.9 ± 61.9 breaths/min, CO 373.0 ± 78.2 breaths/min, N₂ 376.6 ± 73.5 breaths/min; TV: CO₂ (30 % VDR) 0.29 ± 0.03 mL, CO₂ (70 % VDR) 0.30 ± 0.04 mL, CO 0.30 ± 0.04 mL, N₂ 0.29 ± 0.04 mL; **Fig. 3c-d**). After start of the gas, CO₂ (30 % VDR) had the mildest respiratory escalation: RR increased to a maximum of 631.7 ± 69.6 breaths/min (49 % above baseline) and then fell to 273.4 ± 55.8 breaths/min at LOP (**Fig. 3c**). TV rose moderately to a maximum of 0.71 ± 0.13 mL and settled at 0.55 ± 0.07 mL at LOP (**Fig. 3d**). CO₂ (70 % VDR) produced a large but slightly smaller RR surge than CO and N₂ (53 % above baseline, 626.8 ± 82.3 breaths/min), followed by a pronounced fall at the moment of LOP (203.8 ± 109.6 breaths/min; **Fig. 3c**). By contrast, TV showed the strongest rise of all groups (0.89 ± 0.21 mL), remaining elevated at LOP (0.75 ± 0.18 mL; **Fig. 3d**). CO and N₂ showed the largest tachypnea (CO 656.8 ± 91.5 breaths/min; N₂ 658.0 ± 88.6 breaths/min) and remained relatively high at LOP (CO 310.9 ± 140.4 breaths/min; N₂ 322.1 ± 132.6 breaths/min; **Fig. 3c**). TV increased strongly in these groups (**Fig. 3d**), reaching 0.87 ± 0.39 mL (CO) and 0.80 ± 0.16 mL (N₂). At LOP, TV was still elevated (CO 0.68 ± 0.42 mL; N₂ 0.54 ± 0.23 mL), continuing to increase thereafter for 10–15 s until signal collapse.

Time-frequency EEG metrics reveal gas-specific cortical suppression trajectories

To characterize the neurophysiological effects induced by CO₂, CO, and N₂, we plotted baseline-normalized EEG spectrograms during euthanasia (**Fig. 4a**). Exposure to CO₂ (30 % VDR) induced a transient θ and α waves increase (3.5 – 9.5 Hz) at 50 – 55 s before collapsing around 60 s (*cluster-based permutation, $p < 0.05$*). CO₂ (70 % VDR) group exhibited faster EEG suppression starting at 28 s, preceded by an earlier δ to low- β positive cluster (2.5 – 21.5 Hz, 16 – 30 s, $p < 0.05$). In contrast, CO exposure induced a biphasic signature in EEG activity: an early global power fall followed by a brief, significant α increase (13.5 – 17.5 Hz, 33 – 37 s, $p < 0.05$), before dropping towards isoelectric EEG at 40 s. Similarly, N₂ exposure also produced an early broadband negative cluster immediately upon the drop of O₂ inside the chamber below 10 % ($p < 0.05$), followed by a broadband rise with a large positive cluster (0 – 50 Hz, 54 – 70 s, $p < 0.05$) consistent with global agonal excitatory events, and concluded by complete suppression and terminal activity at 70 s (**Fig. 4a**).

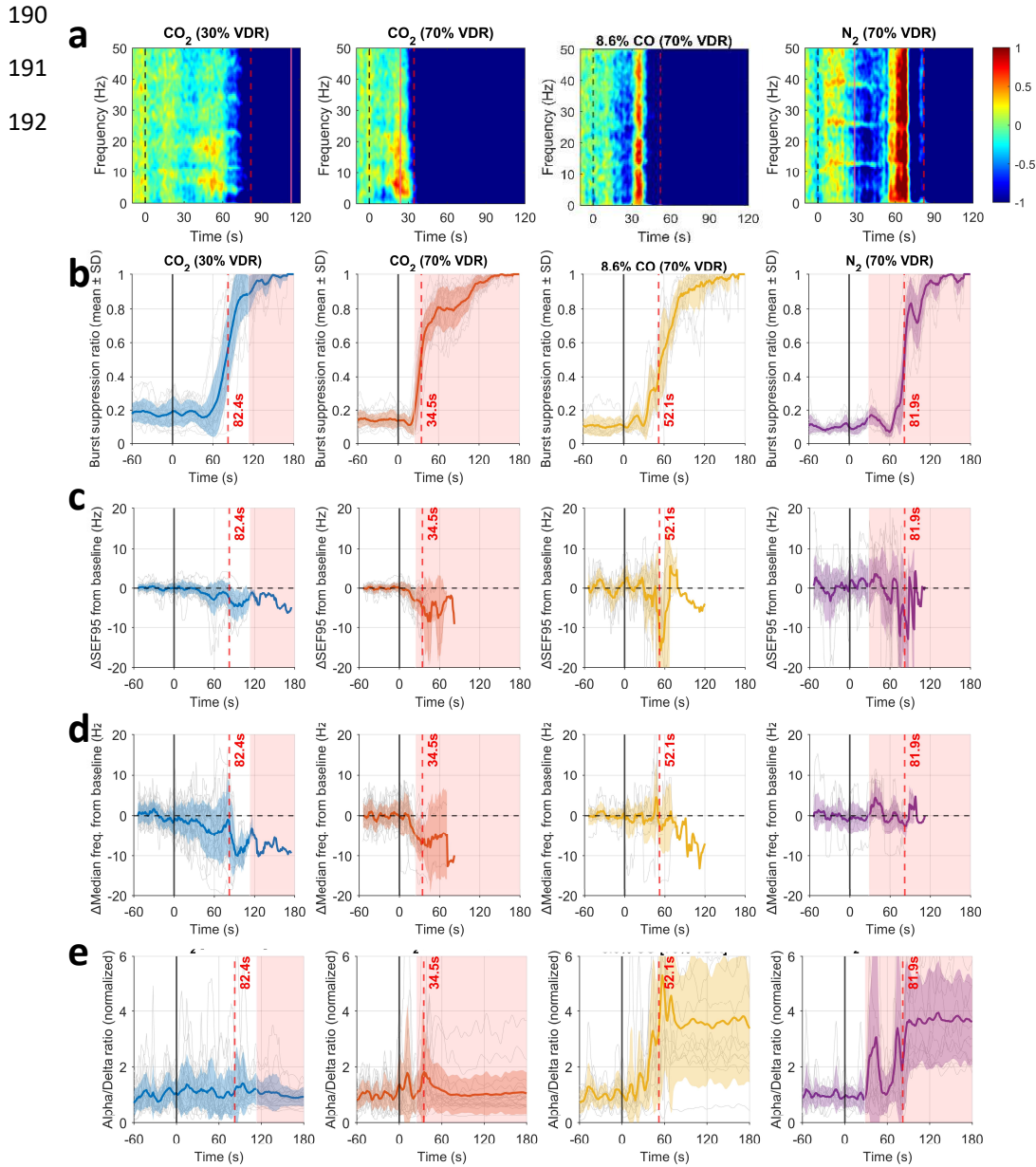


Figure 4: EEG signatures of cortical excitability and suppression after exposure to CO_2 , CO, and N_2 .

Surface EEG was acquired from HD-X02-implanted C57BL/6N mice during controlled atmosphere stunning euthanasia. **a** Group-averaged, baseline z-scored EEG spectrograms (color scale is z-score (0 = baseline mean; $\pm 1 = 1$ SD)) from a few seconds before gas influx to 120 s post-gas exposure. **b** Temporal progression of burst suppression ratio (BSR) for each gas group, including the group-averaged inflection point and corresponding time label, representing time of loss of consciousness (LOC). **c** Deviations of the spectral edge frequency at the 95th percentile (SEF95) from baseline in Hz. **d** Deviations of the median frequency (MF) from baseline in Hz. **e** Time course of the α/δ ratio, normalized to baseline [-60, 0] s, for each gas group. Time–frequency clusters were evaluated with two-sided cluster-based permutation tests (5000 permutations). Group differences in LOC latency, BSR slope, spectral edge frequency at the 95th percentile (SEF95) slope and instability, median frequency (MF) slope, and α/δ ratio were assessed with Kruskal–Wallis tests followed by Dunn–Sidak-corrected Wilcoxon rank-sum tests. Within-animal comparisons to baseline (ΔMF , α/δ ratio) used Wilcoxon signed-rank tests. Vertical black lines mark gas onset ($t = 0$ s), and red dashed lines indicate LOC.

To assess the transition to cortical silence across euthanasia gases, we examined burst suppression ratio (BSR). Both CO₂ groups showed a gradual rise in BSR, with CO₂ (70 % VDR) reaching sustained cortical suppression significantly earlier than the other groups, whereas N₂ induced a delayed but steeper rise (**Fig. 4b**). Quantitatively, start of LOC, marked by the inflexion point in the BSR trace, differed by gas (*Kruskal–Wallis* $H(3)=48.48$, $n=63$, $p<0.001$). Time to LOC were 34.5 ± 1.3 s for CO₂ (70 % VDR), 52.1 ± 4.0 s for CO, 81.9 ± 1.7 s for N₂, and 82.4 ± 2.5 s for CO₂ (30 % VDR): CO₂ (70 % VDR) earlier than CO₂ (30 % VDR) and N₂ (*Dunn–Sidak post hoc*, both $p<0.001$); CO earlier than CO₂ (30 % VDR) ($p<0.01$) and earlier than N₂ ($p<0.05$). Steepness of the BSR, the a measure of how abruptly cortical suppression unfolds, also differed (maximum BSR slope: *H(3)=32.25*, $n=63$, $p<0.001$): N₂ (0.062 ± 0.004 / s) and CO (0.053 ± 0.006 / s) were greater than CO₂ (70 % VDR) (0.030 ± 0.002 / s) and CO₂ (30 % VDR) (0.026 ± 0.003 / s, *Dunn–Sidak post hoc*, all $p\leq 0.021$). Together, CO₂ (70 % VDR) causes sustained early and gradual suppression, whereas N₂ and CO exhibit a more abrupt rise once suppression begins (**Fig. 4b**).

Spectral edge frequency captures instability while median frequency quantifies slowing in gas-specific paths to cortical silence

Spectral edge frequency at the 95th percentile (SEF95), defined as the frequency below which 95% of EEG power resides, was used to assess the stability of the high-frequency component of cortical activity during gas exposure. Fast EEG components in the β – γ range depend on intact cortical network dynamics, thus a decrease in SEF95 reflects cortical disengagement, whereas transient increases indicate intermittent high-frequency excursions and unstable cortical dynamics. In CO₂-exposed mice, SEF95 (baseline-subtracted) progressively decreased over time, particularly in the CO₂ (70 % VDR) condition (**Fig. 4c**). Quantitatively, Δ SEF95 at LOC did not differ between gases (*Kruskal–Wallis* $H(3)=0.211$, $n=49$, $p=0.976$). However, the rate of SEF95 decline in the interval 0–LOC differed across groups (*maximum linear slope*, *Kruskal–Wallis* $H(3)=10.277$, $n=61$, $p<0.05$), with a steeper drop for CO₂ (70 % VDR) than CO₂ (30 % VDR) (-0.14 ± 0.03 vs -0.04 ± 0.01 Hz/s; *Dunn–Sidak* $p<0.01$). SEF95 instability (defined as the median absolute deviation, scaled to the standard deviation of detrended Δ SEF95 in 0–LOC) also differed (*H(3)=34.801*, $n=61$, $p<0.001$). Both CO (1.31 ± 0.13) and N₂ (1.40 ± 0.22) showed greater instability than CO₂ (30 % VDR) (0.44 ± 0.03 , *Dunn–Sidak* $p<0.001$ for both) and CO₂ (70 % VDR) (0.62 ± 0.07 , *Dunn–Sidak* $p<0.01$ for both). These results suggest a more unstable shift toward low-frequency dominance in CO and N₂, whereas CO₂ (70 % VDR) shows the steepest monotonic decline among the CO₂ conditions (**Fig. 4c**).

To determine whether changes in SEF stability were accompanied by a general shift toward lower-frequency activity, we computed the median frequency (MF, baseline-subtracted), which reflects the center of mass of the EEG spectrum. Slowing of MF corresponds to a shift toward δ – θ activity, a hallmark of declining cortical responsiveness. Within-group comparisons against baseline showed that the average Δ MF from gas onset to LOC was significantly negative for both CO₂ groups (CO₂ (30 % VDR) $p<0.01$; CO₂ (70 % VDR) $p=0.001$), but not for CO ($p=0.68$) or N₂ ($p=0.924$), indicating a frequency downshift specific to CO₂ exposure. Between groups, the rate of decline also differed (*maximum linear slope* in the interval 0–LOC: *Kruskal–Wallis* $H(3)=29.87$, $n=61$, $p<0.001$), with CO₂ (70 % VDR) steeper than CO₂ (30 % VDR) ($p<0.001$), N₂ ($p<0.001$), and CO ($p<0.05$). Collectively, CO₂ exposure drives a consistent downward shift in MF, and CO₂ (70 % VDR) does so more rapidly. In contrast, CO and N₂ maintain higher-frequency signaling until LOC, with N₂ signal being more stable than CO (**Fig. 4d**).

α/δ ratio indicates gas-specific mechanisms to LOC

Having established that CO₂ induces progressive cortical slowing while CO and N₂ retains high-frequency rhythms despite instability, we next asked whether these frequency trajectories correspond to distinct mechanisms leading to LOC. Thus, we analyzed the α/δ ratio (ADR, baseline-normalized), which captures whether cortical activity transitions into δ dominance or preserves α -band structure. Within-gas tests against baseline showed no change in ADR from gas onset to LOC for CO₂ (30 % VDR) (*Wilcoxon signed-rank* $p=0.411$) or CO₂ (70 % VDR) ($p=0.737$). CO trended higher ($p=0.054$), whereas N₂ showed a similar pattern ($p<0.1$), indicating a relative α predominance in CO and N₂. Across gases, both the mean ADR and its slope from gas onset to LOC differed significantly (*Kruskal–Wallis* $H(3)=8.661$, $p<0.05$; $H(3)=14.187$, $p<0.01$), with CO and N₂ showing higher ADR levels and positive slopes prior to LOC indicating preservation of α activity despite hypoxia-driven compromise. Under both CO₂ conditions, ADR remained near baseline or decreased, consistent with gradual disengagement of α -band activity (**Fig. 4e**).

Animals euthanized with CO and N₂ display seizure-like bursts shortly before the LOC

To test whether the video-scored seizures (**Fig. 2d**), the high-frequency excursions observed in the spectrograms (**Fig. 4a**) and the upper-edge instability of EEG spectra (**Fig 4c**) under CO and N₂ indeed reflected seizure-like cortical events before LOC, we analyzed γ -band dynamics – γ -band activity is a well-established marker of epileptiform activity. Baseline-z-scored low- γ power (30 – 50 Hz) revealed a marked transient increase across all gases (*Kruskal–Wallis* $H(3)=28.38$, $n=62$, $p<0.001$), with differences in both amplitude and timing. Peak burst amplitude was significantly larger in CO (7.00 ± 1.62) and N₂ (5.07 ± 1.00) than CO₂ (CO₂ (30 % VDR) 0.90 ± 0.14 , CO₂ (70 % VDR) 0.32 ± 0.09 , *post-hoc Dunn–Sidak*, $p<0.05$; **Fig. 5a**). The time-integrated γ excess also differed between gases (AUC above a 95th-percentile baseline threshold: $H(3)=22.67$, $p<0.001$), with CO and N₂ exceeding both CO₂ groups. Burst duration above threshold ($z > 1.645$) was longer with CO (5.0 ± 1.0 s) and N₂ (6.8 ± 2.0 s) than CO₂ (30 % VDR) (0.34 ± 0.15 s) or CO₂ (70 % VDR) (0.49 ± 0.19 s) ($H(3)=9.84$, $p<0.05$). When a sustained-threshold onset could be identified, γ onset preceded LOC by 16.7 ± 3.2 s with CO and 24.5 ± 4.4 s with N₂. Together, CO and N₂ produce large low- γ bursts that terminate at LOC, whereas some animals under CO₂ (70 % VDR) yielded small low- γ increases resolving before LOC and CO₂ (30 % VDR) showed no seizure-like low- γ bursts (**Fig 5b**). To assess the structure of these events at the single-animal level, we traced the Hilbert envelope of the 30 – 80 Hz bandpass-filtered EEG, revealing consistent, seizure-like cortical hyperexcitability periods in all animals in the CO and N₂ groups (**Fig. 5c**).

Isoelectric EEG and EMG timing and pre-collapse dynamics differ by gas

To characterize how CO₂, CO and N₂ drove cortical shutdown and motor quiescence, we quantified the timing, slope, and pre-collapse dynamics of EEG and EMG power (RMS, baseline subtracted). Time to isoelectric EEG differed by gas (*Kruskal–Wallis* $H(3)=43.00$, $n=62$, $p<0.001$): CO₂ (70 % VDR) reached the isoelectric EEG earliest (40.36 ± 3.07 s), followed by CO (67.54 ± 6.07 s), N₂ (87.10 ± 3.01 s), and CO₂ (30 % VDR) (95.99 ± 5.63 s). The decay slope from gas onset to isoelectric EEG also varied across groups ($H(3)=45.15$, $p<0.001$), with the fastest decline under CO₂ (70 % VDR) (-0.481 ± 0.064 μ V/s), compared with CO (-0.250 ± 0.045 μ V/s), CO₂ (30 % VDR) (-0.228 ± 0.025 μ V/s), N₂ (-0.145 ± 0.020 μ V/s). In addition to timing and slope, pre-collapse EEG dynamics differed markedly ($H(3)=28.96$, $p<0.001$). Prior to isoelectric EEG, signal excursions were significantly larger under CO (10.62 ± 1.27) and N₂ (13.55 ± 1.83) than under either CO₂ (70 % VDR) (3.93 ± 0.66) or CO₂ (30 % VDR) (5.06 ± 0.90).

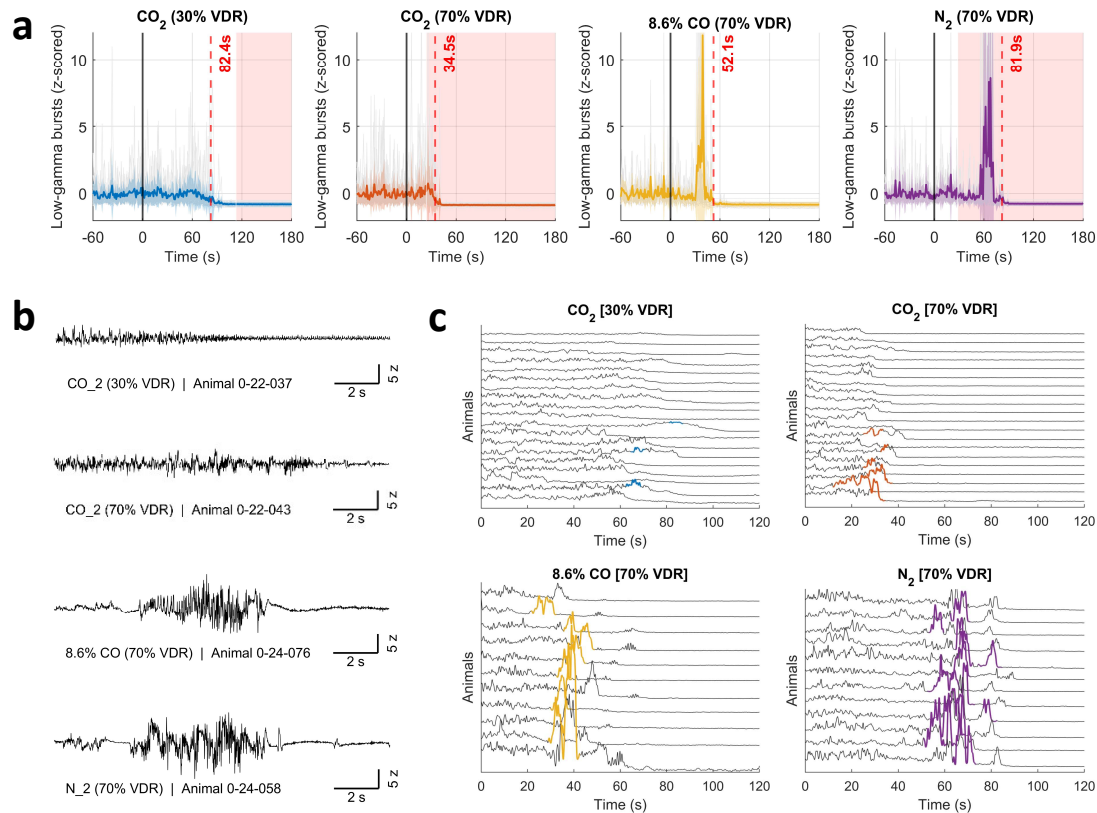


Figure 5: Low- γ power escalation, and γ -burst morphology preceding LOC.

During controlled atmosphere stunning euthanasia in a whole-body plethysmography chamber, surface EEG signal from male and female C57BL/6N mice implanted with an HD-X02 transmitter was recorded for assessment of low- γ power and γ -burst morphology. **a** Low- γ power (30–50 Hz), estimated using a continuous wavelet transform and normalized as baseline z-scores. Thin grey traces show individual animals; colored traces represent group means $\pm$ SEM. Gas onset is marked by a black line and the dashed red line denotes loss of consciousness (LOC). **b** Representative 10-s, baseline-z-scored EEG snippets from one mouse in each group, centered on the peak γ -burst detected in the 20 s preceding LOC. **c** Stacked Hilbert envelopes of the 30–80 Hz band-passed EEG for all animals in each group. Thin black traces show baseline-corrected envelopes; colored segments mark epochs classified as γ -bursts ($z > 3$ for ≥ 0.5 s). Each row represents one animal, ordered by peak burst amplitude. Statistical tests reported in the text include Kruskal–Wallis tests with Dunn–Sidak-corrected pairwise comparisons for amplitude, duration, and AUC differences across gases.

281 Together, CO₂ produced a faster and more orderly EEG collapse, whereas CO and N₂ showed more
 282 pronounced pre-collapse variability (**Fig. 6a**). Similarly, EMG power declined steadily across groups
 283 ($H(3)=52.56$, $n=63$, $p<0.001$), with isoelectric EMG reached fastest under CO₂ (70 % VDR) (37.7 ± 1.3
 284 s, *Dunn–Sidak*, $p<0.05$), followed by CO (76.4 ± 3.2 s), CO₂ (30 % VDR) (89.9 ± 3.7 s), and N₂ ($104.0 \pm$
 285 4.2 s). Brief muscular fasciculations in the final 20 s preceding isoelectric EMG were more apparent
 286 under CO and N₂, consistent with terminal brainstem-mediated movements, but group differences in
 287 fasciculations count and time-integrated EMG activity did not reach statistical significance (both
 288 $H(3)=4.73$, $p>0.1$; **Fig. 6b**).

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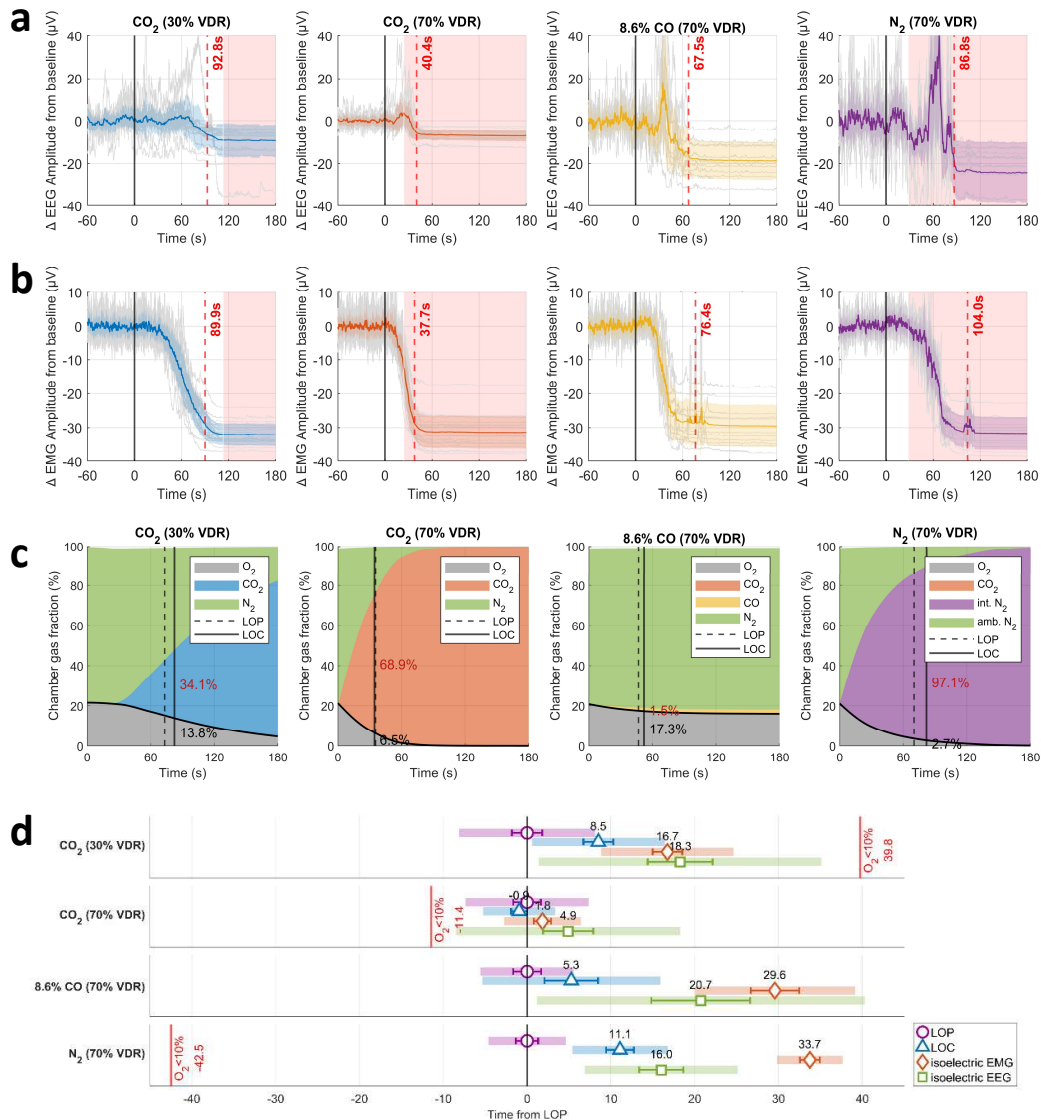


Figure 6: Onset of isoelectric EEG and EMG, composition of the gas mix inside the chamber at LOP and LOC, and temporal sequence of electrophysiological markers in relation to LOP.

During controlled atmosphere stunning euthanasia in a whole-body plethysmography chamber, EEG and EMG temporal deterioration was assessed and related to loss of posture (LOP) and loss of consciousness (LOC). **a-b** Group-averaged EEG (**a**) and EMG (**b**) amplitude, expressed as change from baseline (thin grey = individuals; coloured = mean $\pm$ SEM). Vertical dashed red lines indicate group-average onset of isoelectric EEG or EMG. **c** Reconstructed chamber gas composition for each euthanasia regimen. Stacked areas give the instantaneous fractions of O_2 (grey with overlaid black line) and the delivered (colored). Vertical dashed and solid lines mark mean LOP and LOC, respectively; percentages at LOC report O_2 fraction and euthanasia-gas fraction. **d** Summary alignment of key physiological parameters relative to LOP, including LOC, isoelectric EEG, isoelectric EMG, and the time at which chamber O_2 was reduced below 10%. Symbols denote group means, shaded horizontal bars represent SD and whiskers represent SEM. Group comparisons of latencies and amplitudes were reported in the text body using Kruskal–Wallis tests with Dunn–Sidak-corrected pairwise comparisons.

LOC occurs at markedly different O₂ balances across euthanasia gases

We reconstructed chamber gas composition over time from measured O₂ decay (**Fig. 1b**) to relate (LOP and) LOC to the inhaled gas environment (mixing model of inflow gas with ambient air). The gas fractions present at LOC differed markedly by gas and flow rate. Under CO₂ (30 % VDR), mice lost consciousness while substantial O₂ remained in the chamber (13.8 % O₂), at a CO₂ concentration of 34.1 %. The CO₂ (70 % VDR) protocol reached LOC at roughly double the CO₂ fraction and lower O₂ in the chamber (69.3 % CO₂, 6.4 % O₂). In contrast, during exposure to 8.6 % CO, LOC occurred at very low chamber CO concentrations (1.5 % CO) while ambient oxygen remained near normoxic levels (17.3 % O₂), consistent with CO's high hemoglobin affinity producing tissue hypoxia despite preserved environmental O₂. By comparison, N₂ (70 % VDR) induced LOC in an almost anoxic atmosphere (97.1 % N₂, 2.7 % O₂; **Fig. 6c**).

Temporal sequence of loss of cortical and motor functions is uncoupled from ambient oxygen

To compare physiological trajectories from gas onset to death and visualize the temporal relation between key physiological events, we aligned markers including LOP, LOC, isoelectric EEG and EMG, and the time at which chamber O₂ fell below 10 % (**Fig. 6d**). The latency from LOC to isoelectric EEG did not differ across gases (*Kruskal–Wallis* $H(3)=6.09$, $n=62$, $p>0.1$). In contrast, the relative sequencing of cortical and motor silencing differed robustly across gases ($H(3)=17.22$, $n=62$, $p<0.001$): in the hypoxic gases, cortical activity typically stopped first (CO: -8.85 ± 5.63 s; N₂: -16.91 ± 3.12 s relative to isoelectric EMG), consistent with cortical failure preceding motor quiescence. Under CO₂, isoelectric EEG-EMG were near-synchronous or EMG-first (CO₂ (30 % VDR): 6.10 ± 5.42 s; CO₂ (70 % VDR): 3.07 ± 2.79 s), suggesting an anesthetic-like immobilization. When timelines were aligned to LOP, a clear divergence emerged: CO₂ (70 % VDR) produced a tightly coupled sequence – LOP to LOC to isoelectric-EEG/isoelectric-EMG within circa 6 s – with no apparent dependence on chamber O₂ levels. CO₂ (30 % VDR) euthanasia followed a slower but similarly ordered trajectory, reaching cortical and motor silence before the onset of hypoxia. In contrast, CO and N₂ showed delayed loss of functions: complete EEG collapse occurred 16 – 21 s after LOP, EMG suppression 9 – 17 s thereafter, while in the case of animals under N₂ euthanasia, O₂ level had already dropped below 10 % ~75 s before both isoelectric EEG/EMG (**Fig. 6d**).

ACTH plasma levels higher for CO and N₂

Plasma levels of the stress hormone ACTH were lowest in the CO₂ (70% VDR) group (693.4 ± 326.6 pg/ml, $n = 23$; **Fig. 7a**). ACTH concentrations were higher in the CO₂ (30% VDR) group (1155 ± 452.6 pg/ml, $n = 24$), the CO group (1572 ± 373.4 pg/ml, $n = 11$), and the N₂ group (1546 ± 471.4 pg/ml, $n = 11$). Overall, ACTH levels were significantly increased in the CO (CO vs. CO₂ (30% VDR) $p < 0.05$; CO vs. CO₂ (70% VDR) $p < 0.0001$) and N₂ groups (N₂ vs. CO₂ (30% VDR) $p < 0.05$; N₂ vs. CO₂ (70% VDR) $p < 0.0001$) compared with both CO₂ groups (one-way ANOVA with Tukey–Kramer multiple comparisons test). ACTH levels were also higher in the CO₂ (30% VDR) group than in the CO₂ (70% VDR) group ($p < 0.01$; **Fig. 7a**). Plasma noradrenaline concentrations did not differ between groups (CO₂ (30% VDR): 11.8 ± 4.6 ng/ml, $n = 22$; CO₂ (70% VDR): 10.0 ± 4.2 ng/ml, $n = 24$; CO: 11.1 ± 4.7 ng/ml, $n = 10$; N₂: 13.5 ± 4.9 ng/ml, $n = 13$; *Kruskal–Wallis* test, $p = 0.256$; **Fig. 7b**).

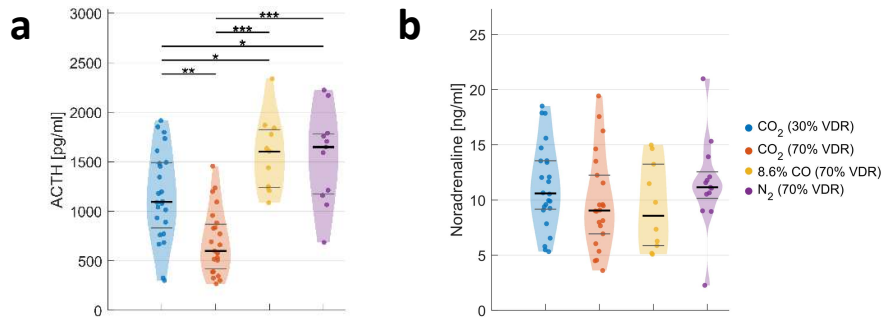


Figure 7: ACTH and Noradrenaline concentration in mice euthanized with CO₂, CO and N₂

Mice were euthanized in a plethysmography chamber, being exposed to 100% CO₂ at 30% volume displacement rate (VDR), 100% CO₂ at 70% VDR, 8.6% CO at 70% VDR or 100% N₂ at 70% VDR. After the euthanasia blood were collected and stored at -80°C. **a** and **b** show the plasma concentration of (a) adrenocorticotrophic hormone [ACTH] and (b) noradrenaline. ACTH was analyzed by one-way ANOVA with Tukey-Kramer multiple comparisons. Noradrenaline was analyzed by Kruskal-Wallis test (global $p = 0.256$; no post hoc). Significance codes: ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. For both hormones, no sex differences were observed.

333

334 Increased lung airspace in mice euthanized with N₂

335 Lung sections (**Fig. 8a**) were analyzed for airspace area, which was significantly increased in mice
 336 exposed to N₂ compared with both CO₂ groups (N₂ vs. CO₂ (30% VDR) $p < 0.01$; N₂ vs. CO₂ (70% VDR) p
 337 < 0.01 ; one-way ANOVA with Tukey-Kramer multiple comparisons test; **Fig. 8b**). Mean airspace values
 338 were $29.8 \pm 6.4\%$ for CO₂ (30% VDR) ($n = 23$), $31.0 \pm 8.4\%$ for CO₂ (70% VDR) ($n = 24$), and $39.4 \pm 7.6\%$
 339 for N₂ ($n = 13$). In the CO group ($37.3 \pm 5.4\%$, $n = 11$), airspace was significantly increased only relative
 340 to the CO₂ (30% VDR) group ($p < 0.05$; **Fig. 8b**). Quantification of erythrocytes in lung tissue revealed
 341 significantly higher values in the CO₂ (70% VDR) group compared with all other groups (CO₂ (70% VDR)
 342 vs. CO₂ (30% VDR) $p < 0.05$; vs. CO $p < 0.01$; vs. N₂ $p < 0.01$; one-way ANOVA with Tukey-Kramer
 343 multiple comparisons test; **Fig. 8c**). Mean erythrocyte area fractions were $4.29 \pm 1.90\%$ for CO₂ (30%
 344 VDR) ($n = 23$), $5.93 \pm 2.77\%$ for CO₂ (70% VDR) ($n = 24$), $2.99 \pm 1.40\%$ for CO ($n = 11$), and $3.23 \pm 1.40\%$
 345 for N₂ ($n = 13$). Acute alveolar hemorrhage was occasionally observed in all groups. Microscopic
 346 analysis of the nasal cavities and sinuses showed no differences in the incidence of nasal hemorrhage
 347 among groups (Fisher's exact test; **Fig. 8d**). In all groups, leukocytes, predominantly neutrophils, were
 348 occasionally observed within pulmonary vessel walls and adjacent interstitial tissue, without a
 349 consistent group-specific pattern.

350

351 CO₂ (30% VDR) ranks best overall despite domain-specific trade-offs in the global comparison of 352 animal distress burden

353 To estimate whole-animal distress, we combined multiple physiological readouts into a heat map
 354 where each parameter is scored on a 0–1 burden scale (0, green, low or minimal distress; 1, red, high
 355 distress) (for details on score calculation see methods). The aggregated animal-distress burden scores
 356 per gas were CO₂ (30 % VDR) = 0.33, CO₂ (70 % VDR) = 0.63, CO = 0.67, and N₂ = 0.70; thus CO₂ at 30
 357 % VDR showed the lowest overall burden (**Fig. 9**). Using parameter-weighted overall scores, groups
 358 differed (Kruskal-Wallis $H(3)=11.68$, $p < 0.01$). Pairwise Wilcoxon tests with FDR correction confirmed

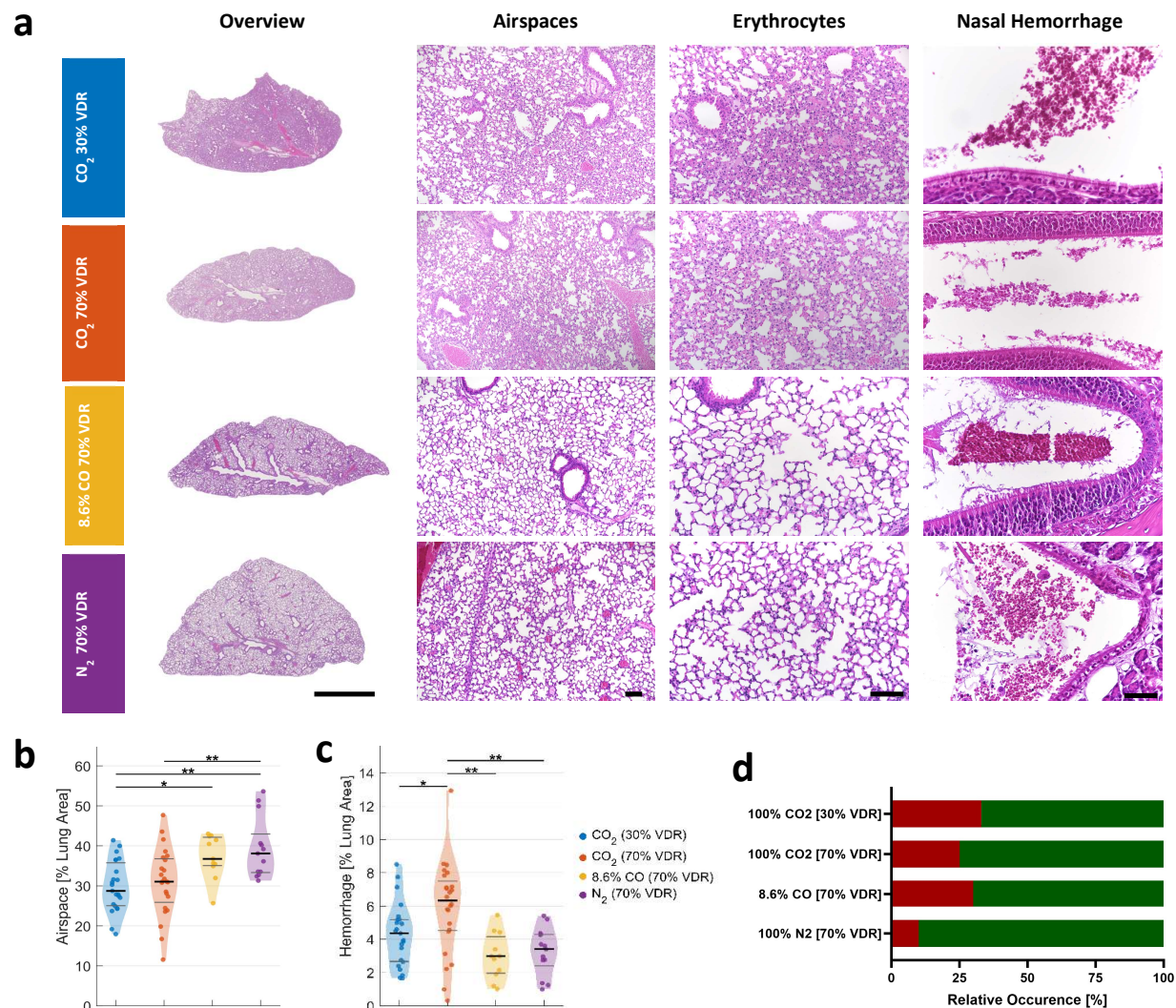


Figure 8: Histopathological analysis of lung and nasal tissue in mice euthanized with CO₂, CO and N₂.

Male and female C57BL6/N mice were euthanized in a plethysmography chamber, being exposed to 100% CO₂ at 30% volume displacement rate (VDR), 100% CO₂ at 70% VDR, 8.6% CO at 70% VDR or 100% N₂ at 70% VDR. After the euthanasia organs were collected, fixed in formalin and processed for histopathological analysis. **a** representative overviews or microscopic images of: lung sections (magnification 1.25x, scale bar 2.5mm), images with lung air space (magnification 10x, scale bar 100µm), images for erythrocytes amount (magnification 20x, scale bar 100µm) or images for the nasal hemorrhage analysis (magnification 40x, scale bar 50µm). **b** analysis of the lung airspace **c** analysis of the erythrocytes amount **d** contingency analysis with a Fisher's exact test of the nasal hemorrhage presence (red = hemorrhage, green = no hemorrhage). Quantitative group comparisons in panels **b** and **c** were analyzed by ordinary one-way ANOVA followed by Tukey-Kramer multiple-comparisons test (significance codes: ns p>0.05; * p≤0.05; ** p≤0.01; *** p≤0.001).

that CO₂ (30 % VDR) produced lower distress than CO ($q = 0.008$) and N₂ ($q = 0.008$) but did not differ from CO₂ (70 % VDR) ($q = 0.42$). The permutation test on the gap between the top two means ($p = 0.016$) supported that this difference was unlikely by chance, even if the CO₂ (30 % VDR) vs. (70 % VDR) contrast remained nonsignificant (*LMM contrast* $p = 0.059$). The mixed-effects model, accounting for gas as a fixed effect and random intercepts for animal, parameter, and system domain, yielded back-transformed marginal means of CO₂ (30 % VDR) = 0.600, CO₂ (70 % VDR) = 0.422, CO = 0.199, and N₂ = 0.088, again ranking CO₂ (30 % VDR) first. A global bootstrap confirmed this lead, with CO₂ (30 % VDR) being the top performer in 93.7 % of resamples (95 % CI: 0.930–0.943). A conservative equal-system sensitivity (each system domain weighted equally per animal) removed the overall difference (*Kruskal–Wallis* $H(3)=1.92$, $p=0.589$), indicating dependence on system weighting; this does not overturn the model-based result, which accounts for the multiparameter, multisystem, structure. Overall, across rank-based, permutation, model-based analyses, and the global bootstrap, CO₂ (30 % VDR) consistently emerged as the option associated with the lowest whole-animal distress burden. For context, cardiovascular outcomes favored CO₂ (30 % VDR); neurophysiological metrics favored CO₂ (70 % VDR) over CO and N₂ but were indistinguishable from CO₂ (30 % VDR); respiratory measures favored N₂ and CO; and behavioral scores showed a modest edge for CO (*family-level FDR-corrected p-values* all ≤ 0.001 , except biochemical/histological $p = 0.235$, data not shown, **Fig. 9**).

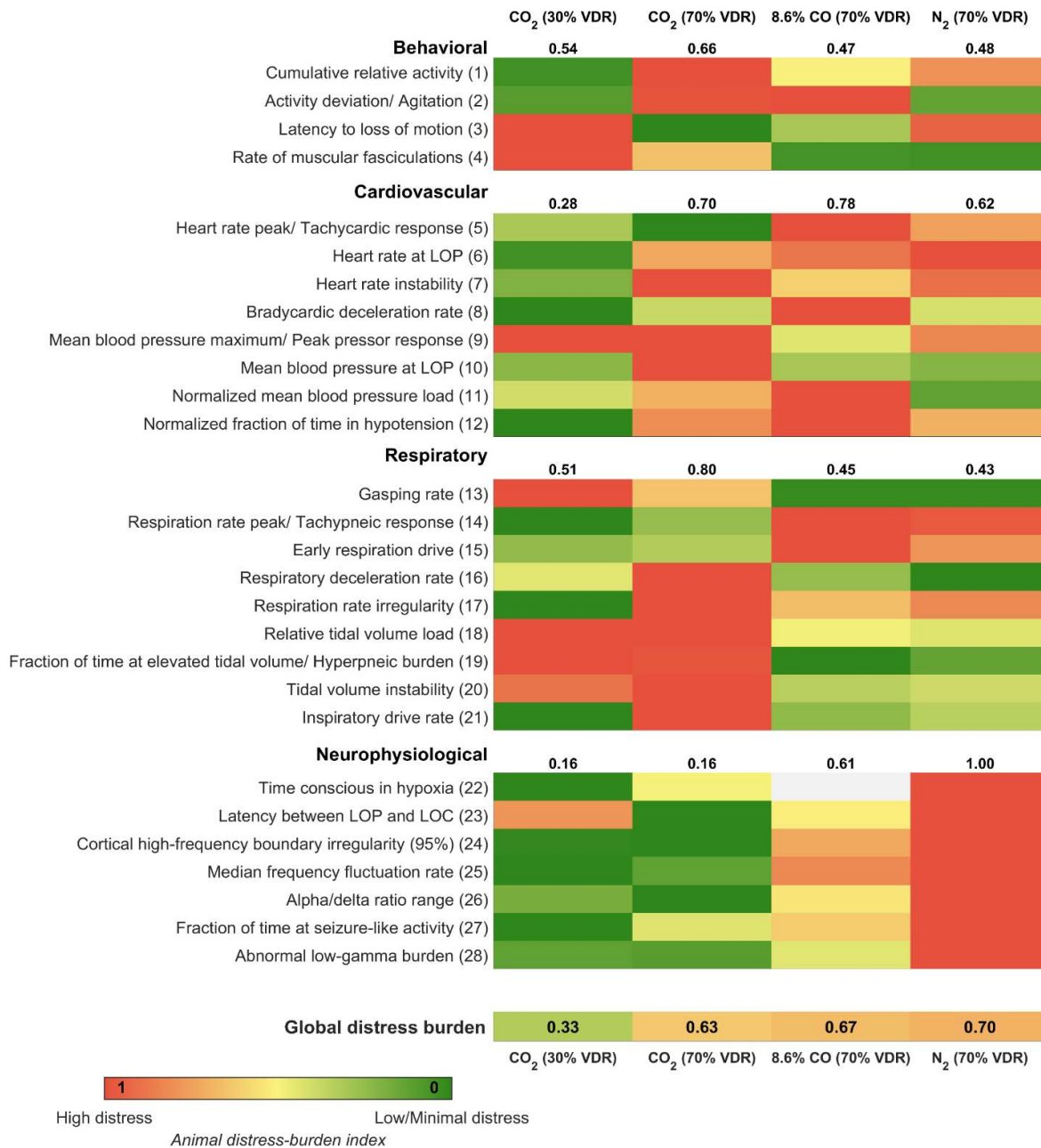


Figure 9: Multisystem distress-burden heatmap of welfare-relevant physiological and behavioral readouts during gas euthanasia.

Each cell shows the normalized distress-burden score (0 = minimal distress, green; 1 = high distress, red) for 28 welfare-relevant parameters spanning behavioral, cardiovascular, respiratory, and neurophysiological domains. Domain-level scores (top of each block) represent the mean burden within that category. Global distress-burden values (bottom) reflect the parameter-weighted aggregation across all systems for each gas. Scores were derived by z-normalizing each parameter relative to its across-group distribution, clipping into a [0–1] scale, and averaging within domains. Group differences in total burden were assessed using Kruskal–Wallis tests with FDR-corrected pairwise Wilcoxon tests; supplementary sensitivity analyses included permutation tests, linear mixed-effects modeling, and bootstrap resampling. All 28 parameters were computed as a baseline-referenced deviation, slope, instability index, or time-fraction measure, with definitions and corresponding equations provided in the Methods. LOP = loss of posture; LOC = loss of consciousness.

DISCUSSION

We aimed to assess objectively the impact of different euthanasia gases on animal welfare and to identify possible alternatives to CO₂ providing a more humane death in rodents. Further, we compared the two threshold VDRs of CO₂ recommended by the AVMA guidelines 2020¹⁴. CO and N₂ have been tested as alternative gases because of being odorless and tasteless, and lacking the irritative and aversive character of CO₂¹⁴. Overall, we believe that animal welfare is affected primarily by the magnitude and duration of distress experienced before LOC, rather than by time to death.

Classical stress markers are of limited value for stress detection during gas anesthesia

Catecholamine and glucocorticoid levels, and cardiovascular parameters are classic markers to quantify stress responses^{34,35}. Stress hormone levels increase with restraint, but also indirect stressors such as predator scent presentation³⁶. In contrast to other studies^{37–39}, we found no significant differences in plasma noradrenaline levels between groups, but we observed significantly higher ACTH levels in the CO and N₂ groups compared to both CO₂ groups. This is consistent with a previous study reporting higher glucocorticoid levels after exposure to hypoxia compared to CO₂⁴⁰. ACTH levels were lower for CO₂ (70 % VDR) than for CO₂ (30 % VDR), but the shorter time to death for CO₂ (70 % VDR) also reduced the time for hormone secretion^{36,41}.

The relationship between stress and elevated cardiovascular parameters is well established for other stressors such as restraint^{42–44}. However, exposure to CO₂, N₂, or CO causes hypercapnia and/or hypoxemia and provokes cardiovascular adaptations to maintain blood pressure and stabilize tissue O₂ and CO₂ levels. These responses may interfere with stress-related effects and must be interpreted with caution.

CO₂ exposure, particularly at higher VDR, induces severe acidosis which affects multiple cardiovascular parameters such as MAP, HR, and cardiac output⁴⁵. The observed increases in MAP, HR, RR and TV may, at least in part, be physiological attempts to restore blood flow and normocapnia^{46,47}. The inverse relationship with a decrease in HR and increase in BP during CO₂ exposure is documented in rats, dogs and humans^{41,48–51}. Thus, the absence of an initial peak in HR in both CO₂ groups may mostly reflect physiological adaptations but not the absence of stress.

In contrast, the immediate initial increase in HR observed with CO and N₂ may be caused by stress. During CO exposure, MAP decreased rapidly consistent with the observation that CO inhalation induces hypotension^{52,53}, mediated by hypoxia-induced vasodilatation⁵⁴. Likewise, MAP was reduced in the CO₂ euthanized mice when oxygen levels in the chamber were low.

All euthanasia methods impact respiration

The respiratory center reacts within seconds to changes in pCO₂ or to a lesser extent pO₂. These features were observed as an immediate initial increase in RR in all four groups, highest in the CO and N₂ groups. TV increased in the N₂, CO and CO₂ (70 % VDR) groups, possibly indicating a stronger respiratory drive triggered by hypoxia. CO₂ exposure induces severe acidosis which triggers central chemoreceptors, causing strong hyperventilation⁵⁵ – visible as instant forced breathing (“gasping”). Both CO and N₂ induce hypoxemia, which also leads to forced breathing⁵⁶. Enforced breathing causes respiratory distress, anxiety, and pain^{6,8,9}. However, in contrast to CO₂ (70 % VDR), high respiratory distress was present after LOC for CO and N₂. CO₂ (30 % VDR) performed slightly better than CO₂ (70 %

VDR), since it presented a lower increase in TV. Importantly, forced breathing before LOC is of more relevance for animal welfare than after LOC. Our study shows that although in all groups most of the forced breathing occurred after LOP, still a considerable amount occurred while the animals were conscious.

In our histopathological analyses we found pulmonary emphysema and enlarged lung airspace in the N₂ and CO groups compared to CO₂ (30 % VDR). The total amount of erythrocytes (related to congestion and hemorrhages) was higher in the CO₂ groups, but differences among groups were minimal. A study showed that C57BL/6N mice are less susceptible to CO₂-induced pulmonary hemorrhage than other strains¹⁵, which could explain the overall minimal findings.

LOC generally occurs a few seconds after LOP

The humaneness and thus suitability of any euthanasia method depends on the experience of pain or distress before LOC. Therefore, it is crucial to accurately determine when animals lose consciousness and the temporal relationship between LOC and the visually detectable LOP. That means, only EEG recordings identify LOC with required precision to provide an accurate assessment of the duration of distress levels during euthanasia. While the time until death is of secondary importance from an animal welfare standpoint, it may be important for the timing of subsequent procedures such as organ removal.

During euthanasia, LOP, cessation of voluntary movement, absence of reflexes, and no observable respiratory activity, are the only observable markers used to infer that the animal is dead. Our results show that this relationship is gas dependent, and that LOP must not always be equated with LOC as LOP most often occurs before LOC. In the CO₂ (30 % VDR), N₂, and CO groups, mice exhibited persistent cortical activity up to 11 seconds after the cessation of visible movements. During this LOP-LOC interval, the EEG may continue to display high-frequency instability, fluctuating ADR and transient seizure-like activity, reflecting ongoing transitions between arousal and suppression, contradicting the assumption of instantaneous loss of brain activity. These observations support longstanding concerns: LOP is a necessary but not sufficient proxy for LOC⁵⁷. To bridge this perceptual gap, more objective physiological markers are needed to reliably indicate the onset of unconsciousness when other behavioral signs fail. Here, the inflection point of the BSR served as a robust electrophysiological indicator of the transition to sustained cortical quiescence. This marker perfectly overlapped with key transition moments in the SEF95, MF and ADR readings in all gases, corroborating studies showing that steady drop into lower-frequency domain and loss of frontal α -waves often predicts LOC^{58–60}. Preservation of cortical reactivity under CO and N₂ suggests that cortical processing capacity may persist during physiologically stressful states, whereas CO₂ induces earlier cortical disengagement, which may have implications for processing aversive stimuli during euthanasia. Although collecting EEG readouts in routine practice is currently impractical, our data provides a basis for better determining LOC during euthanasia or anesthesia. Future studies could correlate these EEG-based markers with loss of reflexes or other physiological signals and guide non-EEG-based refinement of euthanasia protocols.

Timing of cortical suppression reveals fastest LOC in animals exposed to CO₂ (70% VDR), but global neurophysiological distress burden is not lower than with CO₂ (30% VDR)

The temporal dynamics of EEG suppression revealed fundamental differences between euthanasia gases. CO₂ (70 % VDR) induced the fastest transition to unconsciousness, immediately before LOP. Moreover, the ADR signature is consistent with deep cortical silencing and reflects the progression to anesthesia-induced LOC. Such patterns resemble the α/δ suppression reported under GABA anesthetics such as propofol and sevoflurane, where frontal α and slow- δ oscillations dominate just before EEG suppression⁵⁸.

CO₂ (30 % VDR) caused a profile that resulted in a slower onset of EEG/EMG suppression. Thus, even a gradual CO₂ fill can induce unconsciousness prior to severe hypoxia – a key mechanistic distinction supported by previous reports showing that the central depressant effects of CO₂ precede hypoxemia^{10,37}.

In contrast, CO and N₂ exposure delayed EEG suppression, yielding erratic SEF95, MF and ADR trajectories, reflecting EEG instability characteristic of hypoxia⁶¹. Overall, our data provides a temporal framework linking overt visual events (agitation, gasping, seizures-like activity, LOP) with neurophysiological markers (LOC, SEF95, MF and ADR signatures, time to isoelectric EEG/EMG). These identified temporal offsets, as well as the discrepancies in distress levels between behavior and neurophysiological scores within gas groups, underscore that behavior responses and visual endpoints do not reliably map to neural state destabilization^{62,63}.

Differences between the two anesthetic CO₂ regimes and hypoxic gases (CO and N₂)

The observed behavioral and neurophysiological effects reflect the different mechanisms by which each gas affects the central nervous system, namely between anesthesia and hypoxic asphyxia during euthanasia.

CO₂ acts as a rapid anesthetic at higher concentrations by depressing the central nervous system⁶⁴; at CO₂ (70 % VDR), it produced rapid central-nervous-system depression, but the transition into unconsciousness exhibited a greater physiological system disruption than CO₂ (30 % VDR), which was a slowly acting anesthetic. This observation is consistent with the well-characterized “CO₂ narcosis” effect, in which hypercapnia causes respiratory acidosis and central depression, accelerating, respectively, inducing LOC^{64–67}. Rapid LOC comes at a welfare cost: as the CO₂ concentration increases in the chamber, the gas becomes considerably aversive and nociceptive to rodents^{62,63,68}. However, we did not observe overt aversive or “escape-like” behaviors such as jumping or freezing as previously described for neither CO₂ nor CO or N₂¹⁰, which may reflect the gentle tube/cup handling, the small chamber size and prechamber habituation implemented rather than absence of aversion per se. In both CO₂ settings here, muscle fasciculations prior to LOC may indicate distress, but it remains unclear whether they are perceived as stressful or painful by the animals. Mainly, since muscle fasciculations physiologically occur by acidosis-induced hyperkalemia at high CO₂ levels⁶⁹. In summary, while CO₂ (70 % VDR) induces unconsciousness rapidly, it also elicits signs of behavioral, cardiovascular and respiratory distress (**Fig. 9**) and more pulmonary congestion than CO₂ (30 % VDR) (**Fig. 8c**).

In contrast, euthanasia with CO₂ (30 % VDR) extended the time to LOC, but with less signs of behavioral, cardiovascular and respiratory distress, and a less irregular, less fluctuating transition into LOC as displayed by the BSR, SEF95 and MF than all the other groups (**Fig. 4&5**). No signs of seizure-like activity and less pulmonary congestion were observed. Although CO₂ (70 % VDR) induced faster LOC, minimizing the period in which distress and pain can be experienced, we observed occasional epileptiform spikes. These data are in line with the inconsistent presence of seizures with CO₂¹¹. Animals from the CO₂ (70 % VDR) group displayed low intensity episodes of seizure-like discharges,

likely due to CO₂'s CNS-depressant properties⁶⁸. In addition, the slow induction of euthanasia with CO₂ (30 % VDR) resulted first in an increase in RR followed by respiratory depression, strongly resembling isoflurane anesthesia⁷⁰, and further confirming the anesthetic potential of CO₂^{65,71,72}. Thus, CO₂ (70 % VDR) may cause a higher level of distress before LOC compared to CO₂ (30 % VDR) which may outweigh the benefits of a faster LOC. Although taking longer, a slower CO₂ VDR may cause less distress (**Fig. 9**), but additional steps are needed to further minimize discomfort before LOC. Additionally, the isoelectric EEG–EMG sequencing dissociation between CO₂ and CO/N₂ corroborates the argument of two different mechanisms: under hypoxic gases the cortical activity ceases before motor quiescence (cortex-first), whereas with CO₂ the near-synchronous or EMG-first pattern indicates immobilization despite ongoing cortical activity, consistent with hypercapnic, anesthetic-like immobilization (brainstem/motor depression). Finally, the gas fraction required to induce unconsciousness reflects both the gas's physiological mode of action, hypercapnia for CO₂, tissue hypoxia for CO, and environmental hypoxia for N₂, and the VDR, with higher VDR accelerating O₂ wash-out from the chamber and accelerating LOC. At higher VDR, CO₂ induces LOC faster by means of higher chamber CO₂ and lower O₂; CO and N₂ reach LOC with minimal chamber CO or near-complete O₂ wash-out, respectively, matching their distinct hypoxic mechanisms.

Implications for refinement and euthanasia practice

N₂ and CO were associated with more signs of distress, such as an increase in HR and RR responses, as well as higher levels of stress markers and lung damage. In addition, a major welfare concern is the transient low- γ -discharges prior to LOC resembling agonal seizures induced by O₂ deprivation. Indeed, this sudden increase in high-frequency EEG activity preceding cortical silencing reflects terminal depolarization across neocortical networks: as O₂ levels fall, ATP-dependent processes fail, triggering widespread neuronal depolarization and synchronized excitatory firing, manifested as broadband spectral bursts, particularly intense in the γ range. This agonal cortical hyperactivity contrasts with the gradual median frequency decline and δ dominance observed under CO₂, where cortical shutdown is driven by GABAergic hyperpolarization rather than metabolic collapse⁷³. Similar hypoxic convulsions, features more closely related to metabolic collapse than sedation, have been documented by others during inert gas euthanasia^{11,74}. Gent et al. (2018) observed that N₂ and Ar reliably induced brief epileptiform seizures¹¹. These findings directly inform refinement by identifying gas choice and VDR as modifiable parameters that reduce pre-LOC distress without requiring additional instrumentation.

Using a multi-modal, objective approach, our study demonstrates that CO and N₂ are not suitable alternatives to CO₂ for laboratory mice euthanasia, as both cause hypoxia-induced distress before LOC. Therefore, we believe that our data clearly support the validity of banning the use of N₂ for rodent euthanasia by the amended EU directive and the AVMA Guidelines for the Euthanasia of Animals^{14,27}. In addition, we recommend adding an explicit ban for CO for rodent euthanasia to the EU-directive²⁷. CO₂ induces unconsciousness through anesthetic mechanisms, resulting in less overall distress. Although a higher CO₂ VDR (70 %) leads to faster unconsciousness, it causes stronger physiological and behavioral disruption. A lower CO₂ VDR (30 %) provides a slower but progressive and less distressful transition towards LOC. Therefore, CO₂ remains the most humane option, with a 30 % VDR recommended to minimize overall distress.

547 **Material and methods**

548 **Ethical statement:** All animal experiments were designed according to the PREPARE/ARRIVE guidelines
549 and approved by the Swiss cantonal veterinary authorities (license number ZH129/2021).

550 **Animals:** Male and female C57BL/6N mice (Charles River, Germany) aged from 11-19 weeks were used.
551 Mice were habituated to the housing and experimental facility for at least 7 days and tunnel handled
552 throughout the entire experiment.

553 **Housing:** Prior to surgery, animals were housed in groups of 2-4 in standard IVC cages (NexGen
554 Mouse500, Allentown, USA) with wood shavings, shredded paper, paper tissues, a red translucent
555 plastic house (Plexx, the Netherlands) and a transparent plastic tunnel (Physik-Werkstatt, Universität
556 Zürich UZH, Switzerland). Mice had *ad libitum* access to food (Kliba-Nafag product no. 3436, GRANOVI
557 AG, Switzerland) and water. Room temperature was maintained at 22-24°C and the relative humidity
558 at approximately 50% during a 12h/12h light/dark cycle with lights on at 7am. After surgery, mice were
559 housed in a climatic chamber (UniProtect Luftstromschrank, Zoonlab GmbH, Germany), which was
560 warmed to 30°C for three days after surgery and then reduced to 23°C. Male mice were separated if
561 they began to fight, females were kept in pairs until the sacrifice.

562 **Surgery/recovery:** C57BL/6N mice were implanted with either a HD-X11 transmitter (70 animals; DSI,
563 USA) or with a HD-X02 transmitter (72 animals; DSI, USA) under isoflurane anesthesia. The following
564 analgesia regime was used pre-operatively with HD-X11: 5 mg/kg carprofen and with HD-X02: 5 mg/kg
565 carprofen, fentanyl 0.01 mg/kg and Emla-crème in the ears. HD-X11 implantation procedures were
566 performed as previously described by others^{75,76}. For the HD-X02 implantation, a craniotomy was
567 performed on a stereotaxic frame, using predefined coordinates (positive electrode: antero-posterior
568 (AP): +2.0 mm, medio-lateral (ML): +1.5 mm; negative electrode: AP: -2.0 mm, ML: +1.5 mm). EEG
569 leads were attached to screws, secured and insulated with tissue adhesive and dental cement (Els
570 Unibond and Els cem, SAREMCO dental, Switzerland). EMG leads were placed on the neck muscles.
571 For post-operative analgesia, 5 mg/kg carprofen was administered twice daily for three days with fluid
572 (1 ml subcutaneous warmed Ringer's lactate). In addition, mice had access to an intensive care
573 nutrition (glucose-soaked standard chow pellets)^{77,78} in a 30°C thermoneutral cage environment.

574 **Study design:** The study was divided in two parts, the CO₂ VDR comparison and the assessment of CO
575 and N₂ as alternatives to CO₂. The first part focused on the establishment and validation of the
576 methods, with two widely accepted CO₂ euthanasia VDRs. In the second part of the study, CO and N₂
577 as potential alternatives for CO₂ euthanasia were evaluated with the validated setup. In both parts,
578 mice underwent surgical transmitter implantation, followed by a minimum 7-day recovery period.
579 During this period all implanted mice were randomized and assigned to the respective gas group (CO₂
580 30 % or 70 % VDR; CO and N₂). After recovery, mice were euthanized with one of the different gases at
581 the given VDRs and gas concentrations: 100 % CO₂ with a 30 % VDR or with a 70 % VDR, 8.6 % CO (in
582 18.3 % O₂ and 73.1 % N₂) with a 70 % VDR or 100 % N₂ with a 70 % VDR. A schematic representation
583 of the study design is shown in **Fig. 1**. 48 mice per transmitter type were assigned to the CO₂ groups at
584 30 % VDR or 70 % VDR. To actively reduce the number of animals needed for this study, we performed
585 a first analysis of the CO₂ VDR comparison and an updated sample size calculation, which allowed us
586 to reduce the number of mice per transmitter from 48 to 24 for CO and N₂. All mice were randomly
587 assigned to the experimental groups.

588 **Euthanasia protocol:** All mice were euthanized in a whole-body plethysmography (WBP) chamber
589 (Buxco®, DSI, USA) by exposing them to different gases either with a 30 % VDR per minute or with a 70
590 % VDR per minute, using the FinePointe 3.1 software (DSI, USA). All mice were acclimated to the

chamber for at least 15 minutes before baseline recordings. The room air was then (automatically) changed to the respective gas and its corresponding VDRs. All gases (mixtures) were obtained from Linde Gas (Switzerland).

Oxygen measurements and estimation of in-chamber gas composition: A calibrated O₂ sensor (CheckMate 3, MOCON, Denmark) was positioned over the gas-inlet port of an empty WBP chamber. Fractional O₂ was recorded during room-air ventilation and every 30 s after the start of each euthanasia protocol; three replicate runs were performed for every gas mixture. The resulting O₂ traces were imported into MATLAB R2023a (MathWorks, USA), averaged per mixture and interpolated with a cubic spline to obtain a 1-s-resolution curve O₂(t) covering the first 6 min of exposure. Because all chamber O₂ originates from the resident air, the fraction of residual air was calculated as O₂(t)/20.95 %, and its complement was taken as the fraction of the delivered euthanasia gas. These two fractions were multiplied by the known dry-gas composition of air (N₂ 78.08 %, O₂ 20.95 %, CO₂ 0.04 %) and of the test mixtures (100 % CO₂, 8.6 % CO in N₂, or 100 % N₂) to reconstruct the full time-resolved chamber composition for O₂, CO₂, CO and N₂. Severe hypoxia was predefined as ambient O₂ < 10 %, which corresponds to an altitude of approx. 5'800 m.a.s.l., representing an extreme high altitude. The reconstructed O₂(t) and derived gas fractions were aligned to the experimental time base used for behavior/EEG, enabling time-locked annotation of hypoxia.

Behavioral observations and LOP: Offline behavioral analysis was performed on video recordings to assess animal responses after gas exposure with the open-source software BORIS 8.2⁷⁹ in the EEG animal cohort. Briefly, events such as LOP (as the last voluntary movement of the animal), gasping (sudden and forceful breathing efforts, indicating respiratory distress or hypoxia), and muscle fasciculations (or twitches) were assessed from video recordings, and time-stamped as discrete events and reported as counts. Seizures (uncontrollable convulsions reflecting sudden, temporary disruption of the brain's normal electrical activity) were scored as START-and-STOP episodes and reported as cumulative time seizing between gas start and LOP, obtained by summing all START–STOP seizure epochs. For each behavior, the normalized rate (s⁻¹), or frequency, was computed per animal as the total count between gas start and LOP divided by that animal's LOP latency. Analysis was performed by a person blinded to gas condition and VDR assignment; scoring was performed once and reviewed by a second blinded person for internal consistency.

In chamber activity: Activity was recorded using implanted telemetry transmitters (DSI, USA) and acquired with Ponemah 6.51 (DSI, USA) at sampling rate of 1 kHz. The same software triggered synchronous video capture in Media Recorder 6.2 (Noldus, Netherlands). In the EEG animal cohort, the x–y position of each transmitter on the receiver plate was polled once per second and converted to activity counts. Relative activity was calculated by dividing post-gas influx counts by the mean at baseline.

Whole-body plethysmography: Measurements were performed in mouse WBP chambers (Buxco®, DSI, USA) that were shielded from the room to reduce interference from external confounders. Data were collected with FinePointe 3.1 (DSI, USA), which allowed a soft and automatic filtering for outlier measurements according to the manufacture's algorithm and recommendations.

Video data: Each WBP chamber was individually recorded using two Logitech Brio 4K webcams (Logitech, Switzerland) running on Media Recorder 6.2 (Noldus, Netherlands). The cameras were positioned to capture a front and rear view of the chambers. The recordings were synchronized and started with Ponemah 6.51 (DSI, USA).

Telemetric cardiovascular monitoring (ECG & BP): As a baseline, ECG were recorded for at least 15 minutes before gas influx, and from the moment of gas influx until the animal was dead (confirmation done by observing asystole). Raw ECG and MAP traces were reviewed off-line; episodes with motion artefact or signal dropout were removed before analysis. Clean data were binned to 1-s epochs, averaged within treatment groups and visualized in MATLAB R2023a (MathWorks, USA).

Cortical and muscular electrophysiology (EEG/EMG): To verify the effect of each gas on the EEG spectra, EEG/EMG recordings (unilateral 1-channel in differential mode) were performed throughout the experiments. Again, recordings were made for at least 15 min before gas influx to serve as baseline, and from the moment of gas influx until the animal was dead by visual confirmation of cessation of breathing. The EEG/EMG signal was sampled at 500 Hz using Ponemah 6.51 (DSI, USA), amplified after application of an anti-aliasing low-pass filter, synchronously digitized, and stored locally on a workstation (Dell, USA).

EEG postprocessing: Data were exported with the respective recording software and further analyzed in Matlab R2023a (MathWorks, USA). Recordings were inspected visually; regional artefacts, clipping and transient spikes (detected by a custom routine) were rejected. Missing samples were spline-interpolated. The EEG signal was filtered between 0.2 and 50 Hz using a zero-phase bandpass FIR filter (filter order $\approx 10\,000$), designed with a Hamming window to maintain a linear phase response. Finally, residual baseline artifacts were removed using wavelet decomposition. Briefly, EEG signals before gas exposure were decomposed into six levels using Daubechies wavelets (db4) and wavelet coefficients were corrected using an adaptive universal threshold (MAD-based) to suppress noise while preserving the underlying signal characteristics. The EEG signal after gas influx was not corrected to avoid deleting signature effects of gas induction. All the steps were applied using the same criteria, irrespective of experimental group.

EMG postprocessing: EMG was acquired at 500 Hz with the same software used for EEG and processed offline in MATLAB R2023a (MathWorks, USA). After a visual scan for regional artefacts, isolated spikes were removed as described for the EEG. Any missing samples were replaced by cubic-spline interpolation. The trace was then band-pass filtered between 10 and 80 Hz with a linear-phase FIR filter designed with a Kaiser window ($\beta = 5$, fLow order ≈ 1650). Forward-backward (zero-phase) filtering (filtfilt) eliminated phase distortion while preserving temporal alignment. The filtered signal was full-wave rectified and converted to an amplitude envelope with a moving root-mean-square (RMS) window of 50 ms, which constituted the EMG signal for all downstream analyses. All the steps were applied using the same criteria, irrespective of experimental group.

General spectral analysis and α/δ ratio: Wakeful periods during baseline were analyzed with the fast Fourier transform using a 1 024-sample Hamming window, 50% overlap and zero-padding to 2 048 points. Power-spectral density was averaged in six bands: δ (0.5–4 Hz, delta), θ (4–8 Hz, theta), α (8–12 Hz, alpha), σ (12–16 Hz, sigma), β (16–30 Hz, beta) and low- γ (30–50 Hz, low-gamma). For each window, band power was expressed in dB, smoothed with a third-order Savitzky-Golay filter (9-point window) and converted to percentage of the total broadband power at that time point. For each animal, a time-frequency spectrogram (0–50 Hz) was computed with a 4-s Hamming window, 90 % overlap and a 2 048-point FFT, z-normalized to the animal's own baseline (-60 to 0 s), smoothed with a 2-D Gaussian kernel, and finally resampled to a common 0.25-s $\times$ 0.5-Hz grid. A cortical-arousal metric was quantified as the α (8–12 Hz) / δ (0.5–4 Hz) power ratio, moving-average-smoothed, and then expressed as a fold-change relative to each animal's baseline (-60 to 0 s).

Burst suppression and LOC: Continuous EEG was converted to a unipolar amplitude trace and considered suppressed when amplitude fell below 10 % of the baseline mean. A 10 s window, advanced in 1 s steps, yielded the burst-suppression ratio (BSR). LOC was established as the inflection-point of the BSR trace, defined as the earliest post-gas time at which a Savitzky–Golay-smoothed BSR exceeded 0.30 for ≥ 10 s while its slope was $>0.01 \text{ s}^{-1}$; if absent, the halfway point between minimum and maximum BSR in the first 120 s was used as an estimate.

Spectral-edge frequency: each individual EEG was segmented with a 10-s Hamming window advanced in 1-s steps from -60 s to $+180$ s around the onset of gas influx. Power-spectral density was estimated with Welch’s method and its cumulative sum was used to locate the SEF95, i.e. the frequency below which 95 % of the total power resided. The mean SEF95 during the pre-gas baseline (-60 to 0 s) was then subtracted from every subsequent value, yielding a baseline-referenced trace $\Delta\text{SEF95}(t)$, a measure of instantaneous spectral slowing or acceleration.

Isoelectric EEG/EMG: Isoelectric entry was detected independently in EEG and EMG. Each rectified trace was converted to a 1 s RMS envelope (500-sample sliding window) and smoothed with a third-order, 21-point Savitzky–Golay filter. Working backwards from the end of the record, the algorithm located the first 30-s plateau whose RMS satisfied three conditions: it remained below an animal-specific isoelectric threshold (EEG, the larger of $2 \mu\text{V}$ or 20 % of the pre-gas baseline RMS; EMG, the maximum RMS in the final 30 s plus $1 \mu\text{V}$), the within-window RMS standard deviation did not exceed $3 \mu\text{V}$, and fewer than 10 % of samples breached the threshold. For EMG, a $\geq 5 \mu\text{V}$ drop relative to the first-second RMS as also a requirement, ensuring a genuine loss of muscle tone. The start of this qualifying plateau defined the isoelectric-zone onset, and baseline-corrected RMS traces ($\Delta\mu\text{V}$ relative to -60 – 0 s) were averaged across treatment groups.

Gamma (γ) bursts: Low- γ power (30 – 50 Hz) was tracked specifically as a surrogate for brief, seizure-like discharges. A continuous Morlet wavelet ($12 \text{ voices} \cdot \text{octave}^{-1}$) was applied to the EEG; power was averaged across 1-Hz bins within the band and then z-scored to each animal’s pre-gas baseline (-60 to 0 s). Segments in which the z-score exceeded $+3$ for ≥ 0.5 s were tagged as γ -bursts, marking putative ictal-like events. To visualize putative seizure-like discharges on an animal-by-animal basis we focused on the low- γ range (30–50 Hz). Low- γ activity was quantified in four steps: the EEG was band-pass filtered with a fourth-order, zero-phase IIR filter, and its Hilbert envelope smoothed with a 300 ms moving mean to produce a continuous γ -power envelope. That envelope was z-scored to each animal’s own pre-gas baseline (-60 to 0 s); this converts every time-point to the number of baseline standard deviations it exceeds or falls below. Finally, contiguous samples whose z-score exceeded $+3$ were grouped and highlighted in “ γ -bursts.”

Group-panels: For every variable plotted at the group level (among others: activity, HR, RR, BS, SEF-95, γ -power, etc.), the time series from each animal were first aligned to gas onset (defined as $t = 0$ s) and averaged pointwise. The shaded band around each mean curve represents the standard error of the mean (SEM). Where relevant, a vertical dashed line was added at the group-averaged time of LOP or LOC.

Plasma analysis: An ACTH ELISA kit (ab263880, LOT 2101049037, Abcam, United Kingdom) was used to quantify ACTH in the blood plasma according to the manufacturer’s specifications. For noradrenaline quantification, a high sensitivity ELISA kit (EA 633/96, LOT NS 116, Eagle Bio, USA) was used according to the manufacturer’s specifications. All samples were measured in duplicates. Absorbance values were determined using a spectrophotometer (μQuant , BioTek, USA). Extrapolation of the standard curve was performed in GraphPad Prism 10.0.3 (GraphPad Software LLC, USA).

Histopathological analysis: For histological evaluation of the lungs and upper airways, 40 animals with EEG transmitters were randomly selected (10 per gas group, balanced sex ratio). Animals were selected from the EEG group only, because a catheter was placed in the carotid artery and ECG transmitter occupies space in the abdomen, potentially affecting lung expansion. Heads and lungs were harvested immediately after euthanasia and fixed in 10 % neutral buffered formalin for 48 hours. Prior to paraffin embedding, heads were cut in half longitudinally with a diamond saw and decalcified in RDF Mild Decalcifier (CellPath Ltd, Newtown, UK) for five days. Three micrometer thick sections were stained with hematoxylin and eosin according to established protocols. Histological slides were examined in a blinded manner³⁷. Morphological changes were quantitatively analyzed using the Visiopharm 2023.09.3.15043 software (Visiopharm, Hoersholm, Denmark). Customized Analysis Protocol Packages (APPs) were developed in Visiopharm based on a threshold method for hematoxylin and eosin-stained sections. These APPs were used to measure the area occupied by erythrocytes in each lung section as an indicator of congestion and by air spaces, as previously described³⁷.

Multisystem distress-burden heatmap: The multisystem heatmap integrates physiological and behavioral parameters into a unified welfare index. Each parameter was expressed as an *animal cost*, defined as the deviation between animal-level baseline values (–60 to 0 s) and post-gas values within the parameter-specific analysis window (typically (0, t_{LOP}) or (0, t_{LOC})). Physiological signals, counts, and time windows matched those used in the corresponding analyses described elsewhere in this study. Plasma stress hormones and histological endpoints were excluded because reliable baselines cannot be established without blood sampling during baseline or pre-mortem tissue measurements. Each parameter carried an explicit orientation rule defining whether higher, lower, or bidirectional deviations from baseline corresponded to higher distress: baseline-floor (higher = more distress), baseline-ceil (lower = more distress), or two-sided (any divergence implies distress). For peer-based rules, where no absolute baseline reference exists, animal costs were computed at the group level using the mean across animals within each gas condition. To normalize heterogeneous costs across parameters, a two-anchor scaling was used for reference-based rules and a purely relative scaling for peer-based rules. The absolute anchor down-weighted costs that did not exceed the intrinsic variability of each parameter, while the relative anchor preserved cross-group contrast. Peer-based parameters, which lack a baseline anchor by design, were scaled using a standard min–max normalization across gases. All resulting values were mapped to a 0–1 distress scale, where 1 represents high distress and 0 represents minimal distress. System-level scores shown above each block represent the mean of parameter-level welfare scores within that physiological domain. The *global distress-burden index* at the bottom aggregates all parameters into a single welfare value per gas by quantifying the proportion of parameters with minimal distress (scores < 0.45, green zone) versus clearly high-distress outcomes (scores > 0.55, red zone), with intermediate values ignored. The overall welfare index corresponds to the fraction of green scores relative to the total number of green and red scores. Missing group means were excluded from averages and displayed in light grey. This framework was designed to be conservative: it penalizes clear deviations from baseline through the absolute anchor but avoids inflating minor numerical differences through relative scaling. Peer-based parameters convey relative ranking among gases rather than absolute magnitude of distress. For a multi-parameter analyses of the heatmap, we used: (i) per-animal composite burden scores (rank-based), (ii) permutation tests for the observed difference between the top two group means, (iii) bootstrap resampling to estimate the probability that each gas produced the lowest burden, and (iv) linear mixed-effects models with “gas” as a fixed effect and random intercepts for “animal”, “parameter”, and “system domain”. All parameters contributing to the multisystem distress-burden heatmap are listed below:

- 769 **Behavioral**
- 770 1. Cumulative activity — Total activity (relative) counts (0, tLOP) (events)
- 771 2. Activity deviation / agitation — $\langle | \text{smoothed Activity}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{Activity}) | \rangle$ (events/s)
- 772 3. Latency to Loss of posture — Time from gas onset to tLOP (s)
- 773 4. Movement frequency — Frequency during $t = (0, \text{LOP})$ (events/s)
- 774 **Cardiovascular**
- 775 5. Peak heart rate response — $\text{Max}(\text{HR}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{HR}))$ (beats/min)
- 776 6. Heart rate at LOP — $\text{HR}(\text{tLOP}) - \mu_{\text{baseline}}(\text{HR})$ (beats/min)
- 777 7. Heart rate instability — $\langle | \text{smoothed HR}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{HR}) | \rangle$ (beats/min²)
- 778 8. Bradycardic/late heart rate slope — $\text{slope}(\text{HR}(\frac{2}{3} \text{tLOP}, \text{tLOP}))$ (beats/min²)
- 779 9. Peak mean blood pressure response — $\text{max}(\text{MAP}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{MAP}))$ (mmHg)
- 780 10. Mean blood pressure at LOP — $\text{MAP}(\text{tLOP}) - \mu_{\text{baseline}}(\text{MAP})$ (mmHg)
- 781 11. Mean blood pressure deviation from baseline — $\langle \text{MAP}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{MAP}) | \rangle$ (mmHg)
- 782 12. Fraction of time in hypotension — Normalized fraction of time $(\text{MAP}(0, \text{tLOP}) < \mu_{\text{baseline}}(\text{MAP}) - 2\sigma_{\text{baseline}}(\text{MAP})) \cdot \delta(0, \text{tLOP})$ (unitless)
- 783
- 784 **Respiratory**
- 785 13. Breathing frequency — Frequency during $t = (0, \text{LOP})$ (events/s)
- 786 14. Peak respiration rate response — $\text{max}(\text{RR}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{RR}))$ (breaths/min)
- 787 15. Early respiratory drive — $\langle \text{RR}(0, \frac{1}{2} \text{tLOP}) \rangle - \mu_{\text{baseline}}(\text{RR})$ (breaths/min)
- 788 16. Respiratory deceleration — $\text{slope}(\text{RR}(\frac{2}{3} \text{tLOP}, \text{tLOP}))$ (breaths/min²)
- 789 17. Respiration rate irregularity — $\text{SD}(\text{RR}(0, \text{tLOP}))$ (breaths/min)
- 790 18. Tidal volume deviation — $\langle \text{TV}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{TV}) | \rangle$ (mL)
- 791 19. Fraction of time at elevated tidal volume — normalized fraction of time $(\text{TV}(0, \text{tLOP}) > \mu_{\text{baseline}}(\text{TV}) - 2\sigma_{\text{baseline}}(\text{TV})) \cdot \delta(0, \text{tLOP})$ (unitless)
- 792
- 793 20. Tidal volume instability — $\langle | \text{smoothed TV}(0, \text{tLOP}) - \mu_{\text{baseline}}(\text{TV}) | \rangle$ (mL)
- 794 21. Inspiratory drive rate — $\langle \text{max}(\delta \text{TV}(0, \text{tLOP}) / \delta(0, \text{tLOP}), 0) \rangle$ (mL/s)
- 795 **Neurophysiological**
- 796 22. Time conscious in hypoxia — $\text{max}(0, \text{tO}_2 > 10\% - \text{tLOC})$ (s)
- 797 23. Motor-to-consciousness gap — $\text{tLOC} - \text{tLOP}$ (s)
- 798 24. SEF95 irregularity — $\text{SD}(\text{SEF95}(0, \text{tLOC}) - \text{smoothed SEF95}(0, \text{tLOC}))$ (Hz; (0–50 Hz))
- 799 25. Median frequency fluctuation rate — $\langle (\delta \text{smoothed MF}(0, \text{tend}) / \delta(0, \text{tend}), 0) \rangle$ (Hz/s; (0–50 Hz))
- 800
- 801 26. Alpha/delta ratio range — $\text{max}(\text{RAD}(0, \text{tend}) - \text{min RAD}(0, \text{tend})) / \sigma_{\text{baseline}}(\text{RAD})$ (unitless)
- 802 27. Fraction of time at seizure-like activity — Fraction of time $(z\text{-scored low-}\gamma(0, \text{tLOC}) > 1.645) \cdot \delta(0, \text{tLOC})$ (s; 30–50 Hz, z to baseline)
- 803
- 804 28. Abnormal low-gamma burden — $\text{AUC}(z\text{-scored low-}\gamma(0, \text{t}_{\text{LOC}})) \cdot \delta(0, \text{t}_{\text{LOC}})$ (s; 30–50 Hz, z-scored to baseline)
- 805
- 806

807 Symbols and notation are defined as follows: μ_{baseline} and σ_{baseline} denote the mean and
 808 standard deviation during baseline; δ indicates the time derivative; $\langle \cdot \rangle$ indicates temporal averaging
 809 within the specified window; t denotes time from gas onset; t_{end} is the end of the analyzed window;
 810 $t_{\text{O}_2 > 10\%}$ marks the time when oxygen levels drop below 10%; $\text{slope}(a, b)$ indicates the linear slope
 811 between timepoints a and b ; HR = heart rate; RR = respiration rate; MAP = mean blood pressure; TV =
 812 tidal volume; SEF95 = spectral edge frequency 95%; MF = median frequency; RAD = ratio of alpha-to-
 813 delta EEG power; AUC = area under the curve; ACTH = adrenocorticotrophic hormone; SD = standard

deviation within the analyzed window; N.f. = normalized fraction of time meeting a given criterion; z-scored indicates normalization to each animal's own baseline mean and standard deviation.

Data handling and statistical analysis: Continuous variables are summarized in the results section as mean $\pm$ SD for consistency with figures. Non-parametric tests were used when normality and variance assumptions failed. For single-factor comparisons across the four gas conditions, we used Kruskal–Wallis tests with Dunn–Sidak–corrected Wilcoxon rank-sum tests for pairwise comparisons. Within-animal baseline vs post-gas comparisons used Wilcoxon signed-rank tests. For EEG-derived time–frequency metrics, cluster-based permutation tests were used to evaluate group-level deviations in baseline-normalized spectrograms. Comparisons of slopes and instability metrics (e.g. BSR, SEF95, MF, ADR) used Kruskal–Wallis tests with Dunn–Sidak correction. For the multisystem distress-burden analysis, we combined per-parameter burden scores into per-animal indices and compared gases using Kruskal–Wallis tests with Wilcoxon pairwise tests under Benjamini–Hochberg FDR control, linear mixed-effects models with gas as a fixed effect and random intercepts for animal, parameter, and system domain, and nonparametric resampling (permutation tests for best–second-best gaps and bootstrap estimation of win probabilities). All data management, extraction, processing, statistical analyses and plotting were performed either in Excel (version 2310, Microsoft Cooperation, USA), Spyder IDE 5.5.5 (Python 3.8.10), Matlab R2024a (MathWorks, USA) and/or Graph Pad Prism 10.0.3 (GraphPad Software, USA). Full analysis scripts are available from the authors on reasonable request.

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Author Contributions

P.V. conceived, designed and performed experiments (gas exposition, necropsies, ELISA, data acquisition), analyzed/reviewed the data, managed the project, and wrote the manuscript.

C.F.M. performed experiments (EEG surgeries, necropsies), analyzed/reviewed the data, and wrote the manuscript.

M.H. performed experiments (necropsies) and scored behavioral data.

F.P. performed experiments (necropsies, histological processing) and analyzed corresponding data.

C.T.R. designed experiments and obtained funding.

C.A.W. conceived and designed experiments, discussed data, and acquired funding.

C.C. conceived, designed and performed experiments (EEG surgeries, gas exposition, necropsies, data acquisition), reviewed the data and the manuscript.

853 P.S. conceived, designed and performed experiments (ECG surgeries), discussed data, acquired
854 funding, and wrote the manuscript.

855 All authors read, edited, and approved the final manuscript.

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857 **Declaration of interests**

858 The authors declare that the research was conducted without any commercial or financial
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