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1 Mechanisms of respiratory depression induced by the combination of 2 buprenorphine and diazepam in rat

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14
15 **Abbreviated title:** Buprenorphine-diazepam and respiratory depression

17 Abbreviations

18 AUC, area under the curve; BBB, blood-brain barrier; B_{max} , radioligand-targeted receptor regional
19 density; BP_{ND} , non displaceable binding potential; ¹¹C-buprenorphine, carbon-11-radiolabeled
20 buprenorphine; CYP, cytochrome P450; f , respiratory frequency; GABA-R, γ -aminobutyric acid-
21 receptor; K_D , radioligand equilibrium dissociation constant; **NOP, nociception opioid receptor**; OR,
22 opioid receptor; PET, positron emission tomography; P-gp, P-glycoprotein; SRTM, simplified reference
23 tissue model; SUV, standard uptake value; T_E , expiratory time; T_I , inspiratory time; T_{Tot} , total time; V_E ,
24 minute ventilation; V_T , tidal volume.

27 Prior presentations

28 - Vodovar D, Chevillard L, Pottier G, Auvity S, Caillé F, Risède P, Buvat I, Tournier N, Mégarbane B.
29 Investigation of the pharmacodynamic interaction involved in the respiratory depression attributed to
30 diazepam/buprenorphine combination. Réanimation 2018, Paris. *Proceedings of Réanimation 2018, the*
31 *French Intensive Care Society International Congress. Ann Intensive Care. 2018 Feb 5;8(Suppl 1):13.*
32 *doi: 10.1186/s13613-017-0345-7.*

33 - Vodovar D, Pottier G, Auvity S, Buvat I, Caillé F, Tournier N, Mégarbane B. Imaging drug-drug
34 interaction using positron emission tomography (PET) scans: investigating the impact of diazepam on
35 buprenorphine-induced respiratory depression. EAPCCT 2017, Basel. *37th International Congress of*
36 *the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) 16-19 May 2017,*

1 *Basel, Switzerland. Clin Toxicol (Phila). 2017 Jun;55(5):371-544. doi:*
2 *10.1080/15563650.2017.1309792. Epub 2017 Apr 13.*
3 - Vodovar D, Pottier G, Auvity S, Mégarbane B, Buvat I, Caillé F, Tournier N. Imaging drug-drug
4 interaction using PET: investigating the impact of diazepam on buprenorphine-induced respiratory
5 depression. *EANM'16. European Journal of Nuclear Medicine and Molecular Imaging volume 43,*
6 *pages 1–734 (2016).*

7
8

9 **Editor's key points**

10

11 Use of buprenorphine as an analgesic is widely considered to have reduced respiratory depression due
12 to its ceiling effect on opioid receptors, but fatalities occur when combined with benzodiazepines.

13

14 The pharmacological mechanisms involved in the interaction between buprenorphine and diazepam on
15 respiratory depression in a rat model using arterial blood gas analysis, plethysmography, diaphragm
16 electromyography and buprenorphine binding in brain.

17

18 Co-administration of diazepam and buprenorphine induced early-onset sedation and respiratory
19 depression, while diazepam did not alter buprenorphine binding in brain or its interaction with opioid
20 receptors.

21

22 This *in vivo* study supports a **pharmacodynamic** mechanism for the diazepam-buprenorphine interaction
23 on respiratory depression involving their actions their target receptors.

23

1 **Abstract**

2 **Background:** The safety profile of buprenorphine has encouraged its widespread use. However,
3 fatalities have been attributed to benzodiazepine/buprenorphine combinations, by poorly understood
4 mechanisms of toxicity. **Mechanistic hypotheses include i) benzodiazepine-mediated increase in brain**
5 **buprenorphine (pharmacokinetic hypothesis); ii) benzodiazepine-mediated potentiation of**
6 **buprenorphine interaction with opioid receptors (receptor hypothesis); and iii) combined effects of**
7 **buprenorphine and benzodiazepine on respiratory parameters (pharmacodynamic hypothesis).**

8 **Methods:** We studied the neuro-respiratory effects of buprenorphine (30 mg.kg⁻¹, i.p.), diazepam (20
9 mg.kg⁻¹, s.c.) and diazepam/buprenorphine combination in rats using arterial blood gas analysis,
10 plethysmography and diaphragm electromyography. Pretreatments with various opioid and gamma-
11 aminobutyric acid-receptor antagonists were tested. Diazepam impact on brain ¹¹C-buprenorphine
12 kinetics and binding to opioid receptors was studied using PET-imaging.

13 **Results:** In contrast to diazepam and buprenorphine alone, diazepam/buprenorphine induced early-onset
14 sedation ($P<0.05$) and respiratory depression ($P<0.01$). Diazepam did not alter ¹¹C-buprenorphine brain
15 kinetics or binding to opioid receptors. Diazepam/buprenorphine-induced effects on inspiratory time
16 were additive, driven by buprenorphine ($P<0.0001$) and were blocked by naloxonazine ($P<0.01$).
17 Diazepam/buprenorphine-induced effects on expiratory time were non-additive ($P<0.01$), different from
18 buprenorphine-induced effects ($P<0.05$) and were blocked by flumazenil ($P<0.01$).
19 Diazepam/buprenorphine-induced effects on tidal volume were non-additive ($P<0.01$), different from
20 diazepam- ($P<0.05$) and buprenorphine-induced effects ($P<0.0001$) and were blocked by naloxonazine
21 ($P<0.05$) and flumazenil ($P<0.05$). Compared to buprenorphine, diazepam/buprenorphine decreased
22 diaphragm contraction amplitude ($P<0.05$).

23 **Conclusions:** Pharmacodynamic parameters and antagonist pretreatments indicate that
24 diazepam/buprenorphine-induced respiratory depression results from a **pharmacodynamic interaction**
25 **between both drugs on ventilatory parameters.**

26

27 **Keywords:** benzodiazepine; buprenorphine; diazepam; PET imaging; rat; respiratory depression

1 Opioid overdose is a major public health concern worldwide representing the first cause of drug-related
2 death in the US.¹ Buprenorphine is considered as a partial μ -opioid receptor (MOR) agonist with a well-
3 established safety profile due to ceiling respiratory effects.² High-dose buprenorphine is increasingly
4 prescribed for maintenance therapy in patients with opioid use disorder to limit the societal impact of
5 the opioid overdose crisis.³ However, fatalities and ventilatory depression episodes have been reported,
6 especially with benzodiazepine/buprenorphine combination,^{4, 5, 5a} suggesting a deleterious drug-drug
7 interaction of toxicological relevance.

8 Preclinical studies have confirmed the deleterious role of the benzodiazepine/buprenorphine
9 combination on ventilation. In rats, neither buprenorphine alone (up to 80% of its lethal dose-50%) nor
10 benzodiazepines alone induced significant respiratory depression, while their combination did.⁴
11 However, the underlying toxicodynamic mechanisms remain to be elucidated. Buprenorphine is
12 extensively metabolized to norbuprenorphine by cytochrome P450 (CYP)3A4. *In vivo* and *in vitro*
13 studies show that benzodiazepines are unlikely to alter the metabolism and plasma disposition of
14 buprenorphine.⁴ Moreover, brain distribution of norbuprenorphine (but not buprenorphine) is restricted
15 by P-glycoprotein (P-gp) efflux at the blood-brain barrier (BBB).^{6, 7} Norbuprenorphine induced
16 respiratory depression only in P-gp-deficient mice and did not account for buprenorphine-induced
17 toxicity when P-gp was fully functional.⁸

18 Several hypotheses could explain benzodiazepine/buprenorphine-induced respiratory depression.
19 These include increased brain distribution of buprenorphine induced by benzodiazepines
20 (**pharmacokinetic hypothesis**); benzodiazepine-mediated potentiation of buprenorphine interaction with
21 MORs (**receptor hypothesis**); and additive *versus* non-additive interactions of buprenorphine and
22 benzodiazepine effects on ventilatory parameters (**pharmacodynamic hypothesis**).

23 The following study was designed to investigate the neuropharmacokinetic, receptor binding and
24 pharmacodynamic components of the diazepam/buprenorphine interaction *in vivo*. A rat model
25 mimicking respiratory depression observed after diazepam/buprenorphine co-administration was first
26 validated. A multimodal *in vivo* approach including plethysmography, arterial blood gas (ABG)
27 analysis, diaphragm electromyography and ¹¹C-radiolabeled-buprenorphine (¹¹C-buprenorphine) brain
28 positron emission tomography (PET)-imaging was performed. Selective γ -aminobutyric acid-receptor
29 (GABA-R) and opioid receptor antagonists were tested to determine the contribution of each receptor
30 system to the diazepam/buprenorphine interaction leading to respiratory depression.

31

1 **Methods**

2 **Animals**

3 The study was conducted in accordance with the French legislation and European directives on animal
4 use in research. Animal data are reported according to ARRIVE guidelines. The protocol was approved
5 by the local animal ethics committee (P2.BM.128.10 and APAFIS#7466-20-1611-04-1-7049220v2).
6 We used male Sprague-Dawley rats (Janvier, France) weighing 250-350 g on the experiment day. Rats
7 were housed in a temperature- and humidity-controlled facility, with 12 h dark-light cycles and free
8 access to food and water. Surgical procedures were performed using 70 mg.kg⁻¹ ketamine i.p. and 10
9 mg.kg⁻¹ xylazine i.p. PET-imaging experiments were performed under isoflurane anaesthesia (induction,
10 3% followed by 1.5–2.5% isoflurane in O₂). At the end of the experiment, rats were killed by
11 asphyxiation in a CO₂ chamber. A total of 171 rats was used.

12 **Drug preparation and dosing**

13 *Neuro-respiratory effect study:* Buprenorphine hydrochloride (Schering-Plough, city, France) and
14 diazepam (Sigma–Aldrich, city, France) were diluted in Tween®10% (Sigma–Aldrich) to obtain 30 and
15 20 mg.ml⁻¹ solutions, respectively. **Buprenorphine (30 mg.kg⁻¹ i.p.) and diazepam (20 mg.kg⁻¹ s.c.) were**
16 **administered at doses and routes previously shown not to alter ventilation (ABG and plethysmography**
17 **parameters) or the level of consciousness in rats if administered separately.**^{9–11}

18 *Imaging experiments:* Buprenorphine (Buprecare®, Animalcare, city, UK) was diluted in saline to a 0.15
19 mg.ml⁻¹ and administered i.v. by the tail vein at a 0.15 mg.kg⁻¹. ¹¹C-buprenorphine (CEA, city, France)
20 was synthesized inhouse as described and diluted in saline to obtain 42.7±1.6 MBq in 1 ml volume
21 (specific radioactivity 76.2±8.2 GBq.μmol⁻¹).¹²

22 *Antagonists:* Naloxonazine dihydrochloride (μ-OR antagonist), nor-binaltorphimine dihydrochloride
23 (κ-OR antagonist), naltrindole dihydrochloride (δ-OR antagonist), flumazenil (GABA_A-R antagonist),
24 and saclofen (GABA_B-R antagonist) were purchased from Sigma–Aldrich. All except flumazenil were
25 diluted in distilled water to 5 mg.ml⁻¹ naloxonazine, 3 mg.ml⁻¹ nor-binaltorphimine, 2.5 mg.ml⁻¹
26 naltrindole and 10 mg.ml⁻¹ saclofen solutions. Flumazenil was diluted in Tween®10% to obtain a 10
27 mg.ml⁻¹ solution. **Antagonists were administered before diazepam/buprenorphine combinations at times**
28 **and doses known to ensure complete antagonism of various opioids and benzodiazepines in the rat, i.e.**
29 10 mg.kg⁻¹ naloxonazine i.v. 5 min before, 5 mg.kg⁻¹ nor-binaltorphimine s.c. 45 min before, 3 mg.kg⁻¹
30 naltrindole s.c. 6 h before, 10 mg.kg⁻¹ flumazenil i.p. 20 min before and 11.2 mg.kg⁻¹ saclofen i.p. 15
31 min before diazepam/buprenorphine administration.^{11, 13–15}

32 **Study design**

33 Randomisation was carried out by drawing lots. The experimenters were not blind to the treatments.

1 *Study 1: Neuro-respiratory effects of diazepam/buprenorphine combination (n=72 rats):* For each sub-
2 study, rats were randomised into four groups (n=6 per group) to receive diazepam or its vehicle
3 immediately followed by buprenorphine or its vehicle, defining the vehicle/vehicle (control),
4 diazepam/vehicle (diazepam), vehicle/buprenorphine (buprenorphine) and diazepam/buprenorphine
5 (treatment) groups. Thereafter, the neurological, ABG (Study1a), plethysmography (Study1b) and
6 electromyography (Study1c) parameters were studied.

7 *Study 2: Effects of diazepam on ¹¹C-buprenorphine brain distribution and receptor binding (n=14 rats):*
8 To validate the cerebellum as reference region, rats were randomised into two groups (n=2 per group)
9 to receive buprenorphine i.v. (displacement) or saline (control group) 30 min after ¹¹C-buprenorphine
10 administration (study2a). To assess the impact of diazepam on the regional ¹¹C-buprenorphine
11 distribution and receptor binding, rats were randomised into two groups (n=5 per group) to receive
12 diazepam (diazepam/¹¹C-buprenorphine) or its vehicle (control group-vehicle/¹¹C-buprenorphine group)
13 15 min (peak of respiratory effects) before ¹¹C-buprenorphine administration (study2b).

14 *Study 3: Effects of GABA-R and opioid receptor antagonists (n=60 rats):* For each antagonist, rats were
15 randomised into 10 groups (n=6 per group) to receive the antagonist or its vehicle (control groups;
16 distilled water or Tween®10%, as appropriate) followed by the diazepam/buprenorphine combination.
17 Thereafter, the neurological, ABG and plethysmography parameters were studied.

18 **Outcome parameters**

19 *Neurological parameters:* Drug-induced effects on temperature were continuously measured during
20 plethysmography. Sedation, ataxia and tonus were assessed in four levels (0-3; [supplemental material-](#)
21 [1A](#)).¹⁶ Neurological parameters were obtained 30, 15 and 5 min before and 5, 10, 15, 30, 60, 120, 180
22 and 240 min after drug injection.

23 *Arterial blood gas analysis:* Arterial catheterization of rats is described in [supplemental material-1B](#).
24 On the experiment day, the rat was placed in a horizontal Plexiglas cylinder (6.5 cm-internal diameter,
25 up to 20 cm-adjustable length) (Harvard Apparatus, Inc. Holliston, MA, USA), modified by the addition
26 of holes at the cephalic end to prevent CO₂ rebreathing. After a 60 min-period of acclimatization, the
27 catheter was exteriorized, flushed, and its patency verified. Arterial blood samples (100 µl) were
28 immediately analysed using Rapidlab®248 (Bayer Diagnostics, city, Germany) 5 min before and, 5, 10,
29 15, 30, 60, 120, 180 and 240 min after drug injection. Hourly, 200 µL heparinized saline were
30 administered to avoid catheter clotting and compensate for volume loss.

31 *Plethysmography parameters:* Respiratory parameters were recorded in a whole-body plethysmograph
32 by a barometric method ([supplemental material-1C](#)).¹⁷ Implantation of **transmitters** to measure rat body
33 temperature, measurement modalities and validation procedures are described.¹⁷ Treatment was
34 administered after a 60 min period of acclimatization. The rat was gently removed for injection and
35 replaced in the chamber. Respiratory parameters were recorded at 30, 15 and 5 min before and 5, 10,

1 15, 30, 60, 120, 180 and 240 min after drug injection, each recording lasting ~60 s. Tidal volume (V_T),
2 inspiratory time (T_I) and expiratory time (T_E) were measured. Total time ($T_{Tot}=T_I+T_E$), respiratory
3 frequency ($f=1/T_{Tot}$) and minute ventilation ($V_E=V_T*f$) were calculated.

4 *Diaphragm electromyography*: Electromyography signals represent electrical expression of
5 neuromuscular stimulation resulting in muscle contraction. Electromyography measures the
6 spontaneous electrical potential difference between two sites in a muscle, corresponding to a single
7 motor unit action potential.¹⁸ Five days before the experiment, the rat peritoneal cavity was surgically
8 opened under ketamine/xylazine (?) anaesthesia and two insulated multi-stranded stainless-steel wires
9 were fixed on the costal diaphragm using biological glue (3M[®]).¹⁹ Wires were connected to a telemetry
10 transmitter (DSI, city, France) implanted in the peritoneal cavity (as described above). On the
11 experiment day, the rat was placed in a cage upon the telemetry platform (DSI). Signals were recorded
12 continuously during a 60 min acclimatization period. The rat was gently removed for injection and
13 replaced in the chamber. Signals were then recorded for 240 min. The demodulated electrical signal was
14 high-pass filtered at 100 Hz to eliminate electrocardiogram interferences, rectified to get only positive
15 values (half-wave rectification), smoothed using a low-pass filter to obtain an envelope equivalent to
16 the modulating signal (Dataquest-A.R.T.4.3[™], DSI), and converted into digital data. We analysed 6 s
17 segments at 30, 15 and 5 min before and 5, 10, 15, 30, 60, 120, 180 and 240 min after drug injection.
18 To calculate the amplitude of diaphragm contraction, we calculated for each respiratory cycle the
19 difference in amplitude between the highest value at the peak of the curve and the lowest value preceding
20 this peak (Excel[®], Microsoft, Redmond, WA, USA).

21 ¹¹C-buprenorphine PET-imaging: PET-imaging was conducted using an Inveon[®] microPET-scanner
22 (Siemens, city, Germany). After catheterizing the tail caudal vein, the rat was placed into the scanner
23 with its head in the center of the field of view. Dynamic PET acquisition started at the time of i.v. ¹¹C-
24 buprenorphine injection. First, displacement experiments were performed to characterize ¹¹C-
25 buprenorphine specific (displaceable) binding to brain regions and assess the reliability of the
26 cerebellum as a reference region (devoid of specific binding) in rats.²⁰ Then, impact of diazepam
27 exposure on brain distribution and receptor binding of ¹¹C-buprenorphine was investigated *in vivo* (90
28 min-PET acquisition). PET data reconstruction is described in [supplemental material-1D](#). Time activity
29 curves were generated from dynamic PET images in selected brain regions predefined in the template.
30 Radioactivity concentration in each region was normalised by the injected dose and animal weight and
31 expressed as standard uptake value (SUV, unitless). Brain exposure to ¹¹C-buprenorphine was estimated
32 using the area under the curve (AUC) of the time-activity curve from 0 to 90 min. Pharmacokinetic
33 modelling using the simplified reference tissue model (SRTM) with the cerebellum as a reference region
34 was performed to estimate ¹¹C-buprenorphine binding potential to selected brain regions
35 ($BP_{ND}=B_{max}/K_D$, where B_{max} represents the regional receptor density and K_D , the radioligand equilibrium
36 dissociation constant).²¹

1 **Statistical Analysis**

2 Sample size was set based on previous studies using similar methodology and outcome.^{11,15} The analysis
3 was not blind to treatments and measurements. No animal having fully completed the experiments was
4 excluded. Results were expressed as mean (SEM). **For all parameters, T0 was the mean of the baseline**
5 **measurements (30, 15 and 5 min before drug administration) collected during the acclimatization period**
6 **when animals were awake and at rest.** For each parameter and at each time, the measured value was
7 corrected with its corresponding baseline value. To analyse the effects of time (up to 240 min) and
8 treatments on each parameter and for each animal, we performed a two-way analysis of variance
9 (ANOVA) followed by multiple comparison tests with Bonferroni's correction. AUCs were calculated
10 using the trapezoid method to analyse the global effect of each drug on each parameter and in each
11 animal during the first 60 min after administration. The distribution normality was tested using Shapiro-
12 Wilk tests. AUCs were compared using Student *t*-tests, Mann-Whitney tests, one-way ANOVA
13 followed by multiple comparison tests with Bonferroni's correction or Kruskal-Wallis tests followed by
14 multiple comparison tests with Dunn's tests, as appropriate. In the PET-imaging study, we compared
15 the BP_{ND} using Student *t*-tests. To study diazepam/buprenorphine interaction on each parameter, we
16 built a 2x2 (diazepam or not x buprenorphine or not) table of AUCs and performed a two-way
17 ANOVA.²² Tests were performed using Prism-v9.0 (GraphPad Software, Inc., San Diego, CA, USA).
18 **$P < 0.05$ was deemed statistically significant.**

19

20 **Results**

21 **Study1: Neuro-respiratory effects of diazepam/buprenorphine combination**

22 **Neurological temperature findings.** The diazepam/buprenorphine combination deepened sedation
23 ($P < 0.05$), decreased tonus ($P < 0.001$) and increased ataxia compared with other groups ($P < 0.001$;
24 **Table 1**). Effects of diazepam/buprenorphine started from 5 min after injection and peaked at 30 min for
25 hypotonia and at 60 min for sedation and ataxia (**Supplemental material-2**). Diazepam/buprenorphine
26 interaction was additive for tonus (related to both diazepam and buprenorphine, $P < 0.0001$) but not for
27 sedation or ataxia ($P < 0.01$). Diazepam/buprenorphine decreased body temperature compared with
28 buprenorphine ($P < 0.01$). In all groups, decreased in temperature was observed over the 240 min, starting
29 from 5, 10, 15 and 180 min for the diazepam/buprenorphine, diazepam, control and buprenorphine
30 groups, respectively. Diazepam/buprenorphine interaction on temperature was not additive ($P < 0.05$).

31 **ABG analysis.** Diazepam/buprenorphine decreased arterial pH ($P < 0.001$) and increased PaCO₂
32 compared with other groups ($P < 0.05$; **Table 1**). Diazepam/buprenorphine decreased PaO₂ compared
33 with the control ($P < 0.05$) and diazepam groups ($P < 0.01$). An increase in PaO₂ was observed in the
34 diazepam *versus* buprenorphine group ($P < 0.05$). Effects of diazepam/buprenorphine started 5 min after
35 injection, peaked at 15 min but were no longer significant after 30 min (**Fig 1**). Diazepam/buprenorphine

1 interaction was additive for PaO₂ (mostly related to buprenorphine, $P<0.001$) but not for pH ($P<0.001$)
2 or PaCO₂ ($P<0.05$). No significant difference in bicarbonate concentrations was observed.

3 **Plethysmography.** Increases in T_I were observed in the buprenorphine and diazepam/buprenorphine
4 groups compared with the control or diazepam groups ($P<0.01$ and $P<0.0001$, respectively; **Table 1**).
5 Decreases in T_E was observed in all groups compared to the control group ($P<0.05$), with more marked
6 decreases in the buprenorphine *versus* diazepam ($P<0.01$) and diazepam/buprenorphine groups
7 ($P<0.05$). Diazepam/buprenorphine decreased f ($P<0.05$), T_{Tot} ($P<0.05$), V_T ($P<0.05$), and V_E ($P<0.01$)
8 compared with other groups (**Table 1**). An increase in f and T_{tot} was observed in the diazepam/vehicle
9 *versus* control group ($P<0.05$). Effects of diazepam/buprenorphine on V_E started 5 min after injection,
10 peaked at 15 min and was no longer significant after 30 min (**Supplemental material-4**). The
11 diazepam/buprenorphine interaction was additive for T_I (mostly related to buprenorphine, $P<0.0001$)
12 but not for T_E ($P<0.001$), f ($P<0.0001$), V_T ($P<0.01$) and V_E ($P<0.001$).

13 **Diaphragm electromyography** Diaphragm contraction amplitude was increased in the buprenorphine
14 *versus* diazepam ($P<0.05$) and diazepam/buprenorphine groups ($P<0.01$; **Fig 2**; **Supplemental material-**
15 **5**). The diazepam/buprenorphine interaction was additive (mostly related to diazepam, $P<0.001$).

16 **Study 2: Effects of diazepam on ¹¹C-buprenorphine brain exposure and receptor binding**

17 Compared with baseline ¹¹C-buprenorphine brain kinetics, injection of unlabelled buprenorphine
18 induced a pronounced displacement of radioactivity from opioid receptor-rich regions such as the
19 striatum (**Fig 3A-C**). This unveiled the specific binding of ¹¹C-buprenorphine to receptors in this region
20 (**Fig 3B**). Such displacement was not observed in the cerebellum, devoid of μ -opioid receptor in rats.
21 Diazepam pretreatment did not increase ¹¹C-buprenorphine delivery to any tested brain region including
22 the cerebellum and striatum (**Fig 3C**). Diazepam did not induce changes in ¹¹C-BUP BP_{ND} in any brain
23 region (**Fig 3C**).

24 **Study 3: Effects of GABA receptor and opioid receptor antagonists**

25 Flumazenil reduced diazepam/buprenorphine-induced sedation ($P<0.05$) and hypotonia ($P<0.01$, **Table**
26 **2**). Flumazenil and nor-binaltorphimine reduced diazepam/buprenorphine-induced hypothermia
27 ($P<0.05$). Flumazenil and naloxonazine limited diazepam/buprenorphine-related effects on pH ($P<0.05$)
28 and PaCO₂ ($P<0.05$). Naltrindole, nor-binaltorphimine and saclofen did not impact
29 diazepam/buprenorphine-related effects on ABG parameters. Flumazenil and naloxonazine reduced
30 diazepam/buprenorphine-related effects on V_E ($P<0.01$). Flumazenil reduced diazepam/buprenorphine-
31 related effects of on T_E ($P<0.01$), f ($P<0.01$) and V_T ($P<0.05$). Naloxonazine reduced
32 diazepam/buprenorphine-related effects on T_I ($P<0.01$), f ($P<0.01$) and V_T ($P<0.05$). Saclofen,
33 naltrindole and nor-binaltorphimine did not reduce diazepam/buprenorphine-related effects on
34 plethysmography parameters.

35

1 Discussion

2 Compared with each drug administered alone, the diazepam/buprenorphine combination caused marked
3 respiratory depression in the rat, which results from a pharmacodynamic interaction and involves both
4 μ -opioid receptors and GABA_A-receptors. Our *in vivo* PET data do not support diazepam-mediated
5 impact on brain exposure (pharmacokinetic hypothesis) or binding of buprenorphine to μ -opioid
6 receptors (receptor hypothesis).

7 Compared to humans, higher doses of diazepam and buprenorphine are needed to exert CNS and
8 respiratory effects in rodents.²³ The selected doses of buprenorphine and diazepam we used were shown
9 not to induce respiratory depression or affect consciousness when separately administered in rats, thus
10 mimicking nontoxic conditions in humans.^{9, 10} Our results show that both the increase in T_I induced by
11 buprenorphine alone and the limited decrease in V_T induced by diazepam alone were counteracted by a
12 decrease in T_E . Consequently, we observed no change in f with buprenorphine and an increase in f with
13 diazepam compared to the control. V_E and arterial blood gas homeostasis were preserved when
14 administering each individual drug. Conversely, the diazepam/buprenorphine combination decreased
15 V_T and f , resulting in a decrease in V_E , respiratory acidosis and hypoxaemia. Compared with previous
16 studies, differences in intensity and duration of the observed respiratory effects could be attributed to
17 differences in drug regimens, administration routes and specific benzodiazepines.^{8–11, 16, 24–28}

18 Clarification of the mechanisms involved in the interaction between diazepam and buprenorphine is
19 needed. A previous microdialysis study showed that the highly potent benzodiazepine flunitrazepam did
20 not increase buprenorphine concentrations in the rat striatum, although this region is not directly
21 involved in the control of ventilatory function.²⁸ Our ¹¹C-buprenorphine PET imaging showed that
22 diazepam, administered at the dose used to study ventilation, did not alter exposure to buprenorphine in
23 whole brain including regions directly involved in ventilatory control. Brain penetration of the
24 radiometabolite ¹¹C-desmethyl-buprenorphine (¹¹C-norbuprenorphine) is negligible compared with its
25 parent (unmetabolised) compound.⁸ Moreover, benzodiazepines such as diazepam are unlikely to inhibit
26 P-gp function at the BBB.²⁹ Thus, brain PET signals associated with ¹¹C-buprenorphine injection
27 reflected buprenorphine concentrations in brain, suggesting that high-dose diazepam unlikely alters
28 buprenorphine transport across the BBB (pharmacokinetic hypothesis).

29 ¹¹C-Buprenorphine binding to brain regions was shown to predominantly reflect its interaction with
30 μ -opioid receptors rather than other opioid receptor subtypes.¹⁵ We showed the lack of specific
31 (displaceable) binding of ¹¹C-buprenorphine in the rat cerebellum. Pharmacokinetic modeling using the
32 cerebellum as reference region showed that diazepam did not alter specific binding of ¹¹C-buprenorphine
33 to μ -opioid receptors. Previous *ex vivo* studies reported that various benzodiazepines and analogues are
34 able to modulate down-regulation of μ -opioid receptors, observed 24 h after buprenorphine
35 administration in rats.^{30, 31} Our *in vivo* PET-study directly estimated ¹¹C-buprenorphine binding (BP_{ND})
36 15 min after diazepam administration (*i.e.* at the peak diazepam/buprenorphine-induced respiratory

1 effects), thus mimicking what occurs in poisoning. Isoflurane anaesthesia might have impacted the
2 neuropharmacokinetics and binding parameters of ^{11}C -buprenorphine. However, experimental
3 conditions were the same in diazepam- and vehicle-treated rats, and our results do not support the
4 hypothesis of diazepam-induced changes in buprenorphine binding at opioid receptors *in vivo* (**receptor**
5 **hypothesis**).

6 We also addressed the hypothesis suggesting that respiratory depression might result from the
7 combination of the pharmacodynamic effects of diazepam and buprenorphine on ventilation
8 (**pharmacodynamic hypothesis**). The diazepam/buprenorphine combination decreased f , in relation to
9 the combination of buprenorphine-related effects on T_I and diazepam-related effects on T_E . Diazepam
10 alone showed no significant effects on T_I while the specific μ -opioid receptor antagonist naloxonazine
11 prevented diazepam/buprenorphine-induced increase in T_I . Diazepam, buprenorphine and their
12 combination decreased T_E but to a greater extent than buprenorphine, while the decrease in T_E observed
13 with diazepam/buprenorphine did not differ from that observed with diazepam alone. Our results suggest
14 that the ceiling effect of buprenorphine alone on f is due to a compensatory decrease in T_E . Diazepam
15 appears to block this compensatory mechanism, thus precipitating respiratory depression. The GABA_A-
16 R antagonist flumazenil consistently prevented the diazepam/buprenorphine-induced decrease in T_E .
17 Interestingly, mechanisms of diazepam/buprenorphine toxicity differ from the toxicodynamic profiles
18 of other μ -opioid receptor agonists, which typically involve an increase in both T_I and T_E .¹¹

19 Both f and T_I/T_E partition are generated by anatomical structures located in the brainstem.³² This
20 includes a coupling of inspiration/expiration oscillators (the pre-Bötzinger complex and retro-
21 trapezoid/parafacial respiratory groups, respectively) modulated by pons structures (the Kölliker-Fuse
22 nucleus, parabrachial complex and locus coeruleus which control the inspiration/expiration transition or
23 post-inspiratory phase), and peripheral/central chemoreceptors.³² Opioid receptor and GABA_A-R
24 systems are co-expressed in these structures.³³ Opioids and benzodiazepines suppress baseline
25 inspiratory neuronal activity and alter the central inspiratory off-switch mechanism, which controls the
26 duration of inspiration and transition to expiration, respectively.^{32, 34} Moreover, they reduce the
27 chemoreceptor response to hypoxia and hypercapnia.³⁵ **It may therefore be hypothesized that**
28 **simultaneous binding of diazepam and buprenorphine to their respective CNS targets alters respiratory**
29 **times and triggers the deleterious benzodiazepine/buprenorphine interaction. However, it cannot be**
30 **excluded that other effects such as changes in body temperature or sedation, which are not directly**
31 **related to respiratory centres, may also account for the respiratory depression induced by the**
32 **diazepam/buprenorphine combination.**

33 Diazepam/buprenorphine-induced respiratory depression might also result from the combined effects
34 of each individual drug on respiratory muscle activity. Both opioids and benzodiazepines induce upper-
35 airway obstruction by oropharyngeal muscle relaxation.³⁵ Opioids also increase intercostal muscle
36 rigidity, decreasing chest compliance.³⁶ In response to upper-airway obstruction and opioid-induced

1 decrease in chest compliance, diaphragm contraction amplitude is not altered.³⁶ This is consistent with
2 our results showing that buprenorphine increases diaphragm contraction with no impact on V_T .
3 Conversely, benzodiazepines-elicited upper-airway compensatory mechanisms are characterized by an
4 increase in inspiratory intercostal and expiratory abdominal muscle activities whereas diaphragmatic
5 contribution is reduced.³⁷ We show that diazepam induced a slight decrease in diaphragm contraction
6 but did not decrease V_T . The decrease in V_T observed with the diazepam/buprenorphine combination
7 may thus result from worsening upper-airway obstruction in the presence of both drugs associated with
8 a loss of the respective compensatory mechanisms involved when buprenorphine and diazepam are
9 administered alone. In our study, diazepam/buprenorphine decreased diaphragm contraction amplitude
10 in comparison to buprenorphine or diazepam alone. Buprenorphine possibly induced chest rigidity and
11 altered the compensatory activity of accessory respiratory muscles required to counteract the diazepam-
12 induced decrease in diaphragm contraction amplitude. These hypotheses are supported by the reversion
13 of diazepam/buprenorphine-induced decrease in V_T using flumazenil or naloxonazine.

14 We assessed the impact of different pre-treatments on diazepam/buprenorphine-induced respiratory
15 depression. Naloxonazine and flumazenil prevented the decrease in V_E , while saclofen did not, although
16 GABA_B-R expression on human airway smooth muscle cells and contributions to central control of
17 ventilation have been reported.³⁸ Our previous work using PET imaging in rats showed that the selected
18 dose of the irreversible and μ -opioid receptor-specific antagonist naloxonazine³⁹ blocked binding of ¹¹C-
19 buprenorphine to neuroreceptors, while the δ -opioid receptor antagonist naltrindole and the κ -opioid
20 receptor antagonist nor-binaltorphimine did not.¹⁵ Naltrindole had no effects on arterial blood gas and
21 respiratory parameters, although the contribution of μ/δ -opioid receptor heterodimerization remains to
22 be investigated.⁴⁰ The role of κ -opioid receptors in ventilation is also limited and complex. Nevertheless,
23 our study confirms the absence of antagonism of buprenorphine-attributed effects by the κ -opioid
24 receptor antagonist nor-binaltorphimine.¹¹ Altogether, our approach using selected receptor-specific
25 antagonists suggests that diazepam/buprenorphine-induced toxicity predominantly depends on μ -opioid
26 receptors and GABA_A-receptors, rather than of δ/κ -opioid or GABA_B-receptors. Buprenorphine is also
27 an agonist of nociception-related opioid (NOP) receptors, but their contribution to the respiratory
28 depression induced by the diazepam/buprenorphine combination remains to be investigated. NOP
29 receptor agonists were shown to protect against respiratory depression induced by stimulation of μ -opioid
30 receptors using full agonists.⁴¹ However, to the best of our knowledge, benzodiazepines have not been
31 reported as NOP receptor antagonists. Moreover, the change in respiratory times observed with the
32 diazepam/buprenorphine combination did not resemble that of μ -opioid receptor agonists. Altogether,
33 these data do not support a role for NOP receptors in mediating the deleterious diazepam/buprenorphine
34 interaction.

35 Naloxone is poorly effective at reversing buprenorphine overdose.⁵ This was assumed to result from
36 the slow dissociation rate of buprenorphine from opioid receptors, as confirmed using ¹¹C-

1 buprenorphine binding in monkeys, together with the fast plasma clearance of naloxone.^{15, 42}
2 Surprisingly, flumazenil was shown to improve consciousness and bradypnea in most buprenorphine-
3 poisoned patients when benzodiazepines were known as co-ingestants.⁵ This observation is consistent
4 with our findings supporting flumazenil use as second-line antidote in patients presenting with opioid-
5 like overdose not reversed by naloxone, in the absence of contra-indications.

6

7 **Conclusions**

8 Our multimodal *in vivo* study supports a **pharmacodynamic** hypothesis that diazepam/buprenorphine-
9 induced respiratory depression results from their combined effects on ventilation related to actions on
10 GABA_A and μ -opioid receptors.

11

12 **Author's contributions**

13 Study design: BM, NT

14 Data collection: DV, LC, FC, PR, GP, SA

15 Data analysis: DV, LC, BM, NT

16 Data interpretation: DV, LC, BM, NT

17 Drafting of paper: DV, BM, NT

18 Critical revision of paper: BM, NT

19 Final approval of paper: all authors

20

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24 network (grant ANR-11-INBS-0006). They also thank Emilie Jaumain and Maud Goislard (CEA/SHFJ)
25 for their technical assistance.

26

27 **Declarations of interest**

28 There are no conflicts of interest associated with this study.

29

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33

References

1. Wilson N, Kariisa M, Seth P, Smith H, Davis NL. Drug and Opioid-Involved Overdose Deaths - United States, 2017-2018. *MMWR Morb Mortal Wkly Rep* 2020; **69**: 290–7
2. Dahan A, Yassen A, Romberg R, et al. Buprenorphine induces ceiling in respiratory depression but not in analgesia. *Br J Anaesth* 2006; **96**: 627–32
3. Olfson M, Zhang VS, Schoenbaum M, King M. Trends in Buprenorphine Treatment in the United States, 2009-2018. *JAMA* 2020; **323**: 276–7
4. Jones JD, Mogali S, Comer SD. Polydrug abuse: a review of opioid and benzodiazepine combination use. *Drug Alcohol Depend* 2012; **125**: 8–18
5. Mégarbane B, Buisine A, Jacobs F, et al. Prospective comparative assessment of buprenorphine overdose with heroin and methadone: clinical characteristics and response to antidotal treatment. *J Subst Abuse Treat* 2010; **38**: 403–7
- 5a. Torrance N, Veluchamy A, Zhou Y, Fletcher EH, Moir E, Hebert HL, Donnan PT, Watson J, Colvin LA, Smith BH. [Trends in gabapentinoid prescribing, co-prescribing of opioids and benzodiazepines, and associated deaths in Scotland.](#) *Br J Anaesth*. 2020;125:159-167.
6. Brown SM, Campbell SD, Crafford A, Regina KJ, Holtzman MJ, Kharasch ED. P-glycoprotein is a major determinant of norbuprenorphine brain exposure and antinociception. *J Pharmacol Exp Ther* 2012; **343**: 53–61
7. Tournier N, Chevillard L, Megarbane B, Pirnay S, Scherrmann J-M, Declèves X. Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2). *Int J Neuropsychopharmacol* 2010; **13**: 905–15
8. Alhaddad H, Cisternino S, Declèves X, et al. Respiratory toxicity of buprenorphine results from the blockage of P-glycoprotein-mediated efflux of norbuprenorphine at the blood-brain barrier in mice. *Crit Care Med* 2012; **40**: 3215–23
9. Cohier C, Chevillard L, Risède P, Roussel O, Mégarbane B. Respiratory effects of buprenorphine/naloxone alone and in combination with diazepam in naive and tolerant rats. *Toxicol Lett* 2014; **228**: 75–84
10. Nielsen S, Taylor DA. The effect of buprenorphine and benzodiazepines on respiration in the rat. *Drug Alcohol Depend* 2005; **79**: 95–101
11. Chevillard L, Mégarbane B, Risède P, Baud FJ. Characteristics and comparative severity of respiratory response to toxic doses of fentanyl, methadone, morphine, and buprenorphine in rats. *Toxicol Lett* 2009; **191**: 327–40
12. Luthra SK, Pike VW, Brady F, Horlock PL, Prenant C, Crouzel C. Preparation of [11C]buprenorphine--a potential radioligand for the study of the opiate receptor system in vivo. *Int J Rad Appl Instrum A* 1987; **38**: 65–6
13. Akada Y, Mori R, Kato Y, Yamasaki F, Mochizuki H. Analgesic properties of the novel compound M43068 in rat models of acute and neuropathic pain. *Eur J Pharmacol* 2005; **523**: 46–53
14. Korkmaz S, Ljungblad E, Wahlström G. Interaction between flumazenil and the anesthetic effects of hexobarbital in the rat. *Brain Res* 1995; **676**: 371–7
15. Auvity S, Goutal S, Caillé F, et al. Pharmacokinetic neuroimaging to study the dose-related brain kinetics and target engagement of buprenorphine in vivo. *Neuropsychopharmacology* 2021;
16. Pirnay SO, Mégarbane B, Borron SW, et al. Effects of various combinations of benzodiazepines with buprenorphine on arterial blood gases in rats. *Basic Clin Pharmacol Toxicol* 2008; **103**: 228–39
17. Bartlett D, Tenney SM. Control of breathing in experimental anemia. *Respir Physiol* 1970; **10**: 384–95
18. Daube JR, Rubin DI. Needle electromyography. *Muscle Nerve* 2009; **39**: 244–70
19. Chang FC, Harper RM. A procedure for chronic recording of diaphragmatic electromyographic activity. *Brain Res Bull* 1989; **22**: 561–3
20. Holland MJ, Carr KD, Simon EJ. Pharmacokinetics of [3H]-buprenorphine in the rat. *Res Commun Chem Pathol Pharmacol* 1989; **64**: 3–16
21. Lammertsma AA, Hume SP. Simplified reference tissue model for PET receptor studies.

- 1 *Neuroimage* 1996; **4**: 153–8
- 2 22. Slinker BK. The statistics of synergism. *J Mol Cell Cardiol* 1998; **30**: 723–31
- 3 23. Dahan A, Yassen A, Bijl H, et al. Comparison of the respiratory effects of intravenous
4 buprenorphine and fentanyl in humans and rats. *Br J Anaesth* 2005; **94**: 825–34
- 5 24. Gueye PN, Borron SW, Risède P, et al. Lack of effect of single high doses of buprenorphine on
6 arterial blood gases in the rat. *Toxicol Sci* 2001; **62**: 148–54
- 7 25. Gueye PN, Borron SW, Risède P, et al. Buprenorphine and midazolam act in combination to
8 depress respiration in rats. *Toxicol Sci* 2002; **65**: 107–14
- 9 26. Ohtani M, Kotaki H, Nishitaten K, Sawada Y, Iga T. Kinetics of respiratory depression in rats
10 induced by buprenorphine and its metabolite, norbuprenorphine. *J Pharmacol Exp Ther* 1997; **281**: 428–
11 33
- 12 27. Yassen A, Olofsen E, Kan J, Dahan A, Danhof M. Pharmacokinetic-pharmacodynamic
13 modeling of the effectiveness and safety of buprenorphine and fentanyl in rats. *Pharm Res* 2008; **25**:
14 183–93
- 15 28. Megarbane B, Pirnay S, Borron SW, et al. Flunitrazepam does not alter cerebral distribution of
16 buprenorphine in the rat. *Toxicol Lett* 2005; **157**: 211–9
- 17 29. Kalvass JC, Polli JW, Bourdet DL, et al. Why clinical modulation of efflux transport at the
18 human blood-brain barrier is unlikely: the ITC evidence-based position. *Clin Pharmacol Ther* 2013; **94**:
19 80–94
- 20 30. Poisnel G, Dhilly M, Le Boisselier R, Barre L, Debruyne D. Comparison of five
21 benzodiazepine-receptor agonists on buprenorphine-induced mu-opioid receptor regulation. *J*
22 *Pharmacol Sci* 2009; **110**: 36–46
- 23 31. Debruyne D, Quentin T, Poisnel G, Lelong-Boulouard V, Barré L, Coquerel A. Acute and
24 chronic administration of clorazepate modifies the cell surface regulation of mu opioid receptors induced
25 by buprenorphine in specific regions of the rat brain. *Brain Res* 2005; **1052**: 222–31
- 26 32. Del Negro CA, Funk GD, Feldman JL. Breathing matters. *Nat Rev Neurosci* 2018; **19**: 351–67
- 27 33. Kalyuzhny AE, Dooyema J, Wessendorf MW. Opioid- and GABA(A)-receptors are co-
28 expressed by neurons in rat brain. *Neuroreport* 2000; **11**: 2625–8
- 29 34. Masneuf S, Buetler J, Koester C, Crestani F. Role of $\alpha 1$ - and $\alpha 2$ -GABA(A) receptors in
30 mediating the respiratory changes associated with benzodiazepine sedation. *Br J Pharmacol* 2012; **166**:
31 339–48
- 32 35. Webster LR, Karan S. The Physiology and Maintenance of Respiration: A Narrative Review.
33 *Pain Ther* 2020; **9**: 467–86
- 34 36. Campbell C, Weinger MB, Quinn M. Alterations in diaphragm EMG activity during opiate-
35 induced respiratory depression. *Respir Physiol* 1995; **100**: 107–17
- 36 37. Mollieux S, Dureuil B, Montravers P, Desmonts JM. Effects of midazolam on respiratory muscles
37 in humans. *Anesth Analg* 1993; **77**: 592–7
- 38 38. Bongianini F, Mutolo D, Nardone F, Pantaleo T. GABAergic and glycinergic inhibitory
39 mechanisms in the lamprey respiratory control. *Brain Res* 2006; **1090**: 134–45
- 40 39. Ling GS, Simantov R, Clark JA, Pasternak GW. Naloxonazine actions in vivo. *Eur J Pharmacol*
41 **1986**; **129**: 33–8
- 42 40. May CN, Dashwood MR, Whitehead CJ, Mathias CJ. Differential cardiovascular and
43 respiratory responses to central administration of selective opioid agonists in conscious rabbits:
44 correlation with receptor distribution. *Br J Pharmacol* 1989; **98**: 903–13
- 45 41. Linz K, Schröder W, Frosch S, Christoph T. Opioid-type Respiratory Depressant Side Effects
46 of Cebranopadol in Rats Are Limited by Its Nociceptin/Orphanin FQ Peptide Receptor Agonist Activity.
47 *Anesthesiology* 2017; **126**: 708–15
- 48 42. Yassen A, Olofsen E, van Dorp E, et al. Mechanism-based pharmacokinetic-pharmacodynamic
49 modelling of the reversal of buprenorphine-induced respiratory depression by naloxone: a study in
50 healthy volunteers. *Clin Pharmacokinet* 2007; **46**: 965–80

51

52

1 Figure legends

2
3 **Fig 1. Effects of vehicle/vehicle (white), diazepam/vehicle (green), vehicle/buprenorphine (blue) or**
4 **diazepam/buprenorphine (red) on the time-course of arterial pH (1A), PaO₂ (1B), PaCO₂ (1C) and**
5 **bicarbonate (1D).** Each group consisted of 6 rats. Values represent mean (SEM). Comparisons were
6 performed using two-way ANOVA followed by multiple comparison tests using Bonferroni's
7 correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.001$.

8
9 **Fig 2. Typical diaphragm electromyogram obtained at 15 min post-injection (2A) and effects of**
10 **vehicle/vehicle (white), diazepam/vehicle (green), vehicle/buprenorphine (blue) or**
11 **diazepam/buprenorphine (red) on diaphragmatic amplitude contraction (2B).** Each group
12 consisted of 6 rats. Values represent mean (SEM) of the area under the curve (AUC) of each parameter
13 between the time of injection and 60 min post-injection. Comparisons were performed using one-way
14 ANOVA followed by multiple tests using Bonferroni's correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and
15 **** $P < 0.0001$.

16
17 **Fig 3. ¹¹C-buprenorphine PET images obtained without (left) or with (right) pre-treatment with**
18 **diazepam 15 min before (3A). ¹¹C- buprenorphine time activity curves in cerebellum and striatum**
19 **obtained without (control group, white circles) or with buprenorphine (displacement group, black**
20 **squares) administered 20 min after the beginning of PET acquisition (3B).** Each group consisted of
21 2 rats. Results are expressed as mean (SEM). Effects of diazepam on ¹¹C-buprenorphine binding in
22 selected brain regions in rats receiving baseline ¹¹C-buprenorphine followed 15 min later by Tween®
23 10% (vehicle/¹¹C-buprenorphine, blue) or diazepam (diazepam/¹¹C-buprenorphine group, red) (3C).
24 Each group consisted of 5 rats. Results are expressed as mean (SEM). (SUV, standard uptake value).
25 Comparisons were performed using *Student t*-tests.

26
27 **Fig 4. Hypotheses of pharmacodynamic interaction between diazepam and buprenorphine**
28 **causing respiratory depression. (4A) Diazepam induces a decrease in T_E resulting in an increase in f ;**
29 **this observation probably corresponds to a deleterious mechanism on respiration rather than a protective**
30 **adaptation mechanism.³⁴ Diazepam induces a limited but not significant decrease in V_T possibly related**
31 **to the trend toward decreased diaphragmatic contraction observed with diazepam (but not significant**
32 **compared to control) and to possible diazepam-induced upper airway obstruction.³⁵ These deleterious**
33 **effects of diazepam are possibly counteracted in part by an increase in the contractility of accessory**
34 **respiratory muscles.³⁷ Taken together, no change in V_E is observed. (4B) Buprenorphine induces a**
35 **significant increase in T_I counteracted by a decrease in T_E resulting in no change in f . Although not**
36 **significant in comparison to control, buprenorphine tends to increase diaphragm contraction without**
37 **change in V_T , possibly to counteract buprenorphine-induced upper airway obstruction and rib cage**
38 **rigidity.^{35,36} (4C) When diazepam and buprenorphine are co-administered, the effects of diazepam and**
39 **buprenorphine are dominant on T_E and T_I , respectively, resulting in a decrease in f . When diazepam and**
40 **buprenorphine are co-administered, each drug prevents the respective mechanisms of adaptation to the**
41 **deleterious effects of each drug on V_T : the effect of diazepam on diaphragmatic contractility overrides**
42 **the diaphragmatic adaptation observed with buprenorphine; the effect of buprenorphine on accessory**
43 **respiratory muscle tone possibly prevents their contraction reported after benzodiazepine exposure.³⁶ To**
44 **these phenomena may be added a summation or synergy of the effects of the two drugs on the patency**

- 1 of the upper airways. Taken together, all these mechanisms result in a decrease of V_E and thus to the
- 2 onset of respiratory depression.
- 3

1 **Table**

2

3 **Table 1 Effects of vehicle/vehicle, diazepam/vehicle, vehicle/buprenorphine and**
4 **diazepam/buprenorphine on clinical findings, arterial blood gases and plethysmography**
5 **parameters.**

6 Each group consisted of 6 rats. We compared the area under the curve (AUC) of each parameter between
7 the time of injection and 60 min post-injection. Comparisons were performed using one-way ANOVA
8 followed by multiple tests using Bonferroni's correction with the exception of temperature and sedation
9 that were compared using Kruskal-Wallis tests followed by multiple comparison tests with Dunn's test.
10 \uparrow or $\downarrow P < 0.05$, $\uparrow\uparrow$ or $\downarrow\downarrow P < 0.01$, $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow P < 0.001$, • not significant. The direction of the arrow
11 corresponds to the trend of the observed difference: increase \uparrow , decrease \downarrow . Graphical representations
12 of these comparisons can be found in supplemental material-3.

13 PaCO₂, arterial partial pressure of CO₂; PaO₂, arterial partial pressure of O₂; T_I, inspiratory time; T_E,
14 expiratory time; T_{Tot}, total time; *f*, respiratory rate; V_T, tidal volume; V_E, minute volume

15

16 **Table 2 Effects of γ -aminobutyric acid (GABA) receptor antagonists (flumazenil and saclofen) and**
17 **opioid receptor antagonists (naloxonazine, naltrindole and nor-binaltorphimine) on**
18 **diazepam/buprenorphine combination-induced neuro-respiratory effects in the rat.**

19 Each group consisted of 6 rats. We compared the area under the curve (AUC) of each parameter between
20 the time of injection and 60min post-injection. Each pre-treatment was compared to its respective control
21 using Student t-tests or Mann-Whitney tests as appropriate. \uparrow or $\downarrow P < 0.05$, $\uparrow\uparrow$ or $\downarrow\downarrow P < 0.01$, $\uparrow\uparrow\uparrow$
22 or $\downarrow\downarrow\downarrow P < 0.001$, • not significant. The direction of the arrow corresponds to the trend of the observed
23 difference: increase \uparrow , decrease \downarrow .

24 FMZ, flumazenil; SAC, saclofen; NLZ, naloxonazine; NTI, naltrindole; NBNI, nor-binaltorphimine;
25 PaCO₂, arterial partial pressure of CO₂; PaO₂, arterial partial pressure of O₂; T_I, inspiratory time; T_E,
26 expiratory time; T_{Tot}, total time; *f*, respiratory rate; V_T, tidal volume; V_E, minute volume

27

28

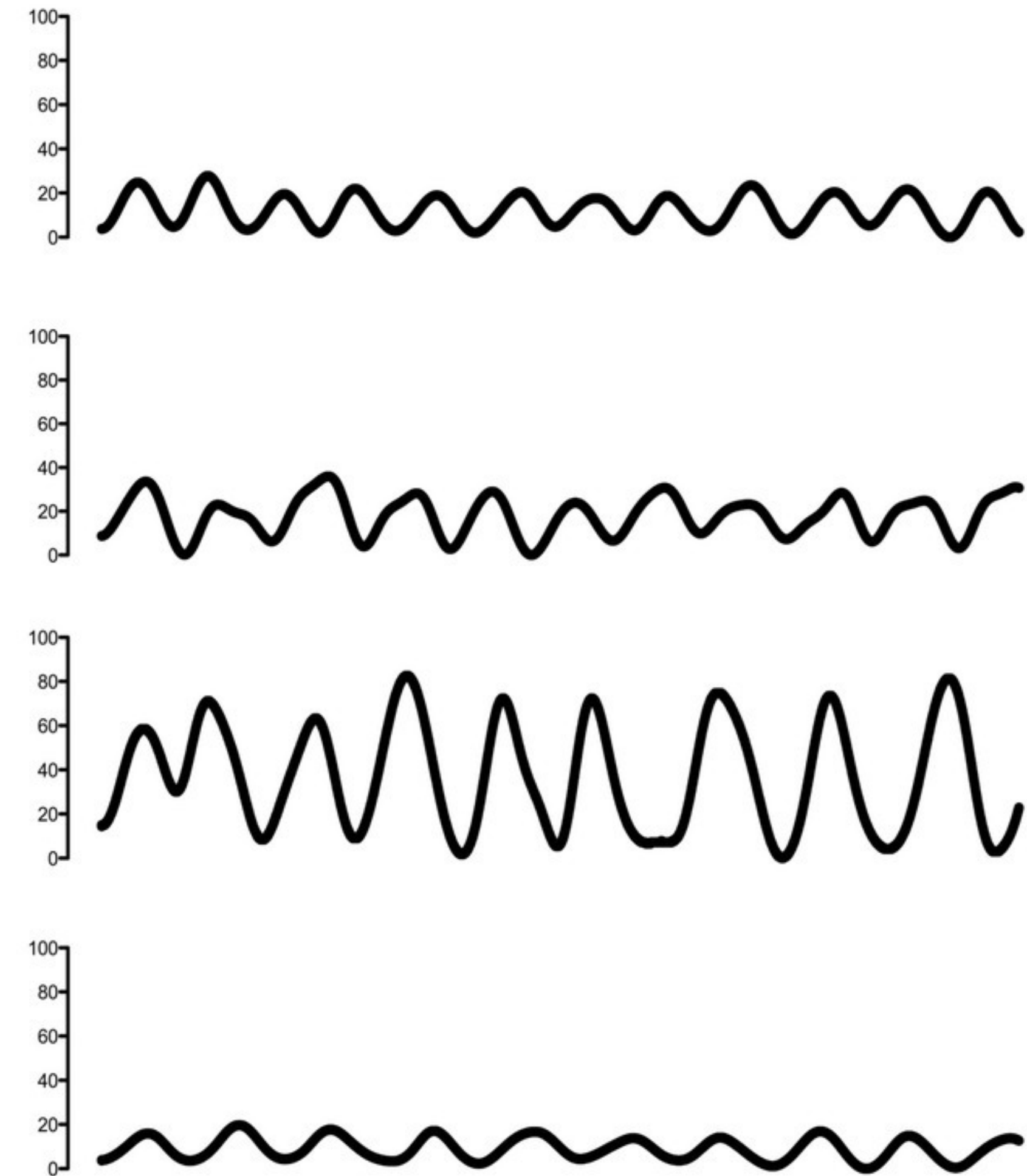
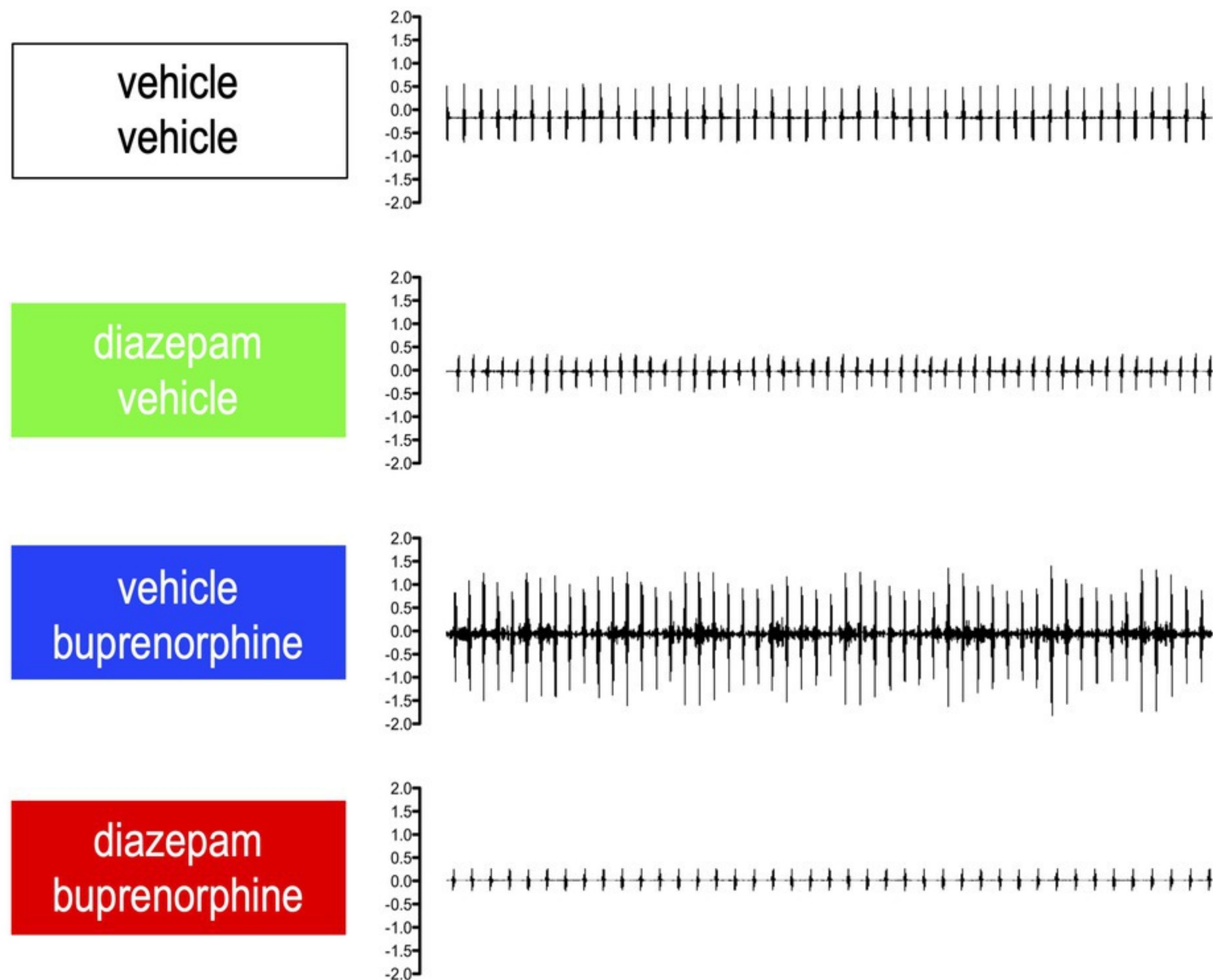
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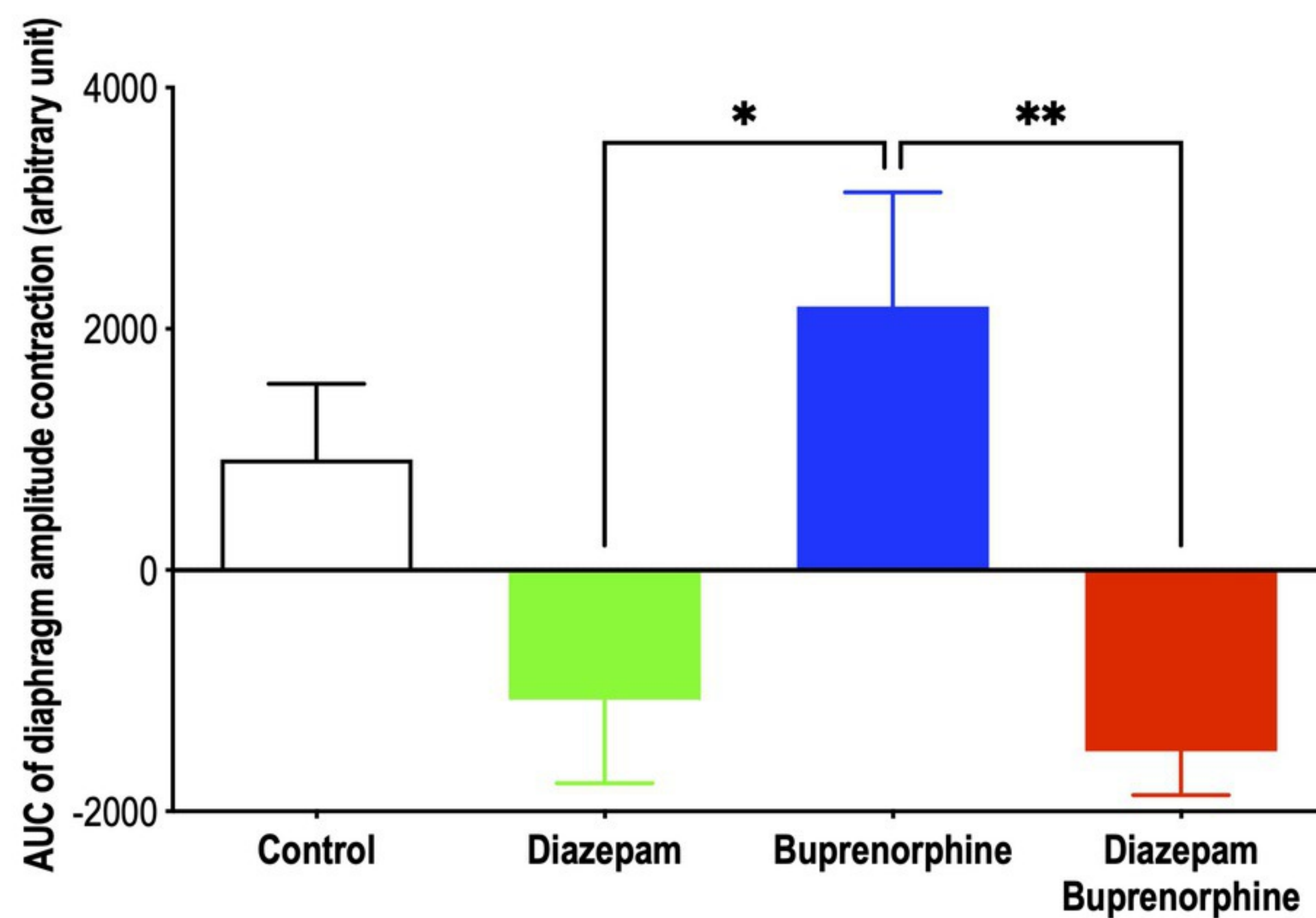
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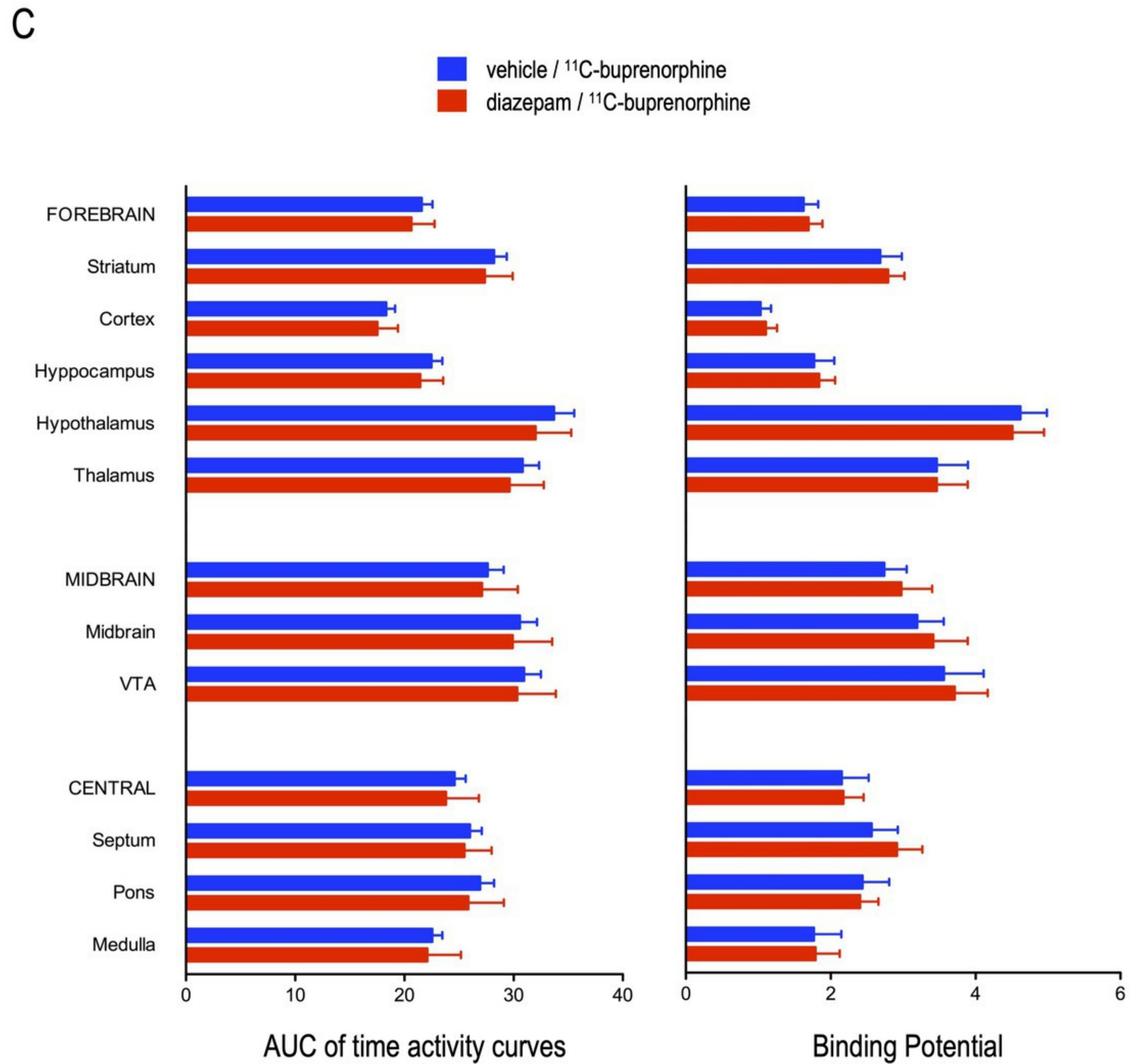
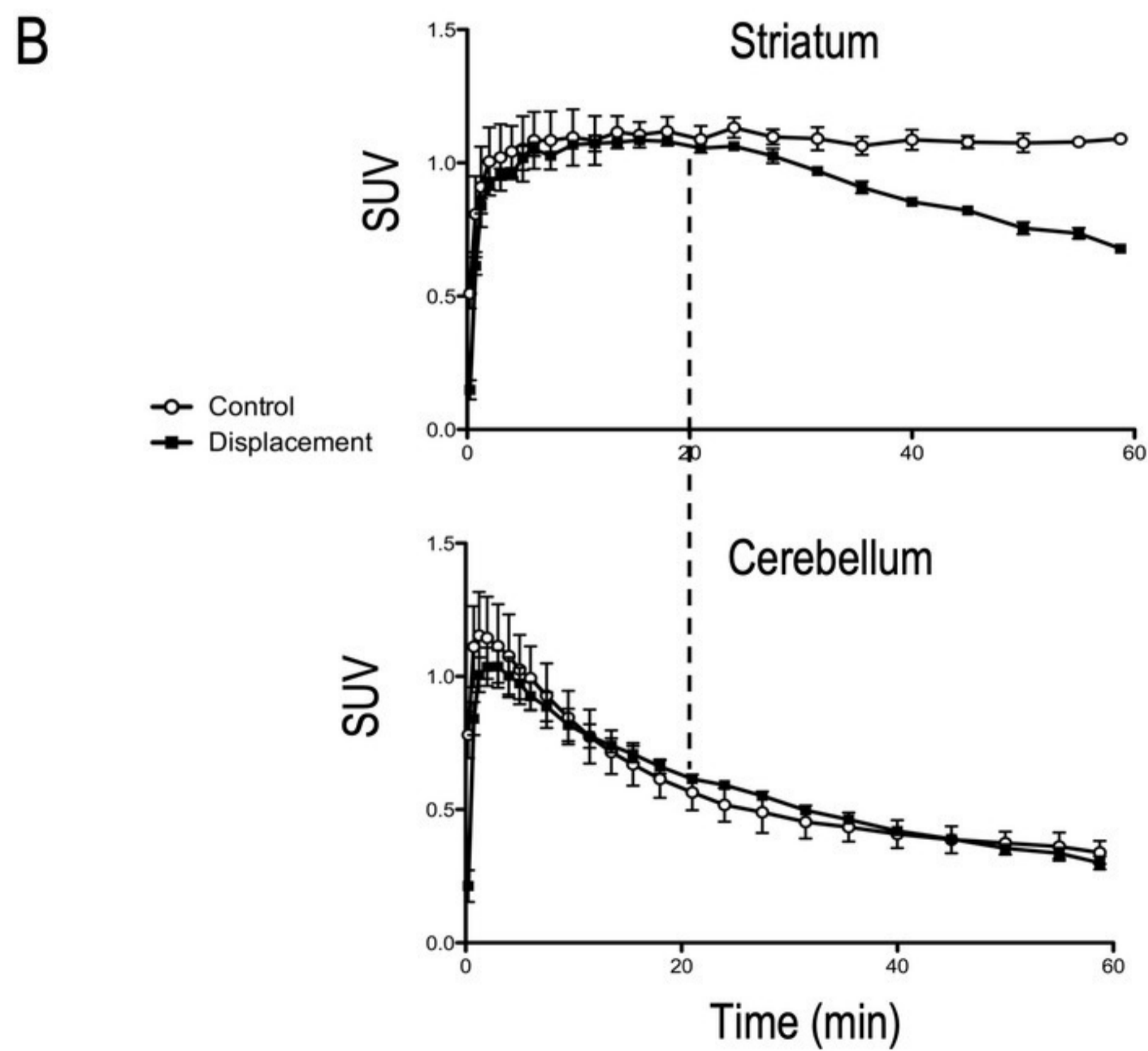
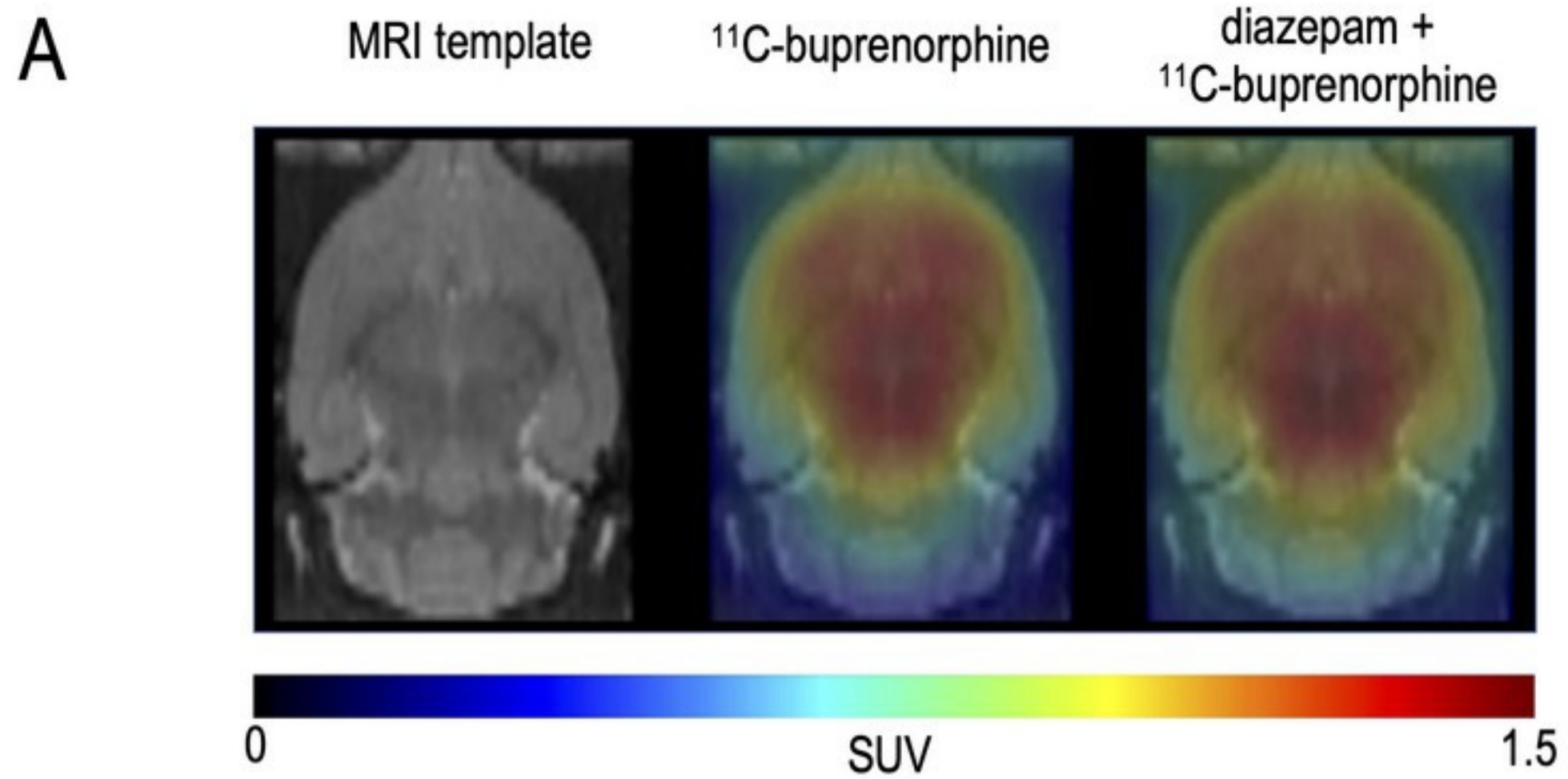
Raw EMG (mV) trace
highpass filtered at 100 Hz
to remove the cardiac signal

EMG trace after highpass filtering (100 Hz),
half-wave rectification, lowpass filtering (3Hz)
and normalisation to the baseline



B





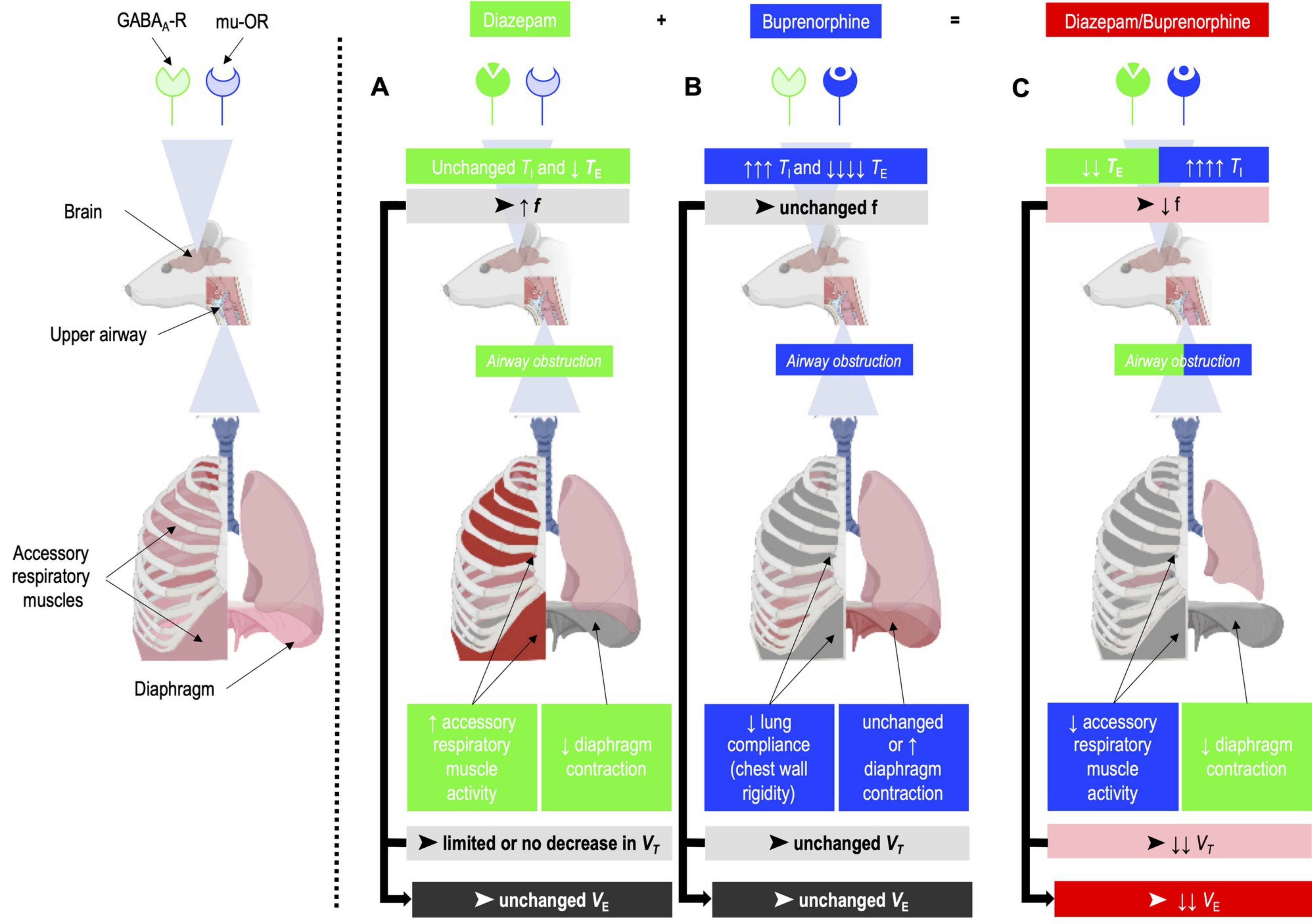


Table 1:

Groups			Clinical findings				ABG				Plethysmography parameters					
			Sedation	Hypotonia	Ataxia	Temperature	Arterial pH	PaO ₂	PaCO ₂	Bicarbonates	T _I	T _E	T _{Tot}	f	V _T	V _E
Diazepam/Vehicle	vs.	Vehicle/Vehicle	•	•	•	•	•	•	•	•	↓	↓	↑	•	•	
Vehicle/Buprenorphine	vs.	Vehicle/Vehicle	•	•	•	•	•	•	•	↑↑↑	↓↓↓	•	•	•	•	
Diazepam/Buprenorphine	vs.	Vehicle/Vehicle	↑↑	↑↑↑↑	↑↑↑↑	•	↓↓↓	↓	↑↑↑↑	•	↑↑↑↑	↓↓	↑	↓	↓↓	
Vehicle/Buprenorphine	vs.	Diazepam/Vehicle	•	•	•	•	•	↓	•	•	↑↑	↓↓	•	•	•	
Diazepam/Buprenorphine	vs.	Diazepam/Vehicle	↑	↑↑↑	↑↑↑	•	↓↓↓↓	↓↓	↑↑↑↑	•	↑↑↑↑	•	↑↑↑↑	↓↓↓↓	↓	
Diazepam/Buprenorphine	vs.	Vehicle/Buprenorphine	↑	↑↑↑	↑↑↑	↓↓	↓↓↓	•	↑	•	↑	↑↑↑	↓↓↓	↓↓↓↓	↓↓↓↓	

↑*P* < 0.05, ↑↑*P* < 0.01, ↑↑↑*P* < 0.001, • *P* > 0.05

ABG, arterial blood gas; FMZ, flumazenil; SAC, saclofen; NLZ, naloxonazine; NTI, naltrindole; NBNI, nor-binaltorphimine; PaCO₂, arterial partial pressure of CO₂; PaO₂, arterial partial pressure of O₂; T_I, inspiratory time; T_E, expiratory time; T_{Tot}, total time; f, respiratory rate; V_T, tidal volume; V_E, minute volume.

Table 2:

		GABA receptor antagonists		Opioid receptor antagonists		
		FMZ	SAC	NLZ	NTI	NBNI
Clinical Findings	Temperature	↑	•	•	•	↑↑
	Sedation	↓	•	•	•	•
	Tonus	↑↑	•	•	•	•
	Ataxia	•	•	•	•	•
ABG	Arterial pH	↑↑	•	↑	•	•
	PaO ₂	↑	•	•	•	•
	PaCO ₂	↓↓	•	↓	•	•
	Bicarbonates	•	•	•	•	•
Plethysmography parameters	<i>T_I</i>	•	•	↓↓	•	•
	<i>T_E</i>	↓↓	•	•	•	•
	<i>T_{Tot}</i>	↓↓	•	↓↓	•	•
	<i>f</i>	↑↑	•	↑↑	•	•
	<i>V_T</i>	↑	•	↑	•	•
	<i>V_E</i>	↑↑	•	↑↑	•	•

↑*P* < 0.05, ↑↑*P* < 0.01, ↑↑↑*P* < 0.001, • *P* > 0.05

ABG, arterial blood gas; FMZ, flumazenil; SAC, saclofen; NLZ, naloxonazine; NTI, naltrindole; NBNI, nor-binaltorphimine; PaCO₂, arterial partial pressure of CO₂; PaO₂, arterial partial pressure of O₂; *T_I*, inspiratory time; *T_E*, expiratory time; *T_{Tot}*, total time; *f*, respiratory rate; *V_T*, tidal volume; *V_E*, minute volume.