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1 **Title: Desogestrel enhances ventilation in Ondine patients: animal data**  
2 **involving serotonergic systems**

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24

**Abstract**

Central congenital hypoventilation syndrome (CCHS) is a neurorespiratory disease characterized by life-threatening sleep-related hypoventilation involving an alteration of CO<sub>2</sub>/H<sup>+</sup> chemosensitivity. Incidental findings have suggested that desogestrel may allow recovery of the ventilatory response to CO<sub>2</sub>. The effects of desogestrel on resting ventilation have not been reported. This study was designed to test the hypothesis that desogestrel strengthens baseline ventilation by analyzing the ventilation of CCHS patients. Rodent models were used in order to determine the mechanisms involved. Ventilation in CCHS patients was measured with a pneumotachometer. In mice, ventilatory neural activity was recorded from *ex vivo* medullary-spinal cord preparations, ventilation was measured by plethysmography and *c-fos* expression was studied in medullary respiratory nuclei. Desogestrel increased baseline respiratory frequency of CCHS patients leading to a decrease in their P<sub>ETCO<sub>2</sub></sub>. In medullary spinal-cord preparations or *in vivo* mice, the metabolite of desogestrel, etonogestrel, induced an increase in respiratory frequency that necessitated the functioning of serotonergic systems, and modulated GABA<sub>A</sub> and NMDA ventilatory regulations. *c-FOS* analysis showed the involvement of medullary respiratory groups of cell including serotonergic neurons of the *raphe pallidus* and *raphe obscurus* nuclei that seem to play a key role. Thus, desogestrel may improve resting ventilation in CCHS patients by a stimulant effect on baseline respiratory frequency. Our data open up clinical perspectives based on the combination of this progestin with serotonergic drugs to enhance ventilation in CCHS patients.

**Keywords:** central congenital hypoventilation syndrome; etonogestrel; *ex vivo* medullary-spinal cord preparations; *in vivo*; progestin; mice.

25

## 1 **Highlights**

2 -Desogestrel enhances basal ventilation in Ondine's curse

3 -Etonogestrel increased respiratory frequency by medullary mechanisms

4 -GABA<sub>A</sub> and NMDA receptors are involved in the respiratory effect of etonogestrel

5 -5-HT systems are implicated on the effect of etonogestrel on basal ventilation

6 -Combining 5-HT and desogestrel may constitute a therapeutic utility in Ondine's  
7 curse

8

9 **Abbreviations:** aCSF, artificial cerebrospinal fluid; CCHS, congenital central  
10 hypoventilation syndrome; CVD, central ventilatory drive; DMSO, dimethylsulfoxide;  
11 DSG, desogestrel; ETO, etonogestrel;  $f_R$ , respiratory frequency; IntC4, integrated C4  
12 burst activity; NMDA, N-methyl-D-aspartate; NTS, nucleus tractus solitaries;  $P_{ETCO_2}$ ;  
13 end-tidal carbon dioxide partial pressure; PBS, phosphate-buffered saline; preBotC,  
14 pre-Botzinger complex; RMg, raphe magnus; ROb, raphe obscurus; RPa, raphe  
15 pallidus; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group;  $\dot{V}_E$ , minute  
16 ventilation; VLM, ventrolateral medullary reticular nucleus;  $V_T$ , volume tidal; 5-HT,  
17 serotonin.

18

## 1 1. Introduction

2 Breathing depends on a rhythmic command originating in a brainstem  
3 neuronal network that is finely tuned to variations of O<sub>2</sub>, CO<sub>2</sub> and pH (Feldman et al.,  
4 2013). Significant disruptions in the neuronal respiratory network or its regulatory  
5 processes are associated with various pathological conditions including central  
6 hypoventilation syndromes (Carroll et al., 2010; Ramanantsoa and Gallego, 2013).  
7 These disorders can be life-threatening and may require mechanical ventilatory  
8 assistance. They expose patients to neural damage (Harper et al., 2014) and impair  
9 their quality of life. No pharmacological treatment is available for central  
10 hypoventilation syndromes.

11 Congenital central hypoventilation syndrome (CCHS) is a neurorespiratory  
12 disease characterized by sleep-related hypoventilation and the absence or reduction  
13 in CO<sub>2</sub>/H<sup>+</sup> chemosensitivity (Amiel et al., 2003; Weese-Mayer et al., 2010) due to  
14 mutations of the *PHOX2B* gene (Amiel et al., 2003). Recovery of CO<sub>2</sub>/H<sup>+</sup>  
15 chemosensitivity was incidentally observed in two adult CCHS women using  
16 desogestrel (DSG) for contraceptive purposes (Straus et al., 2010). In view of the  
17 known effects of progesterone on central ventilatory drive (CVD) and despite the  
18 absence of known effects of progesterone in CCHS (Behan et al.,  
19 2003; Sritippayawan et al., 2002), it was hypothesized that DSG was responsible for  
20 restoring the ventilatory response to CO<sub>2</sub> (Straus et al., 2010). Deliberately  
21 administering DSG to another patient did not induce any recovery of CO<sub>2</sub>/H<sup>+</sup>  
22 chemosensitivity (Li et al., 2013). These contradictory findings may be due to the  
23 complex nature of the actions of progestins on breathing control, idiosyncrasies or  
24 both. The action of DSG (Straus et al., 2010), or rather its metabolite 3-  
25 ketodesogestrel (etonogestrel; ETO), a synthetic progestin derived from testosterone

1 and belonging to the gonane family (Schumacher et al., 2007; Sitruk-Ware, 2008),  
2 may involve multiple pathways, as animal studies have revealed that the ventilatory  
3 action of progesterone and progestins depends on hypothalamus and brainstem  
4 mechanisms (Bayliss et al., 1990; Pascual et al., 2002) related to both genomic  
5 (Schumacher et al., 2007) and non-genomic effects (Belelli and Lambert, 2005; Pang  
6 et al., 2013; Pascual et al., 2002; Ren and Greer, 2006). A better understanding of the  
7 mechanisms of action of DSG and ETO on breathing command and regulation is  
8 fundamental to evaluate the conditions under which these progestins could be used  
9 to treat patients with central hypoventilation. In this context, we recently showed, in  
10 rodents, that ETO enhances the ventilatory response to metabolic acidosis by a  
11 mechanism involving supramedullary structures (Loiseau et al., 2014). Whether or  
12 not DSG and ETO interfere with generation of the respiratory rhythm (namely resting  
13 ventilation) is currently unknown.

14 The present study examined the effect of DSG on resting ventilation in CCHS  
15 women and the actions of its metabolite, ETO, on CVD at the medulla oblongata, the  
16 anatomical region where essential respiratory neural structures are located (Feldman  
17 et al., 2013). The *ex vivo* mouse medullary-spinal cord preparation was used to  
18 identify the medullary effects of ETO (Voituron et al., 2011). Combined co-  
19 applications of ETO and GABA<sub>A</sub>ergic, glutamatergic and serotonergic agonists or  
20 antagonists and c-FOS analysis were used to identify the mechanisms involved.  
21 Some of the results of these studies have been previously reported in the form of  
22 abstracts (Joubert et al., 2014; Perrin-Terrin et al., 2015).

23

## 1 2. Materials and Methods

### 2 2.1. Humans

3 The study in humans was carried out on the ventilatory signal recorded by Straus et  
4 al. (Straus et al., 2010) at the time of the first description of recovery of  
5 chemosensitivity in two CCHS patients taking DSG 75µg daily for contraception. The  
6 previous publication (Straus et al., 2010) reported the ventilatory response of patients  
7 to hypercapnia. In the present study, we analyzed the baseline ventilation recorded  
8 before exposure to hypercapnia.

9 The two patients were regularly assessed in the adult branch of the French reference  
10 Center for CCHS (Straus et al., 2010;Trang et al., 2005), according to current  
11 guidelines (Weese-Mayer et al., 2010). Both patients gave their written consent to  
12 scientific publication of the results obtained from their data (Straus et al., 2010).

13 Briefly, the first patient was a 19-year-old woman, who harbored a 5-alanine  
14 expansion mutation of the *PHOX2B* gene. At the time of the study, she was  
15 dependent on mechanical ventilation only during sleep and presented normal  
16 ventilation during wakefulness at rest.

17 The second patient was a 30-year-old woman, who harbored a 6-alanine expansion  
18 mutation of the *PHOX2B* gene. At the time of the study, she was still tracheotomized  
19 and dependent on mechanical ventilation, but only during sleep. However,  
20 hypoventilation was present during wakefulness at rest ( $P_aO_2 \approx 75$  mmHg;  $P_aCO_2 \approx$   
21  $55$  mmHg).

22 The two patients breathed through a pneumotachometer and their tidal volume ( $V_T$ ),  
23 respiratory frequency ( $f_R$ ), minute ventilation ( $\dot{V}_E$ ), and end-tidal carbon dioxide partial  
24 pressure ( $P_{ETCO_2}$ ) were recorded (Hyp'Air Compact+, Medisoft, Sorinnes-Dinant,

1 Belgium) at different times during 5 respiratory cycles, *i.e.* before, during and after  
2 DSG exposure.

## 3 **2.2. Animals**

4 Experiments were performed on both male and female newborn (0-3 days old;  
5  $2.1\pm 0.1$ g) wild-type mice (*Mus musculus*, OF1 strain; Charles River laboratories,  
6 L'Arbresle, France ([http://www.criver.com/products-services/basic-research/find-a-](http://www.criver.com/products-services/basic-research/find-a-model/of1-mouse)  
7 [model/of1-mouse](http://www.criver.com/products-services/basic-research/find-a-model/of1-mouse))). All experiments were carried out in accordance with Directive  
8 2010/63/EU of the European Parliament and of the Council of 22 September 2010  
9 and French law (2013/118). All efforts were made to minimize the number of animals  
10 used and their suffering. Animals were kept on a 12-hour light-dark cycle with free  
11 access to food and water.

### 12 **2.2.1. Pharmacological agents**

13 Drugs obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France), were prepared  
14 in either saline or dimethylsulfoxide (DMSO) and were dissolved in artificial  
15 cerebrospinal fluid (aCSF) for *ex vivo* preparations and in oil for *in vivo*  
16 experimentations (Garcia-Pelaez et al., 2007; Ren and Greer, 2006). Bicuculline, MK-  
17 801, methysergide, N-methyl-D-aspartate (NMDA), muscimol and serotonin (5-  
18 hydroxytryptamine, 5-HT) were prepared in saline. ETO, like other steroids, was  
19 dissolved in DMSO or oil.

### 20 **2.2.2. Whole body plethysmography**

21 Animals were placed in an experimental chamber (20mL) in which they could freely  
22 move. The chamber was maintained at 33°C, the thermoneutral zone (Gordon and .,  
23 1993), with an external heat source. During the experimental period, the chamber  
24 was continuously flushed at  $0.6\text{L}\cdot\text{min}^{-1}$  for the continuous delivery of air and removal  
25 of expired  $\text{CO}_2$ . Using an adaptation of the barometric method previously described



1 (Bartlett, Jr. and Tenney, 1970), the pressure change induced by the respiratory flow  
2 was recorded with a differential pressure transducer (Valydine MP 45, Northridge,  
3 CA, USA). The pressure signal was digitized through a LabChart data analysis  
4 system (ADInstruments, Castle Hill, Australia). Measurements were made on 15 sec,  
5 at intervals of 5 min.

### 6 **2.2.3. Medullary-Spinal Cord Preparations**

7 Newborn mice were placed under deep cold anesthesia and medullary-spinal cord  
8 preparations were dissected out as previously described. The rostral section was  
9 made at the level of the eighth cranial nerve exit point. The caudal section was made  
10 between the seventh and eighth cervical spinal roots. Preparations were placed in a  
11 recording chamber with the ventral surface facing upward. They were continuously  
12 superfused at a rate of 10 ml/min, at 26°C, with di oxygenated aCSF (129.0 mM NaCl,  
13 3.35 mM KCl, 1.26 mM CaCl<sub>2</sub> 2H<sub>2</sub>O, 1.15 mM MgCl<sub>2</sub> 6H<sub>2</sub>O, 0.58 mM NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O,  
14 21.0 mM NaHCO<sub>3</sub>, 30.0 mM d-glucose) saturated with O<sub>2</sub> and adjusted to pH 7.4 by  
15 bubbling with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (normal pH-aCSF).

16 CVD was analyzed by measuring the electrical activity of a fourth cervical ventral  
17 nerve root (C4) recorded using a suction electrode, filtered (10-3000 Hz), amplified  
18 (x5000), integrated (time constant 100 ms) and digitized by a Spike 2 data analysis  
19 system (CED, Cambridge, UK), at a sampling frequency of 2500 Hz. As previously  
20 reported,  $f_R$  was commonly defined as the burst frequency recorded from C4 over 1  
21 minute. The integrated C4 burst activity (IntC4) was also used as an index of  
22 inspiratory activity (Voituron et al., 2006).

### 23 **2.2.4. Pharmacological applications**

#### 24 *2.2.4.1. Analysis of the effect of ETO on in vivo newborn mice*

25 Newborn mice received *per os* either ETO (10<sup>-3</sup>mg/kg) dissolved in oil, or oil alone.

1 *2.2.4.2. Analysis of the effect of ETO on ex vivo preparations*

2 After completion of the surgical procedure, *ex vivo* preparations were maintained in  
3 normal pH-aCSF superfusion for 30 minutes to stabilize CVD; baseline values were  
4 defined as the mean value over the last 5 minutes of this period. The effect of ETO  
5 was determined under normal pH at 0.05, 0.5, 1 and 2 $\mu$ M (final concentration of  
6 DMSO used to dissolve ETO was 0.01%). After stabilization, ETO or DMSO alone  
7 was added to normal pH-aCSF for 30 minutes.  $f_R$  and IntC4 were then averaged over  
8 successive 5-minutes intervals and expressed as a percentage of baseline values.  
9 Preparations were then either returned to normal pH-aCSF superfusion for 30  
10 minutes or fixed by incubation in 4% paraformaldehyde in 0.1 M phosphate-buffered  
11 saline (PBS; pH 7.4).

12 *Analysis of interactions of ETO with GABA<sub>A</sub> receptors on ex vivo preparations*

13 In a first step, we investigated possible interactions between GABA<sub>A</sub> receptors and  
14 ETO by evaluating changes in the effect of ETO under conditions of GABA<sub>A</sub> receptor  
15 blockade. After stabilization, preparations were successively superfused with normal  
16 pH-aCSF containing bicuculline for 10 minutes followed by normal pH-aCSF  
17 containing bicuculline and supplemented with ETO or DMSO for 30 minutes;  $f_R$ , the  
18 only respiratory variable affected by ETO, was expressed as a percentage of  
19 bicuculline values (values obtained during the last 5 minutes of bicuculline exposure).  
20 Preparations were subsequently returned to normal pH-aCSF for 10 minutes.

21 In a second set of experiments, we tried to characterize the action of ETO on the  
22 GABA<sub>A</sub> receptor effects on  $f_R$  *i.e.* facilitation or moderation. We compared the effect  
23 of muscimol on  $f_R$  in both the presence and the absence of ETO. According to data of  
24 the literature, the IC<sub>50</sub> of muscimol was first determined by examining its effect on  $f_R$   
25 at several concentrations (0.05, 0.10, 0.15, 0.20 and 0.25 $\mu$ M) for 4 minutes.

1 Preparations were exposed to normal pH-aCSF containing either ETO or DMSO and  
2 then to normal pH-aCSF containing ETO or DMSO with muscimol at  $IC_{50}$  for 4  
3 minutes;  $f_R$  was expressed as a percentage of pre-muscimol values (values obtained  
4 during the 5 minutes preceding muscimol exposure). Preparations were subsequently  
5 returned to normal pH-aCSF for 10 minutes.

#### 6 *2.2.4.3. Analysis of interactions of ETO with NMDA receptors on ex vivo preparations*

7 According to data of the literature, the  $EC_{50}$  of the effect of NMDA on  $f_R$  was  
8 determined by examining its effect at several concentrations (8.0, 8.5, 9.0, 9.5 and  
9  $10\mu M$ ) for 10 minutes.

10 First, we evaluated changes in the effect of ETO under conditions of NMDA receptor  
11 blockade. We determined the lowest concentration of MK-801 that totally  
12 antagonized the NMDA effect on  $f_R$ . Preparations were successively exposed to  
13 several concentrations of MK-801 (2.5, 5 and  $10\mu M$ ) for 10 minutes followed by  
14 NMDA at  $EC_{50}$  for 10 minutes; the lowest concentration of MK-801 that totally  
15 antagonized NMDA receptors was found to be  $2.5\mu M$ . Then, after a stabilization  
16 period, preparations were superfused with normal pH-aCSF containing MK-801  
17 ( $2.5\mu M$ ) for 10 minutes followed by normal pH-aCSF containing MK-801  
18 supplemented with ETO for 30 minutes;  $f_R$  was expressed as a percentage of MK-801  
19 values. Preparations were subsequently returned to normal pH-aCSF for 10 minutes.

20 Second, to investigate possible modulation of the effects of NMDA receptors on  $f_R$  by  
21 ETO, we compared the effect of NMDA in both the presence and the absence of  
22 ETO. Preparations were exposed to NMDA at  $EC_{50}$  for 10 minutes after 30 minutes  
23 of exposure to normal pH-aCSF containing either ETO or DMSO;  $f_R$  was expressed  
24 as a percentage of pre-NMDA values (values obtained during the 5 minutes

1 preceding NMDA exposure). Preparations were subsequently returned to normal pH-  
2 aCSF for 10 minutes.

#### 3 *2.2.4.4. Analysis of interactions of ETO with serotonergic systems on ex vivo* 4 *preparations*

5 To study the implication of serotonergic systems on modulation of central  
6 respiratory drive by ETO, we evaluated the effect of ETO in the presence of blockade  
7 of 5-HT<sub>1/2/7</sub> receptors, which are the main 5-HT receptors involved in respiratory  
8 modulation (Hilaire and Duron, 1999).

9 We determined the effect of 5-HT (25µM) for 10 minutes on  $f_R$ . We then determined  
10 the lowest concentration of methysergide that totally antagonized the respiratory  
11 effects of 5-HT. Preparations were successively exposed to several concentrations  
12 (1, 2.5, 5 and 10µM) of methysergide for 10 minutes followed by 5-HT (25µM) for 10  
13 minutes; the lowest concentration of methysergide that totally antagonized the effects  
14 of 5-HT on  $f_R$  was determined to be 5µM.

15 After a stabilization period, preparations were successively superfused with normal  
16 pH-aCSF containing methysergide (5µM) followed by a normal pH-aCSF containing  
17 methysergide supplemented with ETO for 30 minutes;  $f_R$  was expressed as a  
18 percentage of baseline values minus the effect of methysergide alone.

#### 19 **2.2.5. Immunohistochemistry**

20 To identify ETO-induced changes in cell activity, immunohistochemical analysis for c-  
21 FOS was carried out in *ex vivo* medullary-spinal cord preparations exposed to either  
22 ETO or DMSO for 30 minutes (n=32). At the end, preparations were fixed in 4%  
23 paraformaldehyde in 0.1 M PBS (pH 7.4) for 48 hours at 4°C. Preparations were then  
24 cryoprotected for 48 hours in 30% sucrose in 0.1 M PBS and stored at -20°C for  
25 subsequent use. Standard immunohistochemical procedures were used to locate c-

1 FOS on 40µm-thick coronal free-floating sections obtained using a cryostat (Leica  
2 CM 1510S) (Voituron et al., 2011). Briefly, sections were incubated with a rabbit  
3 polyclonal antibody against c-FOS (sc-52; Santa Cruz Biotechnology Inc., Santa  
4 Cruz, CA, USA; 1:2000) in 1% BSA for 48 hours at 4°C. They were then incubated  
5 for 2 hours with a biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories,  
6 Burlington, Canada; 1:500) and then with an avidin-biotin-peroxidase complex (ABC;  
7 Novostain Super ABC kit, Novocastra Laboratories, Newcastle, UK; 1:250) for 1 hour.  
8 Peroxidase activity was detected with 0.02% 3,3'-diaminobenzidine  
9 tetrahydrochloride and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.05M Tris-HCl buffer (pH 7.6).

10 To characterize the cells displaying changes in activity revealed by c-FOS analysis,  
11 dual detections were performed *i.e.* c-FOS and tyrosine hydroxylase (TH) and c-FOS  
12 and 5-HT. Sections were first incubated with a rabbit polyclonal antibody against c-  
13 FOS (sc-253 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:8000; 48  
14 hours; 4°C), then with a biotinylated goat anti-rabbit immunoglobulin (Vector  
15 Laboratories, Burlington, Canada; 1:500; 2 hours) and ABC (1:250). Peroxidase  
16 activity was detected with 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.04%  
17 nickel ammonium sulfate and 0.01% hydrogen peroxide in 0.05M Tris buffer (pH 7.6).  
18 Secondly, sections were incubated with either a mouse polyclonal antibody against  
19 TH (MAB318, Millipore, 1:4000) or a rabbit polyclonal antibody against 5-HT (S5545,  
20 Sigma–Aldrich, Saint-Quentin Fallavier, France; 1:500) for 48 hours at 4°C. Sections  
21 were subsequently incubated for 2 hours with biotinylated horse anti-mouse (Vector  
22 Laboratories, Burlington, Canada; 1:500) or goat anti-rabbit (Vector Laboratories,  
23 Burlington, Canada; 1:500), respectively, and then with ABC (1:250). Peroxidase  
24 activity was detected with 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.01%  
25 hydrogen peroxide in 0.05M Tris buffer (pH 7.6).

1 In all cases, control sections were processed in parallel, but with the omission of  
2 primary or secondary antibodies. No labeling was observed on control sections.  
3 Sections were mounted in sequential caudo-rostral order on silanized slides, air-dried  
4 and coverslipped with Entellan<sup>®</sup> (VWR International S.A.S).  
5 Sections were examined under a light microscope (Leica DM 2000, Leica  
6 Microsystems, Heidelberg, Germany). The distribution of c-FOS, c-FOS/TH and c-  
7 FOS/5-HT immunolabeled cells was plotted onto drawings with the aid of a drawing  
8 tube attached to the microscope (magnification x10). c-FOS and double-labeled cells  
9 were visually counted under the microscope at high magnification (x400) in medullary  
10 structures involved in central respiratory drive using standard landmarks (Paxinos et  
11 al., 2007;Paxinos and Franklin, 2001). Immunolabeled cells were photographed with  
12 a digital camera (Leica DFC450C, Leica Microsystems, Heidelberg, Germany). c-  
13 FOS-positive cells were analyzed in the ventrolateral medullary reticular nucleus  
14 (VLM), nucleus tractus solitarius (NTS), medullary raphe nuclei (raphe magnus  
15 (RMg), obscurus (ROb) and pallidus (RPa)), retrotrapezoid nucleus/parafacial  
16 respiratory group (RTN/pFRG), parapyramidal area (PP), hypoglossal and facial  
17 nucleus. The VLM is a neuronal column ventral to the nucleus ambiguus including  
18 the pre-Botzinger complex (preBotC) and A1C1 group of neurons and extending from  
19 the pyramidal decussation to the caudal edge of the facial nucleus. Using standard  
20 landmarks (Paxinos et al., 2007;Paxinos and Franklin, 2001), a distinction was made  
21 between the caudal part of the RPa and ROb (from the pyramidal decussation to the  
22 rostral edge of the inferior olives) and their rostral part (from the rostral edge of the  
23 inferior olives to the rostral edge of the facial nucleus). Several subdivisions of the  
24 NTS were analyzed, *i.e* the commissural, median and ventrolateral and the

1 commissural and median subdivisions were grouped in a single entity referred to as  
2 the commissural/median NTS (c/mNTS).

### 3 **2.2.6. Statistics**

4 Data were expressed as mean ( $\pm$ SEM) and analyzed with GraphPad (GraphPad  
5 Prism5 San Diego California USA) or Matlab (MATLAB Version: 8.5.0.197613  
6 (R2015a)).

7 For human data, the significance of the effects on the responses ( $f_R$ ,  $V_T$ ,  $\dot{V}_E$   $P_{ETCO_2}$ )  
8 of the fixed effect time factor (before, during or after DSG treatment) and of the  
9 random effect patient factor (two modalities, first or second patient) were tested using  
10 a hierarchical two-way ANOVA, homoscedasticity being assessed by Bartlett's test,  
11 and normality by Lilliefors' variant of Kolmogorov-Smirnov's test. The p-values of the  
12 two-by-two comparisons between different times for each patient were adjusted for  
13 multiple testing using Sidak's correction. For animal data, a single dose of each  
14 tested drug was applied for each animal or preparation. Depending on normality and  
15 homoscedasticity, two-way ANOVA followed by Bonferroni's *post hoc* least squares  
16 differences (PLSD) correction or Kruskal-Wallis test followed by Dunn's PLSD were  
17 used. Differences were considered significant at  $p < 0.05$ .

### 1 3. Results

#### 2 3.1. Effect of DSG on baseline ventilatory variables of two CCHS patients

3 Before DSG exposure, the first CCHS patient displayed a  $\dot{V}_E$  of  
4  $8.33\pm 0.36$  L/min resulting from a  $f_R$  of  $13.06\pm 0.54$ /min and a  $V_T$  of  $0.64\pm 0.03$  L. Her  
5  $P_{ETCO_2}$  was  $37.33\pm 0.23$  mmHg. Eighteen months after starting DSG (Straus et al.,  
6 2010), baseline  $f_R$  increased ( $17.26\pm 0.66$ /min, +32%;  $p<0.01$ ; Fig. 1A),  $P_{ETCO_2}$   
7 decreased ( $34.63\pm 0.37$  mmHg, -7%;  $p<0.01$ ; Fig. 1C), but  $V_T$  remained unchanged  
8 ( $0.66\pm 0.02$  L), resulting in an increase in  $\dot{V}_E$  ( $11.42\pm 0.25$  L/min, +37%;  $p<0.001$ ). Four  
9 months after stopping DSG,  $f_R$  and  $\dot{V}_E$  returned to baseline levels ( $13.34\pm 0.90$ /min,  
10  $8.98\pm 0.29$  L/min;  $p<0.01$ ; Fig. 1A) and  $P_{ETCO_2}$  increased to  $42.53\pm 0.45$  mmHg  
11 ( $p<0.001$ ; Fig. 1C).

12 The second patient presented a similar response profile. Her baseline  
13 ventilatory variables were  $18.02\pm 0.57$ /min,  $0.60\pm 0.03$  L and  $10.69\pm 0.49$  L/min for  $f_R$ ,  $V_T$   
14 and  $\dot{V}_E$ , respectively. Her  $P_{ETCO_2}$  was  $52.90\pm 0.30$  mmHg. Two months after starting  
15 DSG,  $f_R$  increased ( $27.36\pm 1.71$ /min, +52%;  $p<0.001$ ; Fig. 1B), but  $V_T$  remained  
16 unchanged ( $0.61\pm 0.04$  L), resulting in an increase in  $\dot{V}_E$  ( $16.53\pm 0.64$  L/min, +55%;  
17  $p<0.001$ ), while  $P_{ETCO_2}$  decreased ( $46.86\pm 0.45$  mmHg, -11%;  $p<0.001$ ; Fig. 1D). Two  
18 months after stopping DSG,  $f_R$  significantly decreased ( $24.00\pm 0.97$ /min;  $p<0.05$ ; Fig.  
19 1B) and  $\dot{V}_E$  was slightly lower than on DSG ( $16.45\pm 0.61$  L/min) but still increased.  
20  $P_{ETCO_2}$  increased to  $54.36\pm 0.79$  mmHg ( $p<0.001$ ; Fig. 1D).

#### 21 3.2. Effect of ETO on baseline respiratory frequency on *in vivo* newborn mice

22 After exposure to  $10^{-3}$  mg/kg ETO, the baseline  $f_R$  was  $196.6\pm 12.1$  cycle/min  
23 (Fig. 2B,C;  $n=11$ ). This  $f_R$  was significantly increased compared to control mice (oil  
24 exposure;  $146.8\pm 7.2$  cycle/min,  $p<0.001$ ; Fig. 2A,C;  $n=16$ ).



### 1 3.3. Effect of ETO on *ex vivo* preparations

2 Baseline  $f_R$  was  $8.5 \pm 0.2$  bursts/min with no significant differences between  
3 groups.

#### 4 3.3.1. Effect of ETO on CVD

5  $f_R$  was significantly increased after 25 minutes of exposure to 0.05, 0.5, 1 and  
6  $2 \mu\text{M}$  ETO ( $129.7 \pm 5.7\%$ ,  $p < 0.001$ ,  $n = 16$ ;  $129.7 \pm 8.6\%$ ,  $p < 0.001$ ,  $n = 14$ ;  $123.2 \pm 3.2\%$ ,  
7  $p < 0.001$ ,  $n = 24$  and  $147.1 \pm 5.8\%$ ,  $p < 0.001$ ,  $n = 14$ , respectively; Fig. 3C-K), while in  
8 control, DMSO exposure, did not induce any significant changes ( $111.0 \pm 3.7\%$ ,  $n = 18$ ;  
9 Fig. 3A,B,K).  $f_R$  was significantly higher at all ETO concentrations than with DMSO  
10 ( $0.5$  and  $1 \mu\text{M}$   $p < 0.01$ ;  $0.05$  and  $2 \mu\text{M}$ ,  $p < 0.001$ ; Fig. 3K). In addition, the increase in  $f_R$   
11 induced by  $2 \mu\text{M}$  ETO was significantly greater than that observed with lower  
12 concentrations ( $p < 0.01$ , Fig. 3K). After removing the ETO by returning to normal-pH-  
13 aCSF,  $f_R$  returned to baseline values for  $0.05$  and  $0.5 \mu\text{M}$  ETO, but not for  $1$  and  $2 \mu\text{M}$   
14 ETO ( $153.1 \pm 1.2\%$  and  $154.3 \pm 14.3\%$ ,  $p < 0.05$ , respectively).

15 In contrast, the burst amplitude (IntC4) was not modified by ETO ( $100.9 \pm 4.9\%$ ;  
16  $100.4 \pm 1.5\%$ ;  $98.1 \pm 2.7\%$ ;  $94.2 \pm 3.3\%$  for  $0.05$ ,  $0.5$ ,  $1$  and  $2 \mu\text{M}$  ETO and  $99.7 \pm 2.2\%$ ,  
17 for DMSO; Fig. A-J).

#### 18 3.3.2. Interaction of ETO with the $\text{GABA}_A$ receptor

19 After 10 minutes of exposure, bicuculline ( $\text{GABA}_A$  receptor antagonist) induced  
20 a significant increase in  $f_R$  ( $137.7 \pm 8.0\%$ ,  $p < 0.001$ ; Fig. 4A,C,E).

21 Under bicuculline,  $0.05 \mu\text{M}$  ETO still induced a significant increase in  $f_R$   
22 ( $115.7 \pm 2.8\%$ ,  $p < 0.05$ ,  $n = 8$ ; Fig. 4C,D,G) that was significantly different from that  
23 observed with DMSO ( $107.6 \pm 3.3\%$ ,  $p < 0.05$ ,  $n = 10$ ; Fig. 4A,B), but significantly lower  
24 (50%) than the  $f_R$  increase induced by ETO without bicuculline ( $p < 0.05$ ; Fig. 4G).  
25 After removing the ETO,  $f_R$  returned to bicuculline values ( $97.0 \pm 1.8\%$ ). In contrast,

1 under bicuculline, 2 $\mu$ M ETO failed to induce any increase in  $f_R$  (107.6 $\pm$ 1.1%,  
2  $p < 0.001$ ,  $n = 9$ ; Fig. 4E,F,G).

3  $f_R$  was dose-dependently decreased by application of muscimol (GABA<sub>A</sub>  
4 receptor agonist;  $IC_{50} = 0.14 \mu\text{M}$ ,  $n = 28$ ; Fig. 4H). The decrease in  $f_R$  induced by  
5 muscimol ( $IC_{50}$ ) was greater with ETO (39.6 $\pm$ 7.1%,  $p < 0.001$ ,  $n = 14$  and 36.7 $\pm$ 7.0%,  
6  $p < 0.001$ ,  $n = 14$  for 0.05 and 2 $\mu$ M, respectively, Fig. 4K-O) than with DMSO  
7 (66.6 $\pm$ 11.0%,  $p < 0.01$ ,  $n = 9$ ; Fig. 4I,J,O). After return to normal-pH-aCSF,  $f_R$  returned  
8 to pre-muscimol values (102.8 $\pm$ 2.6% for DMSO and 100.6 $\pm$ 3.8% and 112.0 $\pm$ 4.0% for  
9 0.05 and 2 $\mu$ M ETO, respectively).

### 10 3.3.3. Interaction of ETO with the NMDA receptor

11  $f_R$  was dose-dependently increased by NMDA ( $EC_{50} = 9.28 \mu\text{M}$ ,  $n = 20$ ; Fig. 5A).  
12 The optimal concentration of dizocilpine (MK-801, a NMDA receptor antagonist) was  
13 2.5 $\mu$ M. At this concentration,  $f_R$  was not significantly altered (103.3 $\pm$ 6.1%) and the  
14  $EC_{50}$  of NMDA did not modify  $f_R$  (113.5 $\pm$ 7.8%,  $n = 5$ ). Under 2.5 $\mu$ M MK-801, the  
15 increase in  $f_R$  induced by 0.05 $\mu$ M ETO was no longer observed (97.6 $\pm$ 9.1%,  $n = 4$ ; Fig.  
16 5B-D), this was significantly different from that observed without MK-801 ( $p < 0.01$ ,  
17 Fig. 5D).

18 Exposure to the  $EC_{50}$  of NMDA together with DMSO or ETO (0.05 and 2 $\mu$ M)  
19 significantly increased  $f_R$  (130.4 $\pm$ 12.2%,  $p < 0.05$ ,  $n = 10$ ; 153.3 $\pm$ 8.8%,  $p < 0.001$ ,  $n = 11$ ;  
20 and 140.5 $\pm$ 10.2%,  $p < 0.01$ ,  $n = 8$ , respectively; Fig. 5E-K). The increase in  $f_R$  observed  
21 with 0.05 $\mu$ M ETO was significantly greater ( $p < 0.05$ , Fig. 5K) than that observed with  
22 DMSO. After return to normal-pH-aCSF,  $f_R$  returned to pre-NMDA values for 2 $\mu$ M  
23 ETO (91.7 $\pm$ 8.8%), but not for DMSO or 0.05 $\mu$ M ETO (80.2 $\pm$ 5.3%,  $p < 0.05$ ;  
24 60.8 $\pm$ 7.5%,  $p < 0.001$ ; respectively).

### 1 3.3.4. Interaction of ETO with serotonergic systems

2 5-HT (25 $\mu$ M) increased  $f_R$  (146.8 $\pm$ 16.4%,  $p$ <0.01,  $n$ =5; Fig. 6A-C). After  
3 exposure to 5 $\mu$ M methysergide (a 5-HT<sub>1/2</sub> receptor antagonist), mean  $f_R$  was not  
4 significantly modified by 5-HT (90.7 $\pm$ 22.8%,  $n$ =3; Fig. 6C).

5 Under 5 $\mu$ M methysergide, the increase in  $f_R$  induced by both 0.05 and 2 $\mu$ M  
6 ETO was no longer observed (97.3 $\pm$ 2.8%,  $n$ =5, and 92.3 $\pm$ 3.2%,  $n$ =5, respectively,  
7  $p$ <0.001; Fig. 6D).

### 8 3.3.5. Effect of ETO on the number of c-FOS-positive cells - identification of 9 serotonergic and catecholaminergic features

10 0.05 $\mu$ M ETO induced an increase in the number of c-FOS-positive cells in the  
11 commissural and median parts of the NTS (c/mNTS; +221 $\pm$ 75%,  $n$ =12; Table 1; Fig.  
12 7A,E), but not in the ventrolateral part (vINTS;  $n$ =12; Table 1; Fig. 7A,E). Only a very  
13 small proportion of cells was also immunoreactive for TH (3.5 $\pm$ 2.5%; Fig. 8A-C). 2 $\mu$ M  
14 ETO did not induce any change in c-FOS-positive cells in any of the subdivisions of  
15 NTS analyzed ( $n$ =14; Table 1; Fig. 7A,I).

16 Both concentrations of ETO induced a significant increase in c-FOS-positive  
17 cells in the VLM (+181 $\pm$ 55% for 0.05 $\mu$ M and +150 $\pm$ 49% for 2 $\mu$ M; Table 1; Fig.  
18 7B,F,J) and a large proportion of these cells was also immunoreactive for TH  
19 (42.3 $\pm$ 13.5% and 30.7 $\pm$ 6.9% at 0.05 and 2 $\mu$ M, respectively; Fig. 8D-F).

20 ETO induced a significant increase in *c-fos* expression in RPa (Table 1, Fig.  
21 7D,H,L) and ROb (Table 1, Fig. 7C,G,K), but not in RMg (Table 1). This increase was  
22 more marked in the caudal part (from the pyramidal decussation to the rostral edge of  
23 the inferior olives) than in the rostral part (from the rostral edge of the inferior olives to  
24 the rostral edge of the facial nucleus) of the ROb (208 $\pm$ 34% vs 1176 $\pm$ 261% and  
25 344 $\pm$ 83% vs +834 $\pm$ 200% for 0.05 and 2 $\mu$ M ETO, respectively; Table 1, Fig. 7C,G,K),

1 but not in the RPa (+59±18% vs +113±35% and +92±26 vs +74±20% for 0.05 and  
2 2µM ETO, respectively; Table 1, Fig. 7D,H,L). A large proportion of c-FOS-positive  
3 cells in the caudal parts of RPa and ROb was also immunoreactive for 5-HT  
4 (38.4±5.2% and 41.0±9.1% at 0.05µM and 43.6±6.8% and 56.1±6.5% at 2µM, Fig.  
5 9A-F). In contrast, only a few cells in the rostral parts of RPa and ROb were also 5-  
6 HT-positive (5.4±0.9% and 23.6±3.8% at 0.05µM and 9.7±3.8% and 15.0±2.2% at  
7 2µM).

8 ETO exposure also induced a significant increase in *c-fos* expression in facial  
9 (+35±8% and +59±14% for 0.05 and 2µM ETO, respectively; Table 1) and  
10 hypoglossal (+626±185% and +400±103% for 0.05 and 2µM ETO, respectively;  
11 Table 1) nuclei.

12 On the ventral medullary surface, at the level of the RTN/pFRG and PP (cells  
13 located at the lateral edge of the pyramidal tract (Paxinos et al., 2007;Paxinos and  
14 Franklin, 2001)), *c-fos* expression was not modified by ETO exposure (Table 1).

15

## 1 4. Discussion

2 This study was conducted on the basis of clinical observations showing that  
3 exposure to a progestin of the gonane family increases  $f_R$  in CCHS patients and  
4 shows that such molecules can experimentally increase respiratory frequency in  
5 newborn mice *in vivo* and in isolated brainstem. Our data demonstrate medullary  
6 mechanisms, indicating that serotonergic neurons within the medullary raphe nuclei  
7 are involved.

### 8 4.1. DSG accelerates $f_R$ and reduces $PET_{CO_2}$ in CCHS patients

9 The first description of the ventilatory effects of DSG in CCHS patients (Straus  
10 et al., 2010) focused on chemosensitivity and recovery of a perceptual and ventilatory  
11 response to  $CO_2$ , mostly because this finding constituted a major surprise. The first  
12 patient did not know at all that she was taking a potentially effective drug, but the  
13 second patient was aware of the observation made with the first patient and of the  
14 hypothesis concerning her own ventilatory response to hypercapnia. Since the  
15 observations were not part of a blinded study, but fortuitously observed in a clinical  
16 setting, the patients were aware of the drug withdrawal. The effects of DSG on  
17 baseline ventilation were not examined in detail. Review of the data collected  
18 dynamically (before, during and after DSG exposure) from the same two patients  
19 showed that  $f_R$  was higher and  $PET_{CO_2}$  was lower in the presence of DSG compared  
20 to the absence of DSG (Fig. 1). This evidence is undoubtedly fragile (only two  
21 patients, retrospective analysis, absence of control of DSG administration due the  
22 serendipitous nature of the observations). The present re-analysis suggests that the  
23 ventilatory effect of DSG could extend beyond chemosensitivity, as DSG may  
24 increase  $f_R$  and lower  $PET_{CO_2}$  during resting breathing in certain CCHS patients  
25 despite the defective respiratory rhythmogenesis characteristic of this disease.

1 Because progesterone and pregnane progestins increase  $f_R$  in healthy humans  
2 (Behan et al., 2003; Jensen et al., 2008; Skatrud et al., 1978), but not in CCHS  
3 patients (Sritippayawan et al., 2002), our observations suggest that DSG and more  
4 generally gonane progestins could interfere with breathing control via distinct  
5 mechanisms from those involved in the action of pregnanes.

#### 6 *4.2. ETO, the metabolite of DSG, increased the $f_R$ on in vivo newborn mice*

7 *In vivo*, considering the bioavailability of the DSG, the administration of  $10^{-3}$   
8 mg/kg of ETO is the nearest concentration of the human exposure (Timmer et al.,  
9 1999). At this concentration, in newborn mice ETO induced an increase of baseline  $f_R$   
10 compared to control (Fig. 2). Although the developmental stage was different, this  
11 increase is similar to what we observed in adult CCHS patients (Fig. 1).

#### 12 *4.3. ETO, the metabolite of DSG, enhances $f_R$ via medullary mechanisms*

13 On *ex vivo* preparations containing only the medullary regions of the  
14 brainstem, acute exposure to ETO induced a dose-dependent increase in  $f_R$  (Fig. 3)  
15 with no change in IntC4. This finding resembles our observations in CCHS patients  
16 (increased  $f_R$  with no  $V_T$  changes). The  $f_R$  increase in CCHS patients receiving DSG  
17 and in *in vivo* newborn mice receiving  $10^{-3}$  mg/kg of ETO (Fig. 1,2) may therefore be  
18 mediated by medullary mechanisms.

19 c-FOS labeling revealed increased cellular activity in the VLM, with 30-40% of  
20 c-FOS-positive catecholaminergic cells (Fig. 8), suggesting that catecholaminergic  
21 cells are involved in the ETO effects. Numerous data have implicated the A1C1  
22 catecholaminergic cell group in the control of breathing (Erickson and Millhorn,  
23 1994; Johnson et al., 2005; Viemari, 2008). However, at least 60% of VLM c-FOS-  
24 positive cells are not catecholaminergic. Of note, the preBotC, one of the two  
25 medullary respiratory oscillators (Feldman et al., 2013; Smith et al., 1991), is located

1 in the VLM and does not contain catecholaminergic cells (Wang et al., 2001).  
2 Therefore, part of the non-catecholaminergic c-FOS-positive cells could be neurons  
3 of the preBotC. Further experiments are needed to confirm this hypothesis. As we did  
4 not observe any change in the number of c-FOS-positive cells in the RTN/pFRG, the  
5 second medullary respiratory oscillator (Feldman et al., 2013; Onimaru and Homma,  
6 2003), is unlikely to be involved in the progestin effect. Of note, this structure is  
7 missing in transgenic mice harboring the same *Phox2b* mutation as CCHS patients  
8 (Dubreuil et al., 2008) and is therefore also probably missing in CCHS patients.  
9 Nevertheless, their baseline ventilation increased with DSG, which is consistent with  
10 the absence of involvement of the RTN/pFRG in the effects of ETO.

#### 11 4.4. ETO regulates the efficiency of GABA<sub>A</sub>- and NMDA-mediated modulation of $f_R$

12 Under conditions of bicuculline-induced GABA<sub>A</sub> receptor blockade, ETO  
13 facilitation was diminished or abolished (Fig. 4). This result suggests that part of the  
14 ETO effect on  $f_R$  depends on an interaction with GABA<sub>A</sub> receptors, which would be  
15 consistent with data of literature reporting that steroids, including progesterone and  
16 progestins, interact with GABA<sub>A</sub> (Belelli and Lambert, 2005; Park-Chung et al., 1999).  
17 Steroids are known to be either negative, positive or both allosteric modulators of  
18 GABA<sub>A</sub> (Park-Chung et al., 1999; Ren and Greer, 2006). No data are available  
19 concerning the interaction of ETO with GABA<sub>A</sub>. Our experiments show that the  
20 decrease in  $f_R$  induced by the GABA<sub>A</sub> agonist muscimol was markedly enhanced by  
21 ETO exposure, suggesting that ETO exerts a positive modulation of GABA<sub>A</sub>. Two  
22 elements support this hypothesis. First, testosterone, from which ETO is derived, is a  
23 positive modulator of GABA<sub>A</sub> (Park-Chung et al., 1999). Second, steroids that are  
24 negative modulators of GABA<sub>A</sub> are characterized by a negative charge at C-3 (Park-  
25 Chung et al., 1999). This is not the case of ETO that displays a keto-group at this site

1 (Grandi et al., 2014). It is possible that part of the ventilatory effect of ETO depends  
2 on positive modulation of GABA<sub>A</sub> receptors that contribute to CVD. The differential  
3 effects of bicuculline on the ventilatory action of ETO (total or partial blockade) may  
4 be due to an ETO concentration-dependent effect on several types of GABA<sub>A</sub>  
5 receptors, as it has been shown that for some neurosteroids the GABA<sub>A</sub>-evoked  
6 responses mediated by receptors containing  $\alpha_{1/3}$  subunits are enhanced by relatively  
7 low steroid concentrations. In contrast, equivalent receptors that incorporate  $\alpha_{2/4/5/6}$   
8 subunits require higher steroid concentrations (Belelli and Lambert, 2005). According  
9 to this hypothesis, differences in the effects of bicuculline on the ventilatory action of  
10 ETO may be due to a global efficiency of various types of GABA<sub>A</sub> receptors,  
11 particularly  $\alpha_{1/3}$  GABA<sub>A</sub> or  $\alpha_{2/4}$  GABA<sub>A</sub> that have been either located in medullary  
12 respiratory areas or shown to play a role in CVD (Liu and Wong-Riley, 2006; Loria et  
13 al., 2013).

14 Bicuculline-induced GABA<sub>A</sub> receptor blockade did not completely abolish the  
15 facilitation induced by low ETO concentration (Fig. 4). We therefore hypothesized  
16 that other receptors were involved. We focused on NMDA receptors because they  
17 are both involved in CVD and are modulated by steroids (Funk et al., 1997; Greer et  
18 al., 1991; Korinek et al., 2011). Our experiments showed that NMDA blockade totally  
19 abolished the facilitatory influence of ETO on  $f_R$  (Fig 5), suggesting that either ETO  
20 modulates NMDA regulation of  $f_R$ , or that all pathways by which ETO increased  $f_R$   
21 require functional glutamate/NMDA neurotransmission, or both. Our results showing  
22 that ETO potentiated the NMDA-induced increase in  $f_R$  only at low progestin  
23 concentrations support the hypothesis of modulation of NMDA regulation of  $f_R$  by  
24 ETO. c-FOS labeling suggested that ETO modulation of  $f_R$  regulation by NMDA  
25 depends on the c/mNTS, which was the only area displaying an increase in c-FOS-



1 positive cells, at low but not at high ETO concentrations. This hypothesis is supported  
2 by data indicating that NMDA receptors are present on c/mNTS neurons (Lin et al.,  
3 2008) and that the excitatory response of NTS neurons to application of NMDA is  
4 modulated by steroids (Xue and Hay, 2003). As the c-FOS-positive neurons of the  
5 c/mNTS were not catecholaminergic, ETO is likely to influence another cell  
6 population.

#### 7 *4.5. ETO increases $f_R$ via a pathway involving medullary serotonergic systems*

8 Because blockade of serotonergic regulation of  $f_R$  abolished the facilitatory  
9 effect of ETO, we hypothesized that this effect involved serotonergic signaling,  
10 which is in line with published data showing that serotonergic neurons are involved  
11 in the facilitatory influence of progesterone (Behan et al., 2003; Farmer et al., 1996).  
12 The effect of steroids on the release of 5-HT depends on supramedullary regions,  
13 such as pons (Robichaud and Debonnel, 2004) or hypothalamus (Farmer et al.,  
14 1996). Our experiments suggest that ETO interfered with serotonergic systems via  
15 a direct medullary action, revealing a new pathway of interaction between  
16 progesterone and serotonergic systems. More specifically, our c-FOS data  
17 suggest that ETO exerted its facilitatory action by activating serotonergic neurons  
18 located in the caudal parts of RPa and ROb, two areas known to be implicated in  
19 ventilatory control (Cao et al., 2006; Cerpa et al., 2015; Depuy et al., 2011). This  
20 progesterone-serotonin interaction affecting modulation of CVD would be specific to the  
21 gonane family. This putative pathway forms a relevant basis for dedicated  
22 investigations designed to elaborate personalized approaches to treat CCHS patients  
23 with gonane progestins. Otherwise, serotonergic and non serotonergic neurons of  
24 the medullary raphe nuclei and particularly those of the rRPa are involved in the  
25 processes of thermogenesis and heat conservation (McGlashon et al.,

1 2015; Nakamura and Morrison, 2007). Thus, *in vivo*, it is quite conceivable that these  
2 neurons stimulated by ETO could increase the CVD not only by a direct action on the  
3 central respiratory pattern generators but also indirectly by inducing an hyperthermia.

#### 4 **5. Conclusion**

5 To conclude, DSG has been associated with chemosensitivity recovery in  
6 CCHS patients (Straus et al., 2010). Its metabolite, ETO, has been shown to  
7 enhance chemosensitivity in newborn rats via supramedullary mechanisms (Loiseau  
8 et al., 2014). Combined with the present data, these observations suggest that two  
9 distinct pathways are involved in the ventilatory effects of these gonane progestins.  
10 The medullary pathway described here could be relevant to resting breathing CVD,  
11 whereas the supramedullary pathway previously described could be relevant to  
12 chemosensitivity. Medullary and supramedullary mechanisms could coexist in CCHS.  
13 Their effect on resting breathing are particularly pertinent to the issue of ventilatory  
14 support. The present animal data, indicating a medullary serotonergic determinant  
15 of the stimulant effect of ETO, provide a rationale for clinical trials combining DSG  
16 and serotonergic drugs to improve ventilation in CCHS patients.

17

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9

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ACCEPTED MANUSCRIPT



**1 Figure/Table Legends****2 Figure 1**

3 DSG increases baseline  $f_R$  in two CCHS patients. (A-D) Boxplot showing the median  
4 breath-by-breath  $f_R$  (A and C) and median breath-by-breath  $PET_{CO_2}$  (B and D) in two  
5 CCHS patients. † indicates a significant difference between before or after DSG  
6 treatment and during DSG exposure. ANOVA 1 way – Bonferroni post test.  $^{††}p <$   
7  $0.01$ ,  $^{†††}p < 0.001$ ; desogestrel (DSG).

**8 Figure 2**

9 ETO increases baseline  $f_R$  *in vivo* on newborn mice. (A-B) Traces illustrate baseline  
10  $f_R$  of newborn mice after two hours of oil (A) or ETO (B) exposure. (C) Histogram  
11 showing mean value of  $f_R$  of oil (white bar) or ETO (gray bar) exposure. Data are  
12 expressed as mean  $\pm$  SEM. # indicates a significant difference between oil and ETO  
13 values. Student t test;  $^{###}p < 0.001$ .

**14 Figure 3**

15 ETO increases baseline  $f_R$  on *ex vivo* medullary-spinal cord preparations. (A-J)  
16 Traces illustrate ventilatory C4 activity under normal-pH-aCSF; traces illustrate the  
17 central respiratory drive recorded the last five minutes before (A, C, E, G and I) and  
18 during the DMSO (B) or ETO exposure (D, F, H and J). (K) Histogram showing mean  
19 value of  $f_R$  of DMSO (white bar) or ETO (gray bars) exposure. Data are expressed as  
20 mean  $\pm$  SEM \* indicates a significant increase in mean  $f_R$  compared to baseline  
21 values. # indicates a significant difference between DMSO and ETO values. §  
22 indicates a significant difference between ETO 2 $\mu$ M and lower ETO concentrations.  
23 ANOVA 2 way – Bonferroni post test. Integrated activity of C4 ventral nerve root  
24 ( $\int C4$ ); electrical activity of C4 ventral nerve root (C4);  $^{***}p < 0.001$ ,  $^{##}p < 0.01$ ,  $^{###}p <$   
25  $0.001$ ,  $^{§§}p < 0.01$ ; etonogestrel (ETO).

**1 Figure 4**

2 ETO regulates the efficiency of GABA<sub>A</sub> modulation of  $f_R$ . (A-F) Ventilatory C4 activity  
3 under bicuculline exposure during the last five minutes preceding (A, C and E); and  
4 during (B, D and F) DMSO or ETO exposure. (G) Histogram showing mean value of  
5  $f_R$  of ETO exposure without and in presence of bicuculline. (H) Dose response curve  
6 of changes in mean  $f_R$  in response to exposure to muscimol. (I-N) Traces illustrate  
7 the ventilatory C4 activity under DMSO and ETO exposure during the last five  
8 minutes preceding (I, K and M) and the last two minutes during (J, L and N) muscimol  
9 application. (O) Histogram showing mean value of  $f_R$  observed under muscimol and  
10 DMSO (white bar) or ETO (gray bars) application. Data are expressed as mean  $\pm$   
11 SEM. \* indicates a significant increase in mean  $f_R$  compared to bicuculline or pre-  
12 muscimol values. # indicates a significant difference between ETO and ETO-BIC or  
13 DMSO exposures. ANOVA 2 way – Bonferroni post test. Integrated activity of the C4  
14 ventral nerve root ( $\int$ C4); electrical activity of the C4 ventral nerve root (C4); \*\* $p <$   
15 0.01, \*\*\* $p <$  0.001, # $p <$  0.05, ## $p <$  0.01; etonogestrel (ETO); bicuculline (BIC).

**16 Figure 5**

17 ETO regulates the efficiency of NMDA modulation of  $f_R$ . (A) Dose response curve of  
18 changes in mean  $f_R$  in response to exposure to the NMDA receptor agonist NMDA.  
19 (B-C) Traces illustrated ventilatory C4 activity under MK-801 exposure during the last  
20 five minutes preceding (B) and during (C) ETO exposure. (D) Histogram showing  
21 mean value of  $f_R$  observed under MK-801 and ETO (gray bars) application. (E-J)  
22 Traces illustrated ventilatory C4 activity under DMSO and ETO exposure during the  
23 last five minutes preceding (E, G and I) and during (F, H and J) NMDA application.  
24 (K) Histogram showing mean value of  $f_R$  observed under NMDA and DMSO (white  
25 bar) or ETO (gray bars) application. All values are expressed as mean  $\pm$  SEM. \*

1 indicates a significant increase in mean  $f_R$  compared to bicuculline or ETO values. #  
2 indicates a significant difference between ETO and DMSO or ETO and ETO-MK 801  
3 exposure. ANOVA 2way – Bonferroni post test. Integrated activity of C4 ventral nerve  
4 root ( $\int C4$ ); electrical activity of C4 ventral nerve root (C4); \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ## $p$   
5  $< 0.01$ ; etonogestrel (ETO).

## 6 **Figure 6**

7 Medullary serotonergic systems are involved in ETO ventilatory effect. (A-B) Traces  
8 illustrated ventilatory C4 activity during the last five minutes preceding (A) and during  
9 (B) the 5-HT exposure. (C) Histogram showing mean value of  $f_R$  observed under 5-  
10 HT exposure without and in presence of methysergide (5 $\mu$ M). (D) Histogram showing  
11 mean value of  $f_R$  over under ETO exposure without and in presence of methysergide.  
12 All values are expressed as mean  $\pm$  SEM. \* indicates a significant increase in mean  
13  $f_R$  compared to pre-5-HT or baseline values. # indicates a significant difference  
14 between presence or absence of methysergide. Kruskal-Wallis – Dunn post test.  
15 Integrated activity of C4 ventral nerve root ( $\int C4$ ); electrical activity of C4 ventral nerve  
16 root (C4); \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , # $p < 0.05$ , ### $p < 0.001$ ; etonogestrel (ETO);  
17 serotonin (5-HT); methysergide (MET).

## 18 **Figure 7**

19 ETO increased the c-fos expression in medullary respiratory areas.  
20 Photomicrographs illustrating the c-Fos immunoreactivity after DMSO (A-D) and ETO  
21 (E-L) exposure in the c/mNTS (A, E and I), the VLM (B, F and J), the obscurus (C, G  
22 and K) and pallidus (D, H and L) raphe nuclei. Scale bar = 100 $\mu$ m. Nucleus of the  
23 tractus solitarius, commissural and median parts (c/mNTS) and ventrolateral part  
24 (vINTS); dorsal motor nucleus of vagus (DMX); hypoglossal nucleus (XII); ambiguus

1 nucleus (Amb); ventrolateral medullary reticular nucleus (VLM); obscurus (ROb) and  
2 pallidus (RPa) raphe nuclei; etonogestrel (ETO).

### 3 **Figure 8**

4 Catecholaminergic character of c-FOS-positive cells under ETO. (A and D) Drawings  
5 illustrating the distribution of cells immunoreactive for c-FOS alone and for both c-  
6 FOS and TH in the c/mNTS (A) and VLM (D) after ETO exposure (0.05 $\mu$ M); Scale  
7 bar = 100 $\mu$ m; white and black point represent respectively c-FOS-positive neurons  
8 and c-FOS-positive neurons also immunoreactive for TH. (B and E)  
9 Photomicrographs of sections double-immunolabelled for c-FOS and TH in c/mNTS  
10 (B) and VLM (E); Scale bar = 100 $\mu$ m; (C and F) Photomicrographs representing an  
11 enlargement of B and E; Scale bar = 10 $\mu$ m. Nucleus of the tractus solitarius, caudal  
12 and median parts (c/mNTS); ventrolateral medullary reticular nucleus (VLM); tyrosine  
13 hydroxylase (TH); ETO (etonogestrel).

### 14 **Figure 9**

15 Serotonergic character of c-FOS-positive cells under ETO. (A and D) Drawings  
16 illustrating the distribution of cells immunoreactive for c-FOS alone and for both c-  
17 FOS and 5-HT in the pallidus and obscurus raphe nuclei; Scale bar = 100 $\mu$ m; white  
18 and black point represent respectively c-FOS-positive neurons and c-FOS-positive  
19 neurons also immunoreactive for 5-HT. Photomicrographs of sections double-  
20 immunolabelled for c-FOS and 5-HT in the pallidus (B and E) and obscurus (C and F)  
21 raphe nuclei after 0.05 $\mu$ M (B and C) or 2 $\mu$ M (E and F) ETO-normal pH-aCSF  
22 exposure; Scale bar = 10 $\mu$ m; etonogestrel (ETO); serotonin (5-HT).

### 23 **Table 1**

24 *c-fos* expression in medullary structures of *ex vivo* preparations.

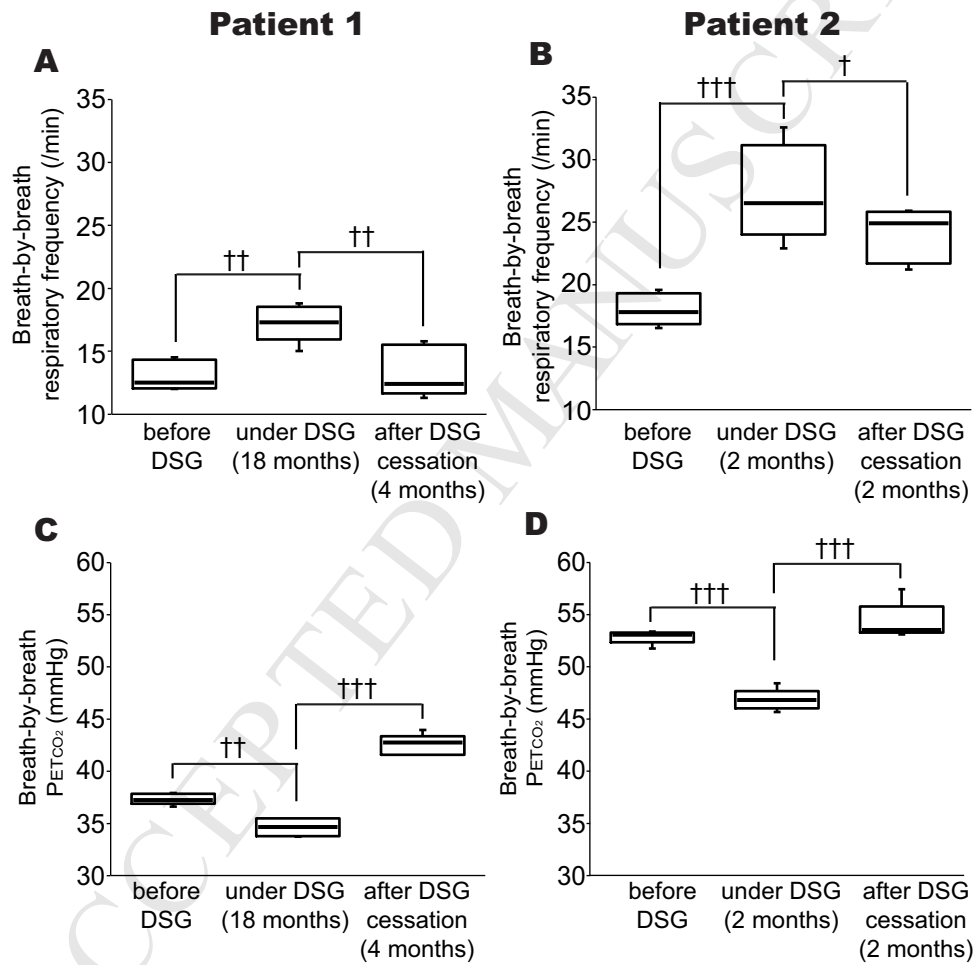
1 Values are expressed as total number of c-FOS positive cells per structure  $\pm$  SEM. \*  
2 indicates a significant increase in total number of c-FOS positive cells per structure  
3 compared to DMSO values. # indicates a significant difference between 0.05 and  
4 2 $\mu$ M of ETO. Kruskal-Wallis – Dunn post test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ### $p$   
5  $< 0.001$ . Nucleus of the tractus solitarius, commissural and median parts (c/mNTS),  
6 ventrolateral part (vINTS); ventrolateral medullary reticular nucleus (VLM); pallidus  
7 raphe nucleus, caudal part (cRPa) and rostral part (rRPa); obscurus raphe nucleus,  
8 caudal part (cROb) and rostral part (rROb); magnus raphe nucleus (RMg);  
9 hypoglossal nucleus (XII); facial nucleus (7N); retrotrapezoid nucleus/parafacial  
10 respiratory group (RTN/pFRG); parapyramidal area (PP).

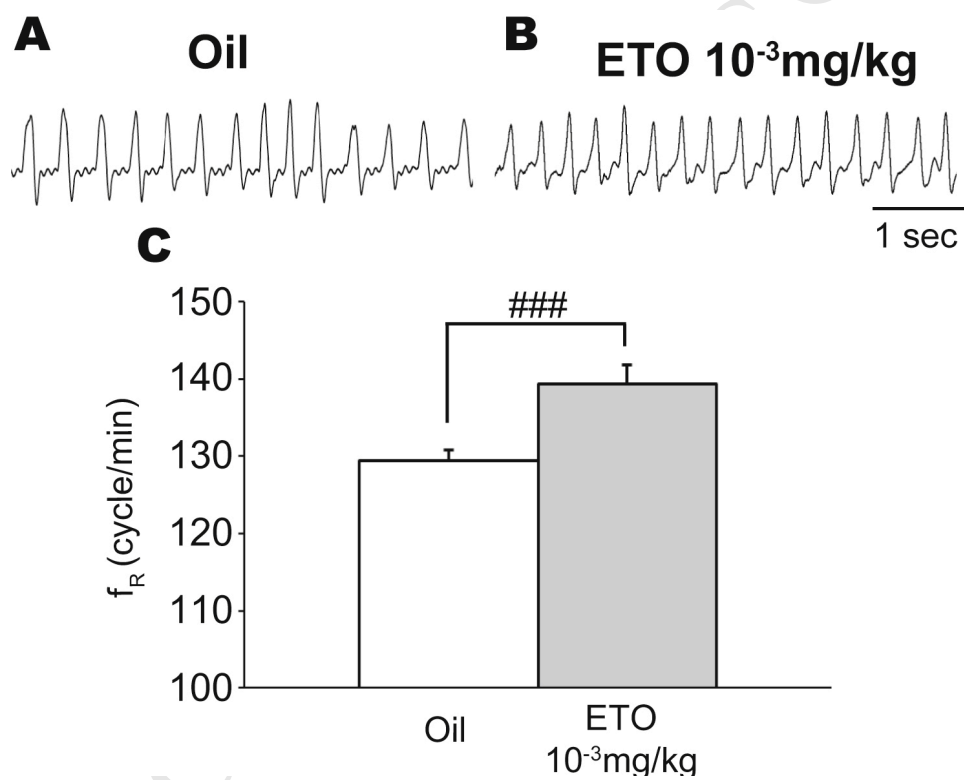
11

**Table 1.** *c-fos* expression in medullary structures of *ex vivo* preparations

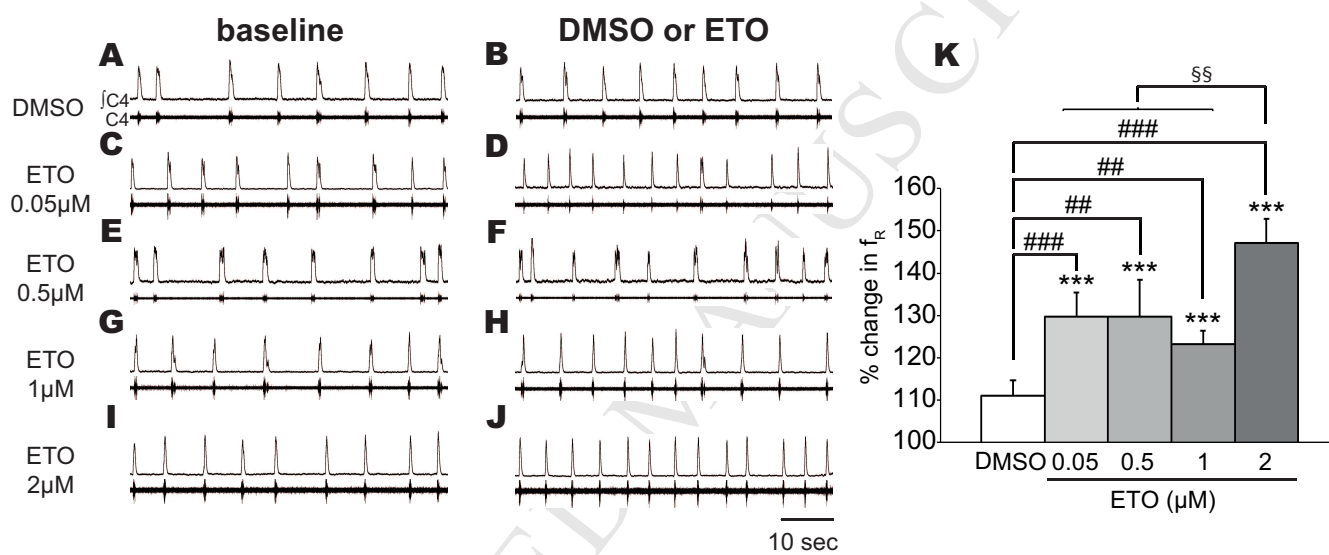
	preparations					
	DMSO		ETO			
			0.05 $\mu$ M		2 $\mu$ M	
	<i>n</i> = 8	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12
c/mNTS	37.6 $\pm$ 11.7	120.7 $\pm$ 28.0*	28.1 $\pm$ 6.8###			
vINTS	6.7 $\pm$ 2.6	4.5 $\pm$ 0.8	3.8 $\pm$ 1.0			
VLM	90.3 $\pm$ 21.1	253.5 $\pm$ 49.5*	225.3 $\pm$ 44.0*			
cRPa	35.6 $\pm$ 8.3	56.5 $\pm$ 6.3	68.5 $\pm$ 9.1*			
rRPa	14.0 $\pm$ 1.8	29.8 $\pm$ 4.9*	24.3 $\pm$ 2.8			
cROb	43.9 $\pm$ 16.6	135.1 $\pm$ 14.9*	194.7 $\pm$ 36.6***			
rROb	3.8 $\pm$ 1.7	48.5 $\pm$ 9.9***	35.5 $\pm$ 7.6**			
RMg	9.4 $\pm$ 1.7	11.8 $\pm$ 2.8	9.2 $\pm$ 2.3			
XII	19.3 $\pm$ 3.3	140.2 $\pm$ 35.7**	96.6 $\pm$ 19.8**			
7N	134.0 $\pm$ 7.6	181.1 $\pm$ 10.8*	213.5 $\pm$ 18.5**			
RTN/pFRG	6.3 $\pm$ 0.9	7.3 $\pm$ 1.4	8.5 $\pm$ 1.3			
PP	8.8 $\pm$ 2.8	10.5 $\pm$ 1.7	13.5 $\pm$ 2.5			

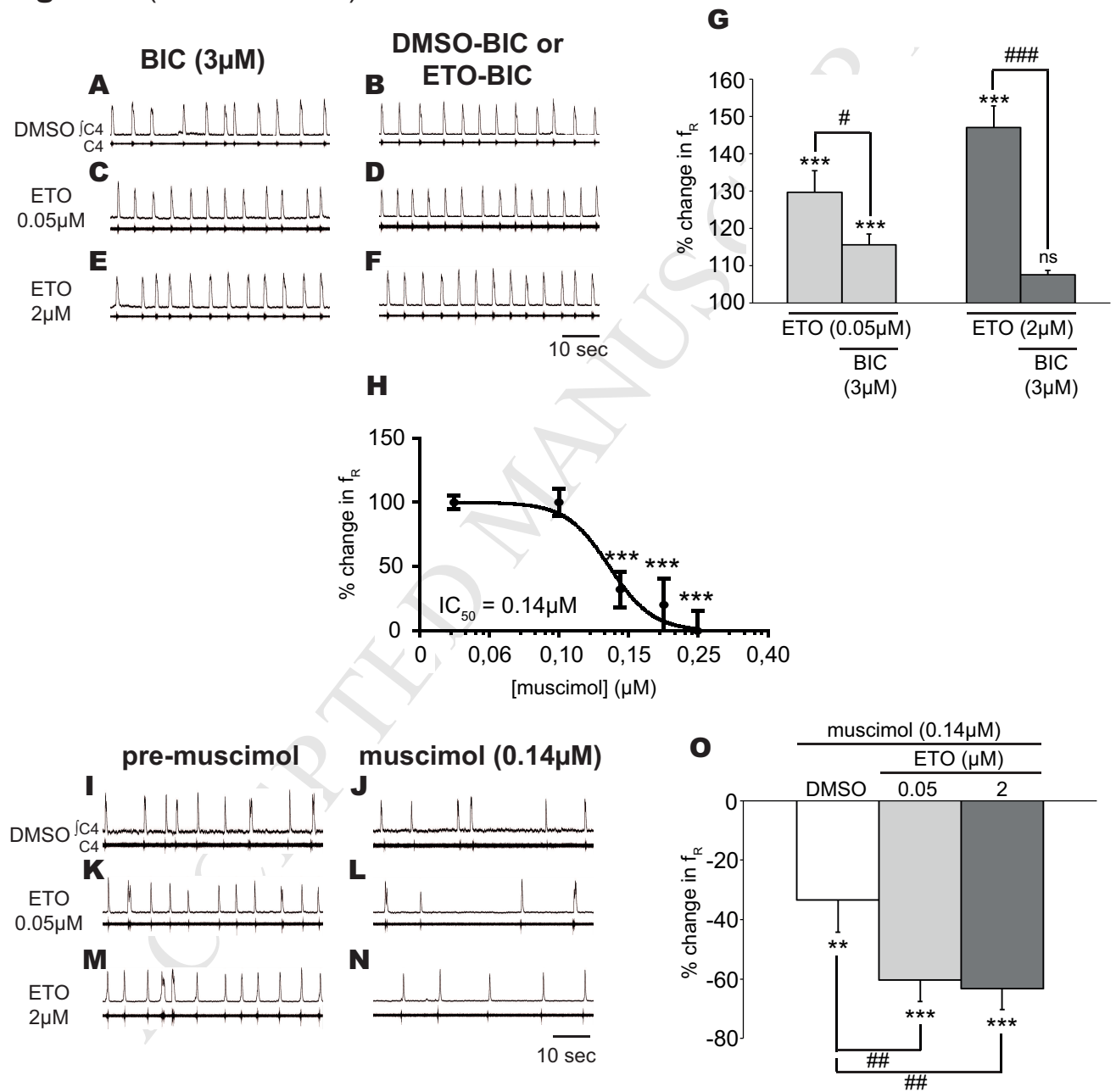
Values are expressed as total number of c-FOS positive cells per structure  $\pm$  SEM. \* indicates a significant increase in total number of c-FOS positive cells per structure compared to DMSO values. # indicates a significant difference between 0.05 and 2 $\mu$ M of ETO. Kruskal-Wallis – Dunn post test. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, ###*p* < 0.001. Nucleus of the tractus solitarius, commissural and median parts (c/mNTS), ventrolateral part (vINTS); ventrolateral medullary reticular nucleus (VLM); pallidus raphe nucleus, caudal part (cRPa) and rostral part (rRPa); obscurus raphe nucleus, caudal part (cROb) and rostral part (rROb); magnus raphe nucleus (RMg); hypoglossal nucleus (XII); facial nucleus (7N); retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG); parapyramidal area (PP).

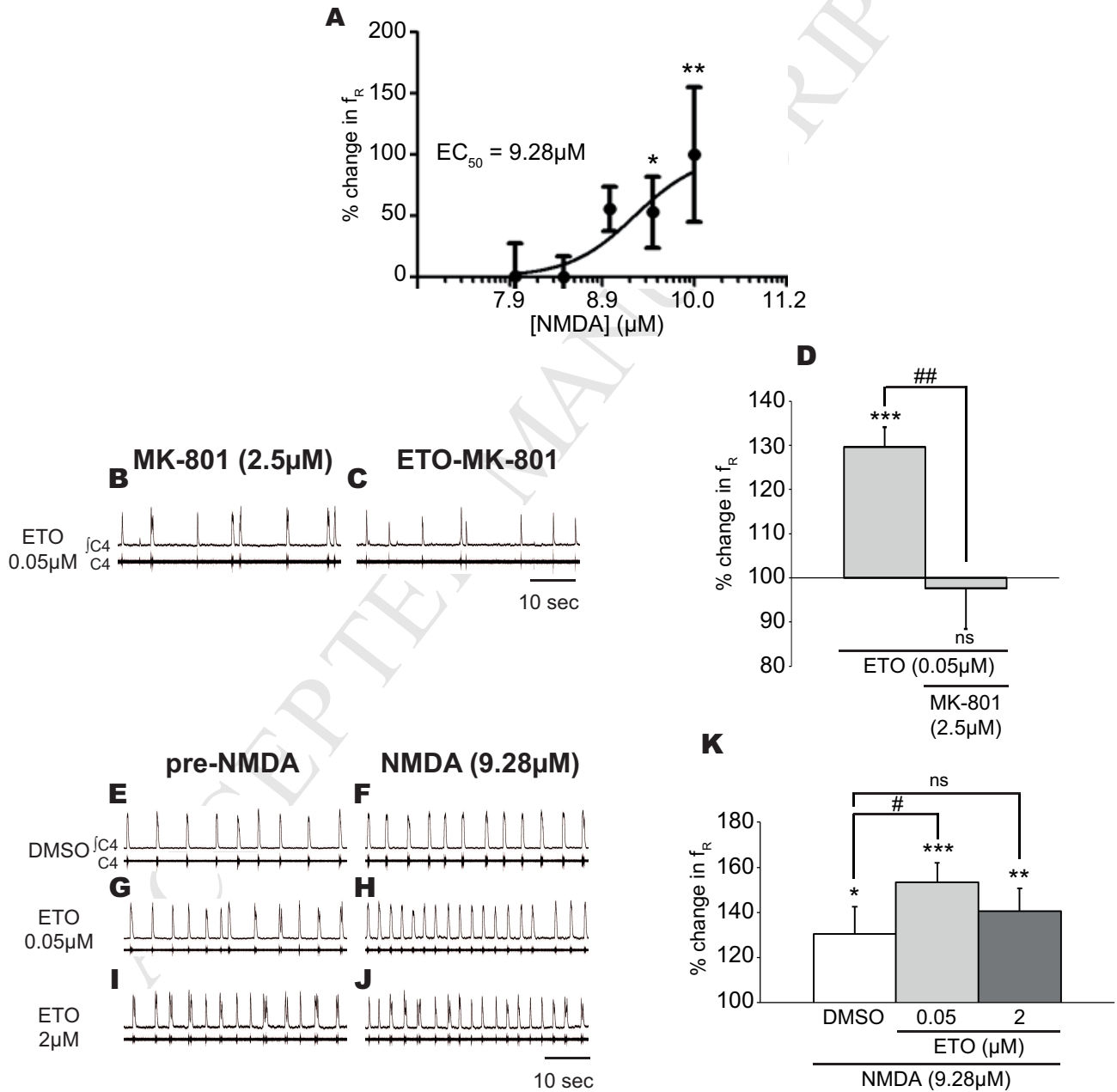
**Figure 1** (1.5 column)

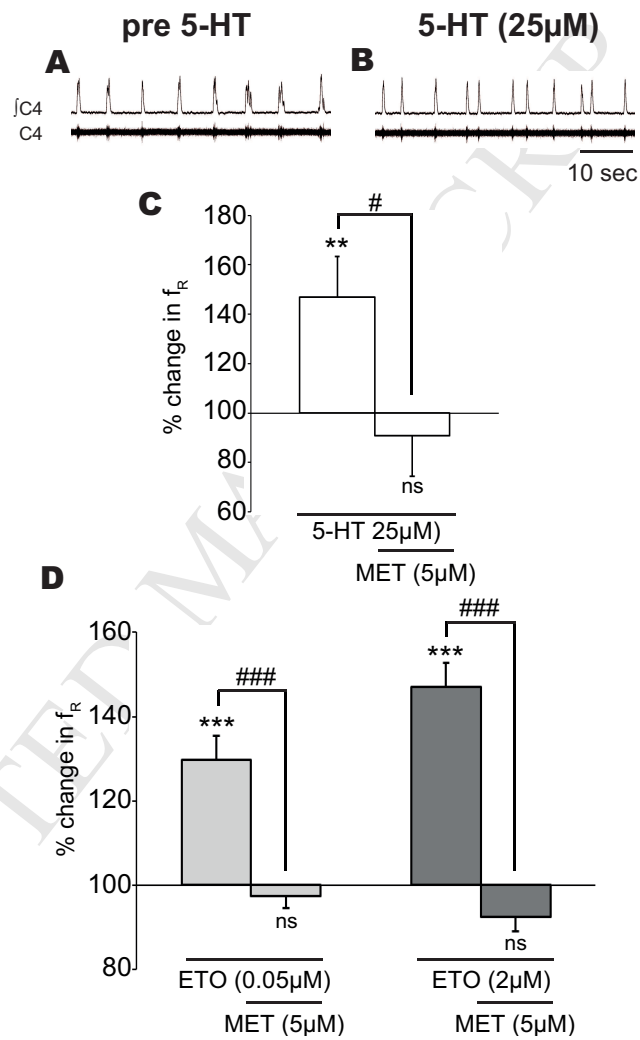


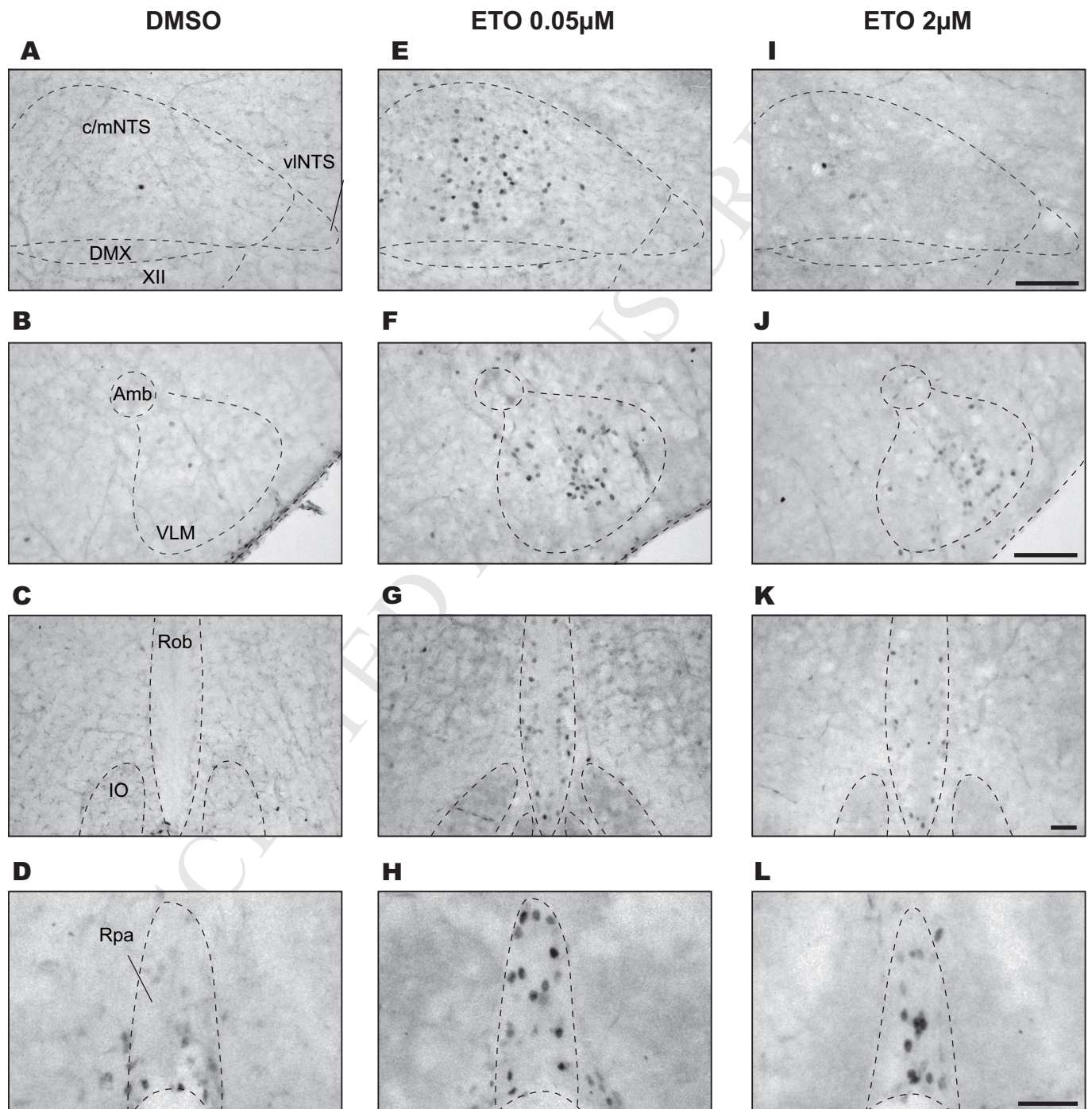


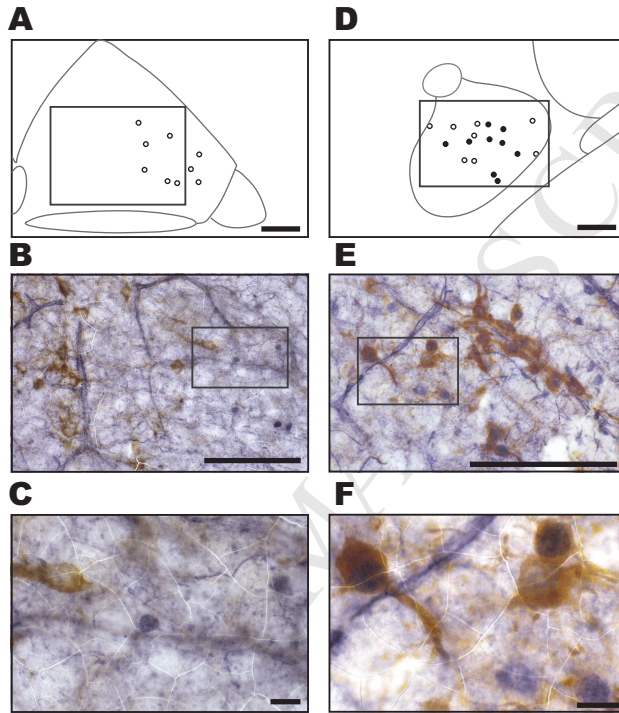
**Figure 3** (double column)

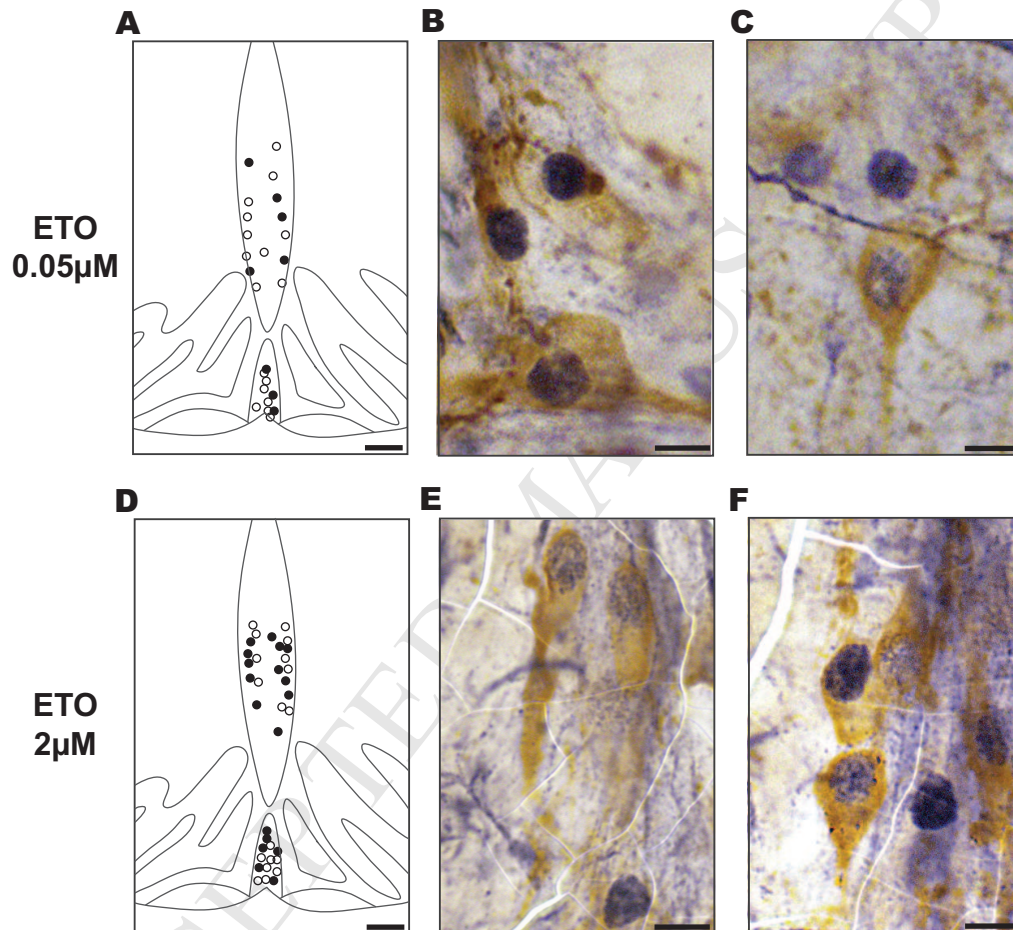
**Figure 4** (double column)

**Figure 5** (double column)

**Figure 6** (single column)

**Figure 7** (double column)

**Figure 8** (single column)

**Figure 9** (1.5 column)

**Highlights**

- Desogestrel enhances basal ventilation in Ondine's curse
- Etonogestrel increased respiratory frequency by medullary mechanisms
- GABA<sub>A</sub> and NMDA receptors are involved in the respiratory effect of etonogestrel
- 5-HT systems are implicated on the effect of etonogestrel on basal ventilation
- Combining 5-HT and desogestrel may constitute a therapeutic utility in Ondine's curse