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► **To cite this version:**

Fanny Joubert, Anne-Sophie Perrin-Terrin, Emilienne Verkaeren, Philippe Cardot, Marie-Noëlle Fiamma, et al.. Desogestrel enhances ventilation in ondine patients: Animal data involving serotonergic systems. *Neuropharmacology*, 2016, 107, pp.339-350. 10.1016/j.neuropharm.2016.03.041 . hal-01299162

HAL Id: hal-01299162

<https://hal.sorbonne-universite.fr/hal-01299162>

Submitted on 7 Apr 2016

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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 CO₂/H⁺ chemosensitivity. Incidental findings have suggested that desogestrel may allow recovery of the ventilatory response to CO₂. 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_{ET}CO₂. 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_A 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_A 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 O₂, CO₂ 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 CO₂/H⁺ chemosensitivity (Amiel et al., 2003; Weese-Mayer et al., 2010) due to
14 mutations of the *PHOX2B* gene (Amiel et al., 2003). Recovery of CO₂/H⁺
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 CO₂ (Straus et al., 2010). Deliberately
21 administering DSG to another patient did not induce any recovery of CO₂/H⁺
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_Aergic, 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 ± 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 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 CaCl₂ 2H₂O, 1.15 mM MgCl₂ 6H₂O, 0.58 mM NaH₂PO₄H₂O,
14 21.0 mM NaHCO₃, 30.0 mM d-glucose) saturated with O₂ and adjusted to pH 7.4 by
15 bubbling with 95% O₂ and 5% CO₂ (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 (10⁻³mg/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 μ 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_A receptors on ex vivo preparations

13 In a first step, we investigated possible interactions between GABA_A receptors and
14 ETO by evaluating changes in the effect of ETO under conditions of GABA_A 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_A 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 IC₅₀ 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 μ 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_{1/2/7} 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 (25 μ M) 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 10 μ M) of methysergide for 10 minutes followed by 5-HT (25 μ M) 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 5 μ M.

15 After a stabilization period, preparations were successively superfused with normal
16 pH-aCSF containing methysergide (5 μ M) 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% H₂O₂ 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[®] (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 ± 0.36 L/min resulting from a f_R of 13.06 ± 0.54 /min and a V_T of 0.64 ± 0.03 L. Her
5 P_{ETCO_2} was 37.33 ± 0.23 mmHg. Eighteen months after starting DSG (Straus et al.,
6 2010), baseline f_R increased (17.26 ± 0.66 /min, +32%; $p<0.01$; Fig. 1A), P_{ETCO_2}
7 decreased (34.63 ± 0.37 mmHg, -7%; $p<0.01$; Fig. 1C), but V_T remained unchanged
8 (0.66 ± 0.02 L), resulting in an increase in \dot{V}_E (11.42 ± 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 ± 0.90 /min,
10 8.98 ± 0.29 L/min; $p<0.01$; Fig. 1A) and P_{ETCO_2} increased to 42.53 ± 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 ± 0.57 /min, 0.60 ± 0.03 L and 10.69 ± 0.49 L/min for f_R , V_T
14 and \dot{V}_E , respectively. Her P_{ETCO_2} was 52.90 ± 0.30 mmHg. Two months after starting
15 DSG, f_R increased (27.36 ± 1.71 /min, +52%; $p<0.001$; Fig. 1B), but V_T remained
16 unchanged (0.61 ± 0.04 L), resulting in an increase in \dot{V}_E (16.53 ± 0.64 L/min, +55%;
17 $p<0.001$), while P_{ETCO_2} decreased (46.86 ± 0.45 mmHg, -11%; $p<0.001$; Fig. 1D). Two
18 months after stopping DSG, f_R significantly decreased (24.00 ± 0.97 /min; $p<0.05$; Fig.
19 1B) and \dot{V}_E was slightly lower than on DSG (16.45 ± 0.61 L/min) but still increased.
20 P_{ETCO_2} increased to 54.36 ± 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 ± 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 ± 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 ± 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 GABA_A receptor

19 After 10 minutes of exposure, bicuculline (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 μ 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_A
4 receptor agonist; IC₅₀=0.14 μ M, n =28; Fig. 4H). The decrease in f_R induced by
5 muscimol (IC₅₀) 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 μ 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 μ M ETO, respectively).

10 3.3.3. Interaction of ETO with the NMDA receptor

11 f_R was dose-dependently increased by NMDA (EC₅₀=9.28 μ M, n =20; Fig. 5A).
12 The optimal concentration of dizocilpine (MK-801, a NMDA receptor antagonist) was
13 2.5 μ M. At this concentration, f_R was not significantly altered (103.3 \pm 6.1%) and the
14 EC₅₀ of NMDA did not modify f_R (113.5 \pm 7.8%, n =5). Under 2.5 μ M MK-801, the
15 increase in f_R induced by 0.05 μ 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 EC₅₀ of NMDA together with DMSO or ETO (0.05 and 2 μ 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 μ 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 μ M
23 ETO (91.7 \pm 8.8%), but not for DMSO or 0.05 μ 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 μ M) increased f_R (146.8 \pm 16.4%, p <0.01, n =5; Fig. 6A-C). After
3 exposure to 5 μ M methysergide (a 5-HT_{1/2} 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 μ M methysergide, the increase in f_R induced by both 0.05 and 2 μ 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 μ 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 μ 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 μ M and +150 \pm 49% for 2 μ 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 μ 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 μ 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 P_{ETCO_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 P_{ETCO_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 P_{ETCO_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_A- and NMDA-mediated modulation of f_R

12 Under conditions of bicuculline-induced GABA_A 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_A receptors, which would be
15 consistent with data of literature reporting that steroids, including progesterone and
16 progestins, interact with GABA_A (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_A (Park-Chung et al., 1999; Ren and Greer, 2006). No data are available
19 concerning the interaction of ETO with GABA_A. Our experiments show that the
20 decrease in f_R induced by the GABA_A agonist muscimol was markedly enhanced by
21 ETO exposure, suggesting that ETO exerts a positive modulation of GABA_A. Two
22 elements support this hypothesis. First, testosterone, from which ETO is derived, is a
23 positive modulator of GABA_A (Park-Chung et al., 1999). Second, steroids that are
24 negative modulators of GABA_A 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_A 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_A
5 receptors, as it has been shown that for some neurosteroids the GABA_A-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_A receptors,
11 particularly $\alpha_{1/3}$ GABA_A or $\alpha_{2/4}$ GABA_A 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_A 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

1 **Acknowledgements:** F Joubert was supported by the "Fonds de dotation pour la
2 Recherche en Santé Respiratoire 2012". E Verkaeren was supported by the "Fonds
3 de dotation pour la Recherche en Santé Respiratoire 2014". The present work was
4 supported by a grant from the "Association Française pour le Syndrome d'Ondine",
5 by a "Legs Poix" grant from the "Chancellerie de l'Université de Paris" and by the
6 French government - Institut Hospitalo-Universitaire - A - Institut du cerveau et de la
7 Moelle épinière (IHU-A-ICM) "Investissement d'avenir" ANR-10-IAIHU-06 program.
8 We thank Anthony Saul for the English style.
9

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- 3
- 4
- 5

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 μ 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_A 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 μ 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 μ 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 μ M); Scale
7 bar = 100 μ 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 μ m; (C and F) Photomicrographs representing an
11 enlargement of B and E; Scale bar = 10 μ 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 μ 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 μ M (B and C) or 2 μ M (E and F) ETO-normal pH-aCSF
22 exposure; Scale bar = 10 μ m; etonogestrel (ETO); serotonin (5-HT).

23 **Table 1**

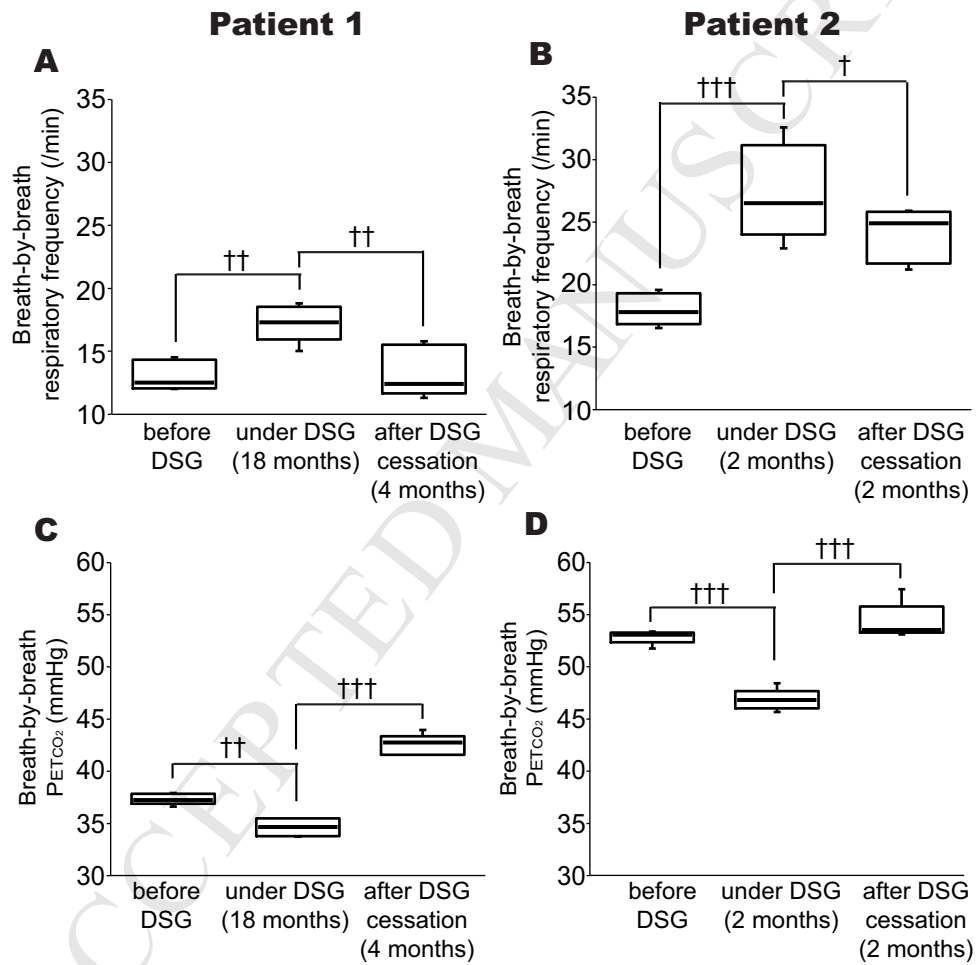
24 *c-fos* expression in medullary structures of *ex vivo* preparations.

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 μ 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 μ M		2 μ 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 μ 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)

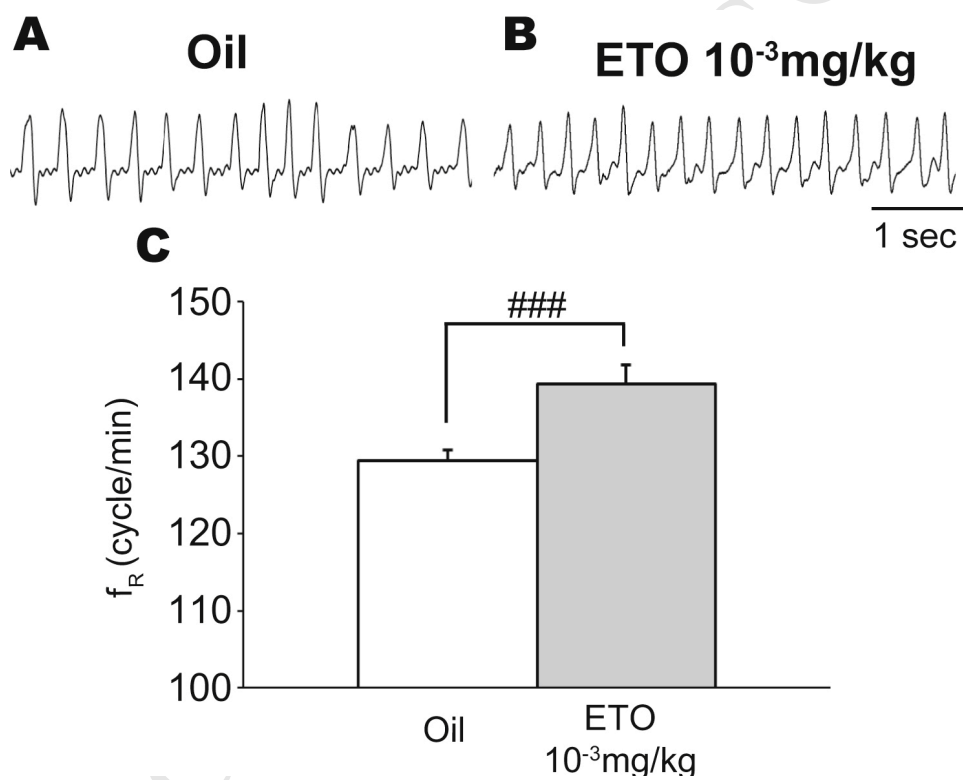


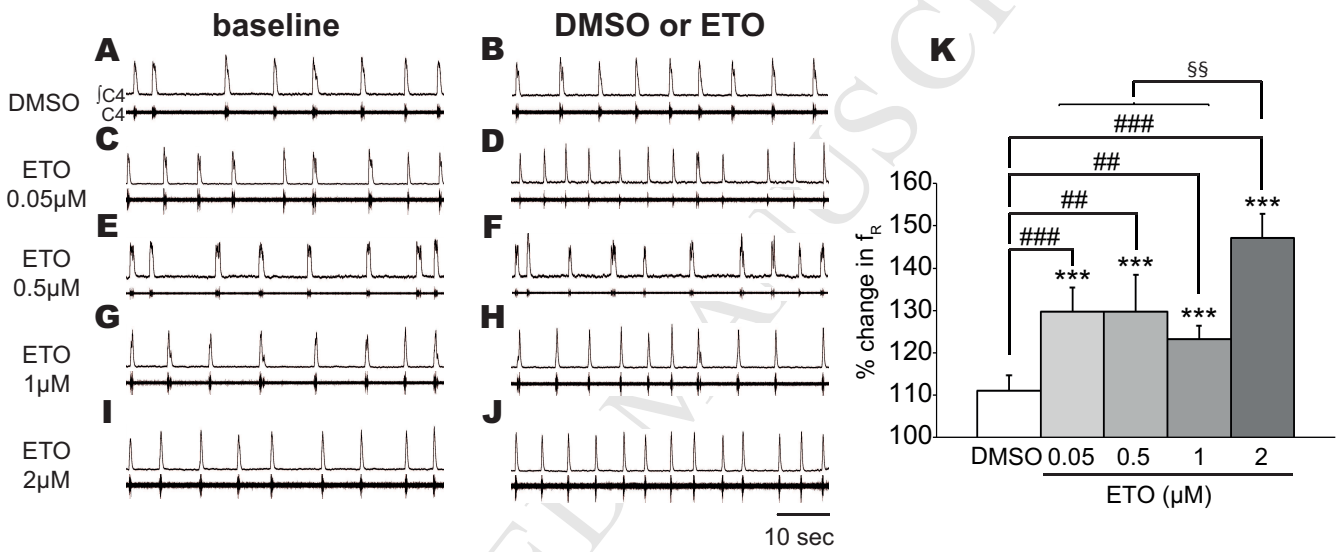
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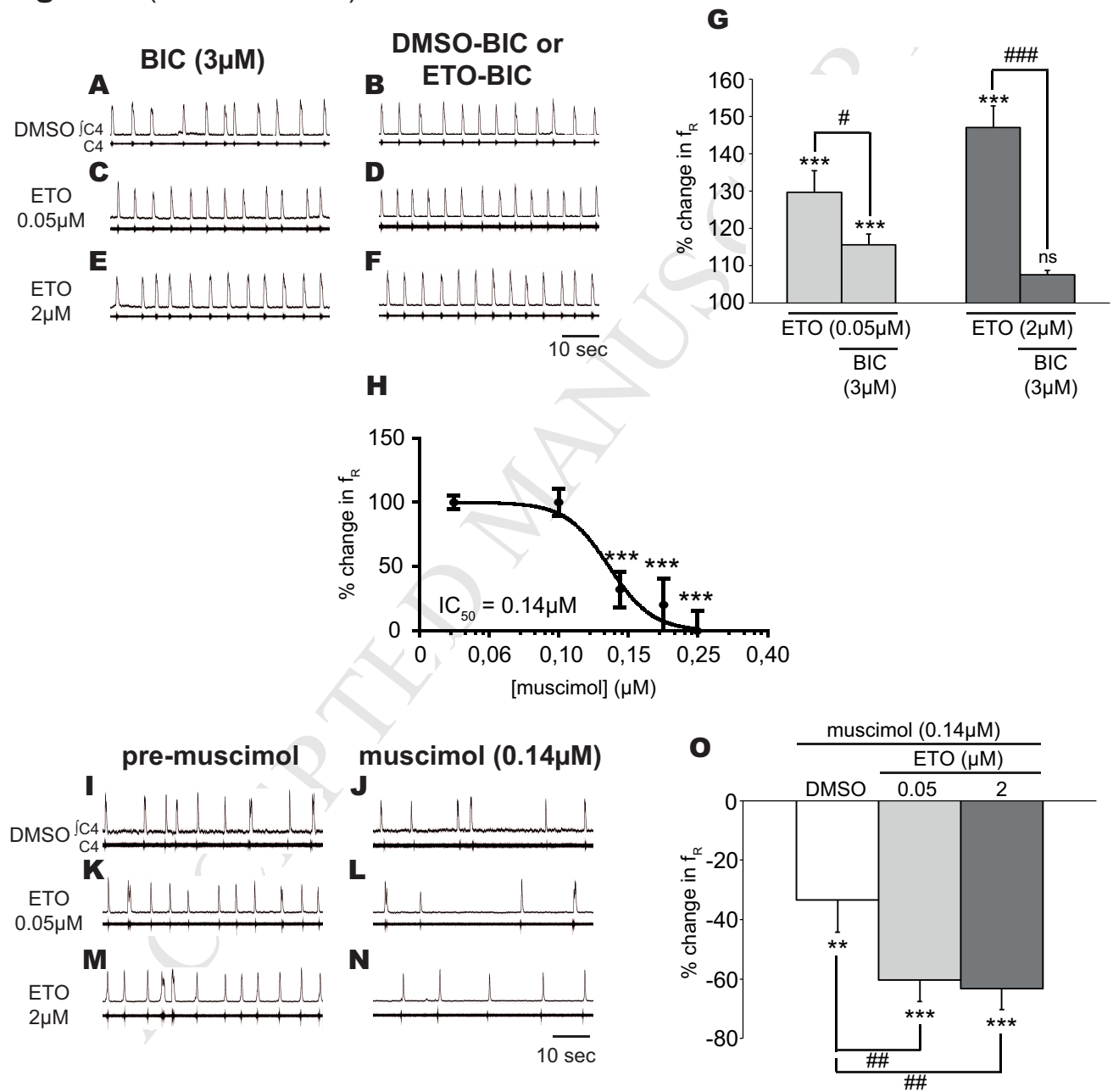
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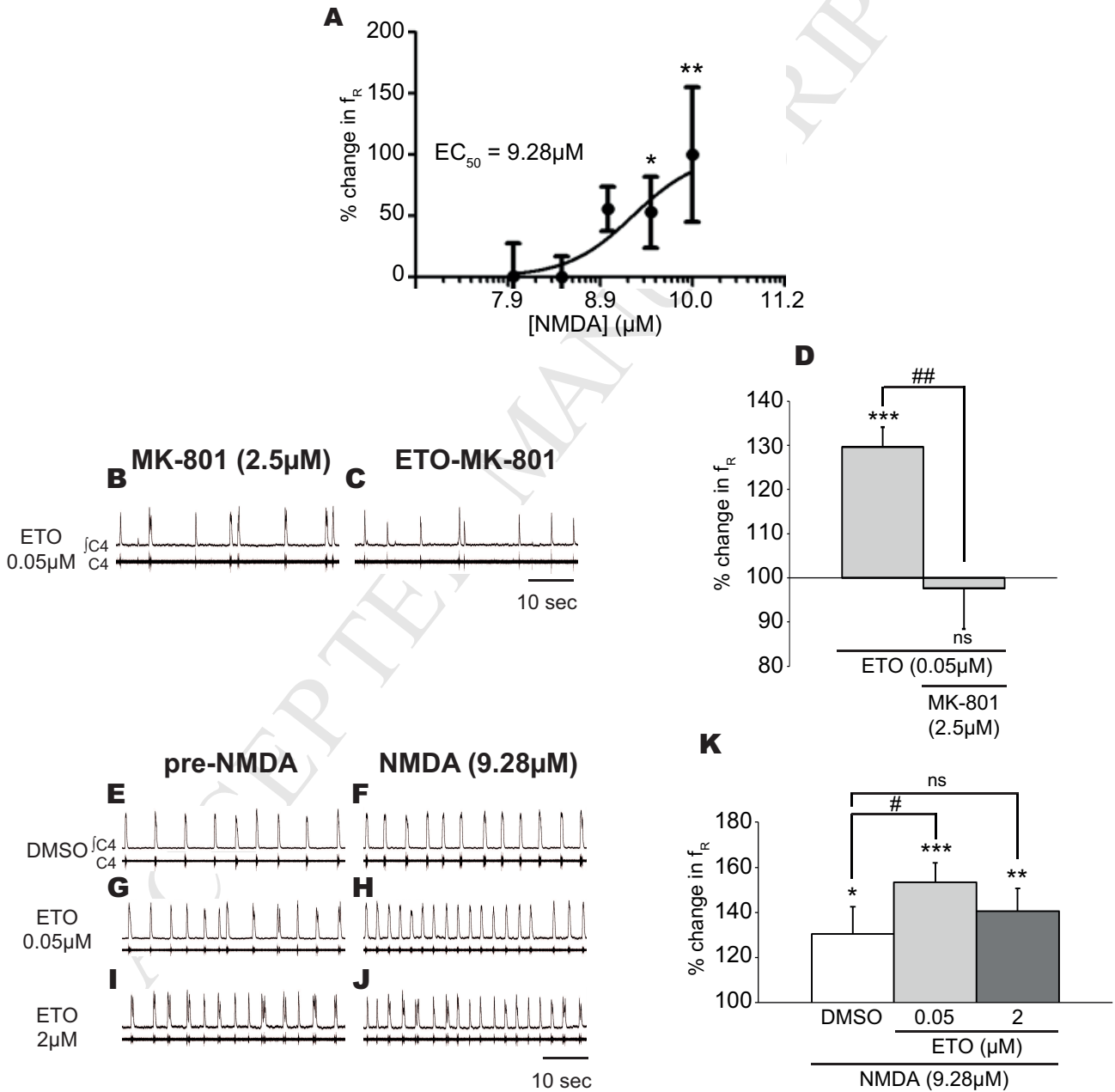
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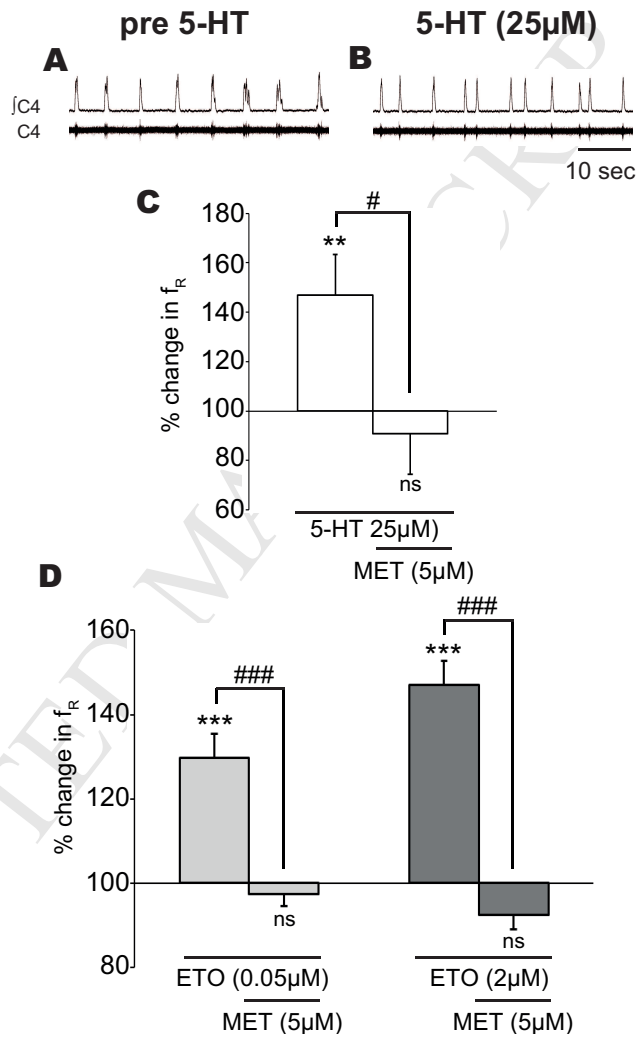
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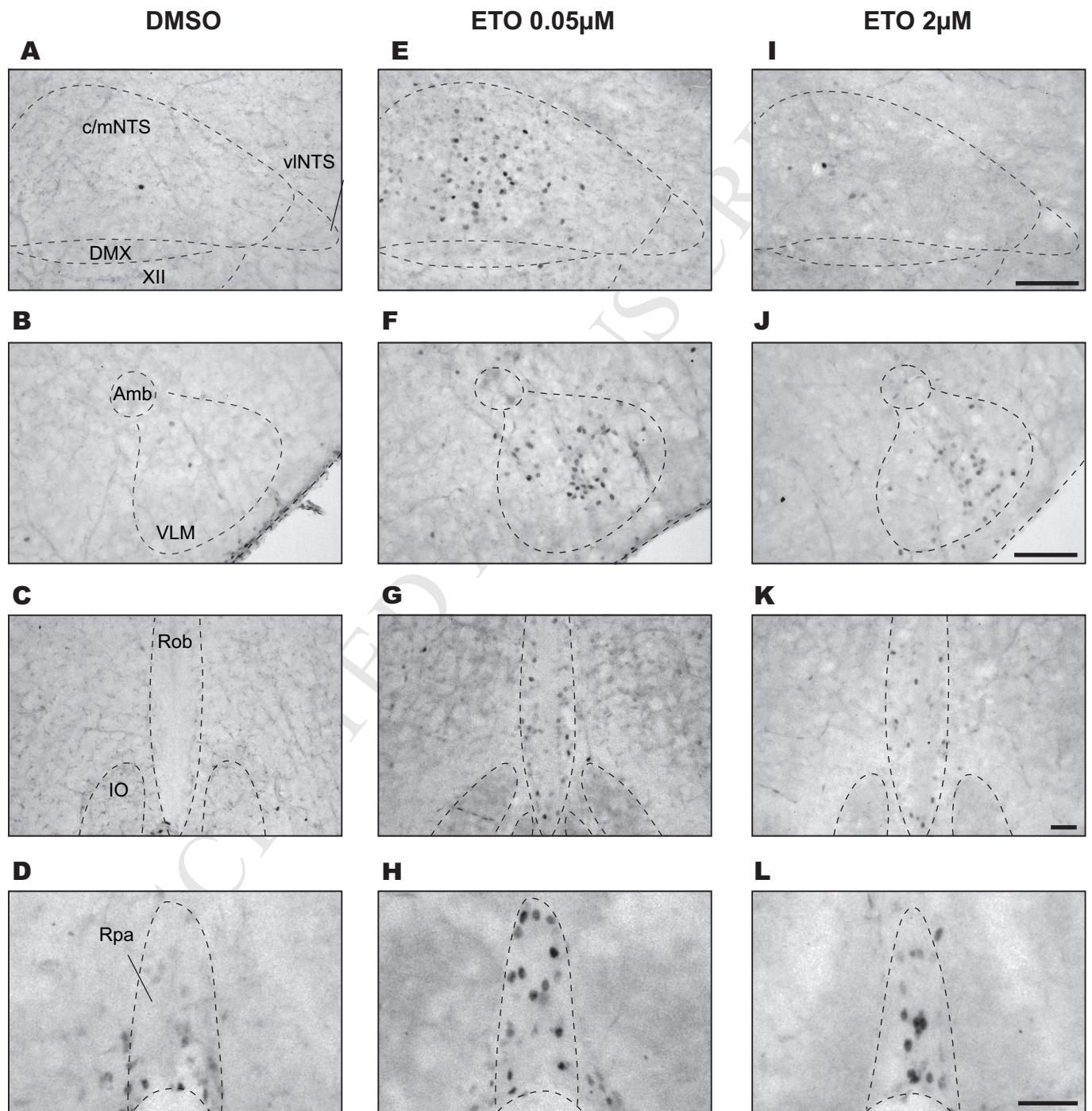
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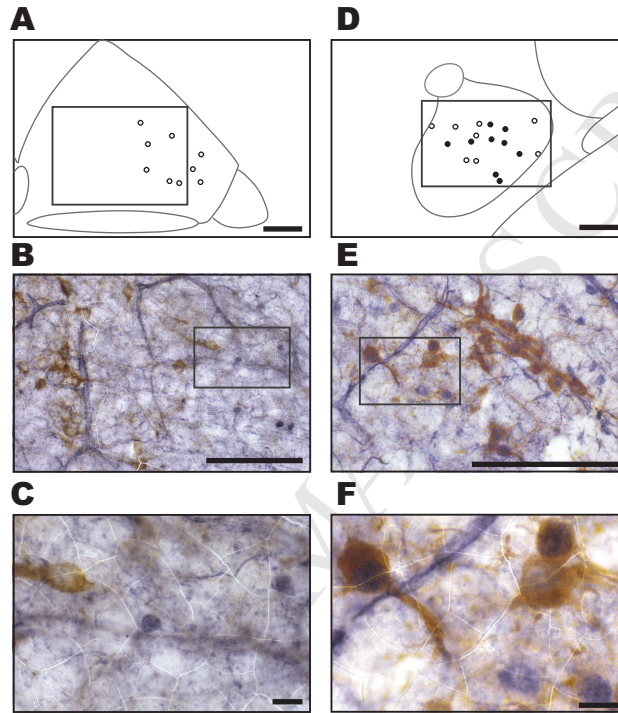
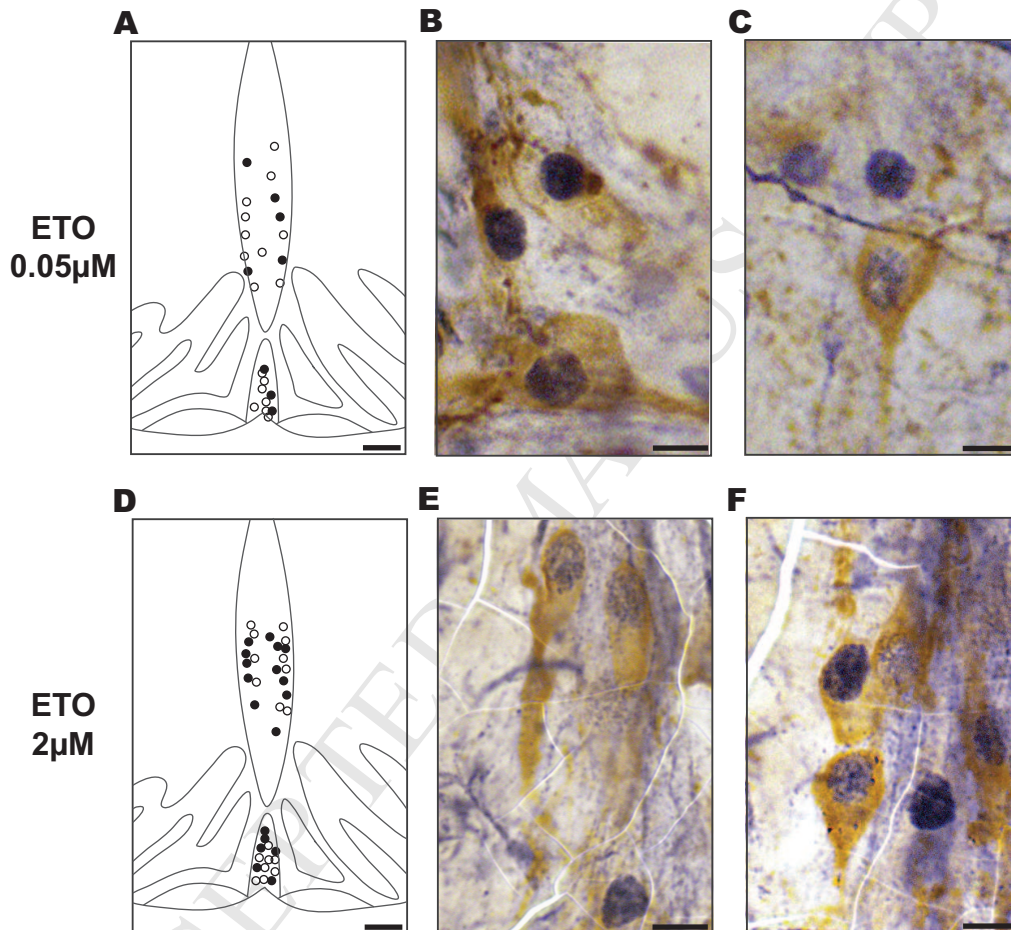
Figure 8 (single column)

Figure 9 (1.5 column)

Highlights

- Desogestrel enhances basal ventilation in Ondine's curse
- Etonogestrel increased respiratory frequency by medullary mechanisms
- GABA_A 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