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Tabinda Zafar, Charly Brouillard, Laurence Lanfumey, Caroline Sévoz-Couche. A hypothalamo-midbrain-medullary pathway involved in the inhibition of the respiratory chemoreflex response induced by potassium cyanide in rodents. *Neuropharmacology*, 2017, 10.1016/j.neuropharm.2017.09.036 . hal-01615236

**HAL Id: hal-01615236**

**<https://hal.sorbonne-universite.fr/hal-01615236>**

Submitted on 12 Oct 2017

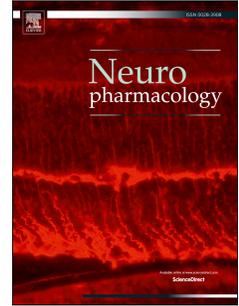
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# Accepted Manuscript

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PII: S0028-3908(17)30453-7

DOI: [10.1016/j.neuropharm.2017.09.036](https://doi.org/10.1016/j.neuropharm.2017.09.036)

Reference: NP 6880

To appear in: *Neuropharmacology*

Received Date: 18 May 2017

Revised Date: 7 September 2017

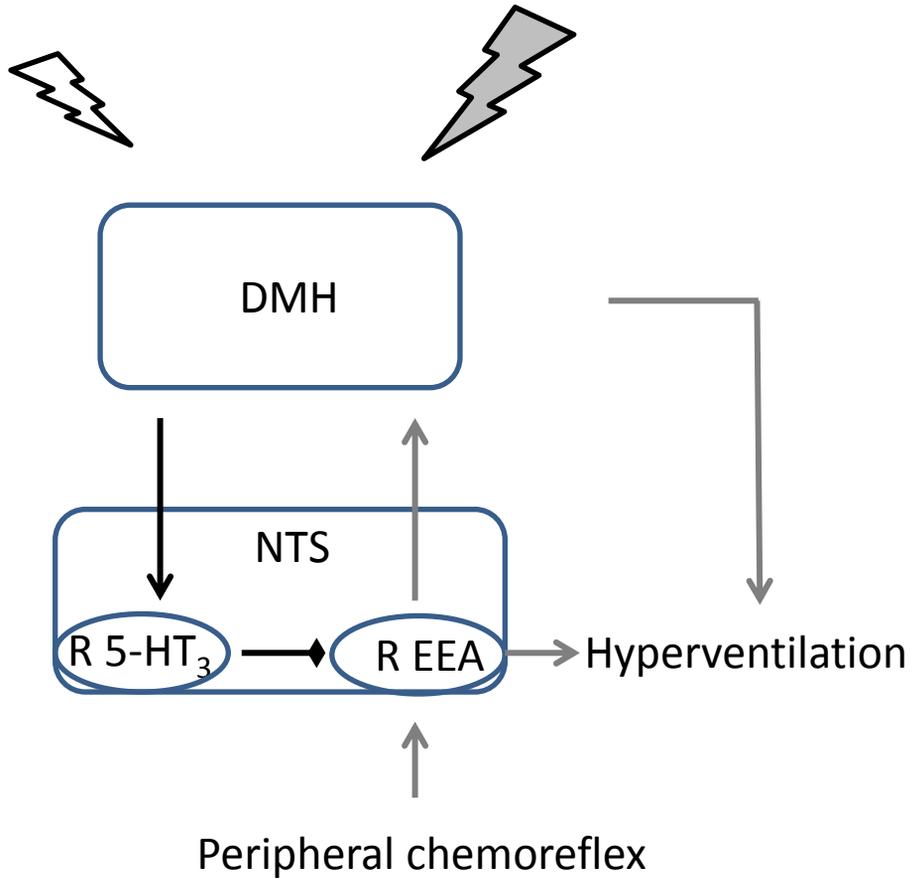
Accepted Date: 26 September 2017

Please cite this article as: Zafar, T., Brouillard, C., Lanfumey, L., Sévoz-Couche, C., A hypothalamo-midbrain-medullary pathway involved in the inhibition of the respiratory chemoreflex response induced by potassium cyanide in rodents, *Neuropharmacology* (2017), doi: 10.1016/j.neuropharm.2017.09.036.

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HMM circuit

HPM circuit



- Excitatory inputs
- Inhibitory inputs
- ⚡ Mild stimulation
- ⚡ Strong stimulation

**A hypothalamo-midbrain-medullary pathway involved in the inhibition of the respiratory chemoreflex response induced by potassium cyanide in rodents**

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**Total word-count : 7484**

**Abstract**

Recent studies have demonstrated that a mild stimulation of the dorsomedian nucleus of the hypothalamus (DMH), a defense area, induces the inhibition of the carotid chemoreflex tachypnea. DMH activation reduces the cardiac chemoreflex response via the dorsolateral part of the periaqueductal grey matter (dIPAG) and serotonin receptors (5-HT<sub>3</sub> subtype) in the nucleus tractus solitarius (NTS). The objectives of this study were to assess whether dIPAG and subsequent NTS 5-HT<sub>3</sub> receptors are involved in chemoreflex tachypnea inhibition during mild activation of the DMH. For this purpose, peripheral chemoreflex was activated with potassium cyanide (KCN, 40 µg/rat, i.v.) during electrical and chemical minimal supra-threshold (mild) stimulation of the dIPAG or DMH. In both situations, changes in respiratory frequency (RF) following KCN administration were reduced. Moreover, pharmacological blockade of the dIPAG prevented DMH-induced KCN tachypnea inhibition. Activation of NTS 5-HT<sub>3</sub> receptors also reduced chemoreflex tachypnea in a dose-dependent manner. In addition, blockade of NTS 5-HT<sub>3</sub> receptors with granisetron (2.5 but not 1.25 mM), or the use of mice lacking the 5-HT<sub>3a</sub> receptor (5-HT<sub>3a</sub> KO), prevented dIPAG-induced KCN reductions in RF. A respiratory hypothalamo-midbrain-medullary pathway (HMM) therefore plays a crucial role in the inhibition of the hyperventilatory response to carotid chemoreflex.

Keywords: carotid chemoreflex, hypothalamus, respiratory responses, nucleus tractus solitarius, 5-HT<sub>3</sub> receptor

## 1. Introduction

Several nuclei in the hypothalamus, including the dorsomedian nucleus of the hypothalamus (DMH), constitute a brain aversive system that coordinates aggressive or defense-type behavioral patterns (Bandler et al., 2000; Sévoz-Couche et al., 2003). The defense reaction, which is elicited in anesthetised animals by either electrical or chemical stimulation of the hypothalamic sites, triggers most of the effect observed in awake animals (Sévoz-Couche et al., 2003); examples include exophthalmos and whisker movements associated with blood flow redistribution among organs, and both hypertension and tachycardia in response to sympathetic activation (Bandler and Carrive, 1988; Bandler et al., 2000; Smith et al., 1990). The dorsolateral part of the periaqueductal grey (dIPAG) also plays a pivotal role in the defense reaction (Bandler and Carrive, 1988). The defense reaction is associated with an inhibition of the baro- and chemo-reflex bradycardia, via activation of nucleus tractus solitarius (NTS) 5-HT<sub>3</sub> receptors (Netzer et al., 2009, 2011). Both DMH and dIPAG acute and intense stimulation also mediate a tachypnea, possibly by direct projections to dorsal pons respiratory centers (Hayward and Castellanos, 2003; Johnson et al., 2008).

Clinical studies have shown that panic disorder is associated with symptoms that include palpitations, shortness of breath, sweating and hyperventilation (Abelson et al., 2010). High levels of anxiety-related behaviour in rats are also associated with an elevation of the resting respiratory rate (Carnevali et al., 2013), whilst an intense neonatal emotional stress such as maternal separation, triggers a long-term decrease in breathing rate during non-REM sleep (Kinkead et al., 2009). However, recent results have shown that social defeat (a validated model of repeated confrontation in rats inducing ultrasonic vocalisations and freezing) generates a decrease in basal respiratory frequency, mediated by low and long-term DMH-induced NTS 5-HT<sub>3a</sub> receptor stimulation (Brouillard et al., 2016). As a result of chemodenervation, basal breathing frequency is diminished (Hayward, 2001; Izumizaki et al., 2004). Chronic reduction of chemoreflex respiratory responses may occur in social defeat and be the origin of the long-term decrease in respiratory frequency (RF).

We have recently demonstrated that minimal DMH supra-threshold stimulation depresses the respiratory component of the peripheral chemoreflex induced by KCN administration (Zafar et al., 2015).

The purpose of the present study was to evaluate in rodents i) the role of the dIPAG in DMH-induced inhibition of the carotid respiratory response, and ii) the role of NTS 5-HT<sub>3a</sub> receptors in the inhibitory effect of dIPAG stimulation on the KCN-induced respiratory response.

## 2. Material and methods

### 2.1 General procedures

All animals were kept under controlled environmental conditions ( $22 \pm 1^\circ\text{C}$ ; 60% relative humidity; alternating circadian rhythms with light (7 h–19 h) and dark (19 h–7 h); access to food and water (*ad libitum*). Procedures involving animals and their care were all conducted in accordance with institutional guidelines, which are in compliance with European Directives (2010/63/EU). Experimental permission (N° 75855) was given by the Ministère de l'Agriculture et de la Forêt, Service Veterinaire de la Sante et de la Protection Animale, to C. Sévoz-Couche.

Experiments were mostly performed in male Sprague-Dawley rats weighing 330–350g (n=76, Breeding Center R. Janvier, Le Genest St.Isle, France). Some experiments were also performed using male homozygous 5HT<sub>3a</sub> KO (n=5) and wild-type (WT, n=5) littermates mice born from heterozygous mutants on a C57BL/6J genetic background (>10 generations), and genotyped as described by Zeitz (Zeitz et al., 2002). Animals were anaesthetised using urethane (1.5 g/kg, i.p.). The depth of anaesthesia was regularly assessed by pinching a hind paw and monitoring the stability of arterial blood pressure and heart rate recordings. Rectal temperature was maintained at 37°C using a thermostatically-controlled heating blanket. In all animals, polyethylene cannulas were introduced into the femoral artery for recording haemodynamic variables and controlling animal stability, and into the vein for administration of KCN or additional doses of urethane. In cases of withdrawal reflex and/or significant variations of these parameters, a supplementary dose of urethane was given (1 mg/kg, i.v.)

### 2.2 Respiratory measurements

The trachea was cannulated to monitor respiration (Zafar et al., 2015). The cannula was connected to a pneumotachograph (Fleisch 0000), which related to a volume transducer (Digitimer Neurolog, NL 905). Respiratory signals were relayed to a 1401 interface (1401 Plus, CED, UK) and processed by Spike 2 Software (6.14, CED, UK). Both RF and tidal volume ( $V_T$ ) were derived from the ventilatory flow signal.

### 2.3 DMH and dIPAH

Anaesthetised rats were placed in a stereotaxic frame, and the dorsal surface of the brainstem was exposed such that the skull was horizontal between bregma and lambda. Stimulations of the DMH and the dIPAG were performed in rats at the following stereotaxic coordinates (from bregma): AP -3.0, L 1.0, V 8 and AP -7.0, L 1.0, V 4, respectively (Sévoz-Couche et al., 2003). Mild ("minimal suprathreshold") DMH and dIPAG activation, sufficient to induce small modifications in heart rate (HR) and arterial blood pressure (ABP) but no arousal respiratory responses, was elicited by low (30  $\mu$ A) electrical stimulation (bipolar electrode, 15 sec, 50 Hz, 1 ms pulse duration). Because of the numerous GABAergic inputs these regions receive and in particular the DMH, slight chemical disinhibition seemed to be appropriate to produce minimal excitation (Zaretskaia et al., 2008). Thus we microinjected locally bicuculline methiodide to stimulate these regions but at a lower dose (5 pmol in 100 nl) than used before (Comet et al., 2004; Netzer et al., 2011). In WT and 5-HT<sub>3a</sub> KO mice, mild (20  $\mu$ A) electrical current (15 sec, 50 Hz, 1 ms pulse duration), sufficient to induce small cardiovascular modifications but no arousal respiratory responses, was applied to activate dIPAG (AP -4.2, L 0.5, V 1.8 mm from bregma).

Inhibition of the dIPAG in rats was obtained by bilateral microinjection of muscimol (500 pmol in 100 nl), a selective agonist of GABA<sub>A</sub> receptors (Sévoz-Couche et al., 2003). Sham electrical and chemical procedures were performed using zero electrical current or vehicle (saline) microinjection, respectively.

### 2.4 NTS microinjections

NTS 5-HT<sub>3</sub> receptors were activated by bilateral microinjection of m-chlorophenylbiguanide (CPBG, 0.1 to 1.6 nmol in 100 nl, Sigma Aldrich, St Louis, USA), or blocked

by bilateral microinjection of granisetron (125 and 250 pmol in 100 nl, SmithKline-Beecham, Harlow, UK), at the following coordinates from the calamus scriptorius (P -0.5, L 0.5 and V 0.5 mm) (Sévoz et al., 1997). In all cases, the microinjected vehicle was saline. Only one dose of CPBG or granisetron was microinjected per rat.

## 2.5 Carotid chemoreceptor reflex activation

This reflex was induced in rats and mice by injections of KCN (40 µg/rat and 4 µg/mice, 0.1 ml) into the femoral and jugular vein, respectively, at least 30 min after induction of anesthesia. Activation of carotid bodies results in hyperventilation, assessed by an increase in RF and  $V_T$ . KCN was given either during electrical (3 sec after the beginning of application) or after chemical (5 min after bicuculline microinjections) activation of the studied area, when the small cardiovascular modifications reached their maximal values. The time interval between tests was greater than 10 min to achieve reproductibility of chemoreflex respiratory responses. We verified that chemodenervation (bilateral resection of carotid bodies) abolishes respiratory responses, as we have demonstrated previously (Zafar et al., 2015).

## 2.6 Experimental design (Fig 1 and 2)

**Experiment 1: Does dIPAG blockade reverse the inhibitory action of low electrical (A) and chemical (B) stimulation of the DMH on KCN-induced respiratory responses in rats?**

1A. KCN was administered during sham or experimental DMH electrical stimulation in the same animal, either 10 min after intra-dIPAG vehicle or 10 min after intra-dIPAG muscimol.

1B. KCN was administered during sham or experimental DMH chemical stimulation in the same animal, 10 min after either intra-dIPAG vehicle or intra-dIPAG muscimol.

**Experiment 2: Does low electrical (A) or chemical (B) dIPAG stimulation exert an inhibitory action on KCN-induced respiratory responses in rats?**

2A. KCN was administered: firstly, during sham dIPAG electrical stimulation, then, in the same animal, during experimental dIPAG electrical stimulation.

2B. KCN was administered: firstly, 5 min after sham dIPAG chemical stimulation, then, in the same animal, 5 min after experimental dIPAG chemical stimulation (bicuculline microinjection).

**Experiment 3: Does NTS 5-HT<sub>3</sub> receptor inhibition reverse the negative influence exerted by low electrical stimulation of the dIPAG on KCN-induced respiratory responses in rats?**

KCN was administered during sham or experimental dIPAG electrical stimulation in the same animal, 10 min after either intra-NTS vehicle or intra-NTS granisetron.

**Experiment 4: Does the 5-HT<sub>3a</sub> receptor exert a role in the effects of low electrical activation of the dIPAG on KCN-induced respiratory responses in mice?**

4A. KCN was administered: firstly, during sham dIPAG electrical stimulation, then, in the same animal, during experimental dIPAG electrical stimulation in WT mice.

4B. KCN was administered: firstly, during sham dIPAG electrical stimulation, then, in the same animal, during experimental dIPAG electrical stimulation in genetically modified mice lacking the 5-HT<sub>3a</sub> receptor (5-HT<sub>3a</sub> KO).

**Experiment 5: Does NTS 5-HT<sub>3</sub> receptor activation exert an inhibitory effect on KCN-induced respiratory responses in rats?**

5A. KCN was administered in different animals 10 min after either intra-NTS vehicle or different doses of CPBG .

5B. KCN was administered: firstly, 10 min after intra-NTS vehicle, then, in the same animal, 10 min after intra-NTS CPBG preceded (2 min) by intra-granisetron.

## **2.7 Histological localization of microinjection sites**

At the end of the experiments, the animals were killed with an i.v. overdose of urethane. The brain was removed and fixed in 10% formalin solution and cryoprotected in 20% sucrose solution for 5 days. Microinjection sites were identified in 50 µm thick sections

of brain tissue by the the track of the micropipette in the DMH or deposit of Pontamine sky blue (Netzer et al., 2011) in the dIPAG and the NTS. The dye spread was inferior or equal to 500  $\mu\text{m}$ , as found previously (Comet et al., 2004, 2007; Sévoz-Couche et al., 1998).

## 2.8 Data Analysis

Normality was evaluated by the Shapiro-Wilk test. In rats and mice, respiratory responses to repeated KCN administrations in the same animal were compared using paired *t* test ANOVA for two, and one way RM ANOVA for three KCN injections. Multiple Scheffé's comparison was used to evaluate the dose-dependent effects of CPBG (one dose per animal) on KCN respiratory responses. The effects of active substances on baseline respiratory parameters in rats were performed using unpaired *t* test ANOVA. Comparisons of baseline respiratory parameters and KCN responses in WT and 5-HT<sub>3a</sub> KO mice were performed using unpaired *t* test ANOVA.

Analyses were performed using SigmaPlot 12.0. All results were considered significant if  $p < 0.05$ . All values are means  $\pm$  SEM.

## 3. Results

Basal cardiovascular and respiratory parameters of the rats and mice are noted in Table 1.

### **Experiment 1: Does dIPAG blockade reverse the inhibitory action of low electrical (A) and chemical (B) stimulation of the DMH on KCN-induced respiratory responses in rats?**

Microinjections of bicuculline into the DMH, and muscimol into the dIPAG, were performed in the following experiments. None of these treatments produced modifications in baseline cardiorespiratory parameters (Table 2).

Experiment 1A: When intra-dIPAG vehicle was used, the reflex increase in RF induced by intravenous administration of KCN was lower when induced simultaneously to minimal (30  $\mu\text{A}$ ) suprathreshold activation (mild electrical stimulation) of the DMH than during sham DMH activation ( $+10 \pm 2$  and  $+42 \pm 6$  cpm, respectively,  $p = 0.004$ ,  $n = 6$ , Fig 3A). The inhibitory effect of the DMH was prevented by muscimol microinjection into the dIPAG ( $p = 0.70$ , Fig

3A). In the same animals, the reflex increase in  $V_T$  was similar during sham or electrical DMH activation after intra-dIPAG vehicle ( $+1.3\pm 0.3$  and  $+1.1\pm 0.4$  ml, respectively,  $p=0.25$ ), and during electrical DMH activation after intra-dIPAG vehicle or muscimol ( $+1.4\pm 0.3$  and  $+1.3\pm 0.5$  ml, respectively,  $p=0.68$ , Fig 3A).

Experiment 1B: When intra-dIPAG vehicle was used,  $\Delta RF$  induced by KCN was lower when induced 5 min after bicuculline injection into the DMH vs vehicle ( $-5\pm 2$  and  $+38\pm 4$  cpm, respectively,  $p=0.001$ ,  $n=6$ , Fig 3B). This inhibitory effect induced by DMH activation was prevented when muscimol was microinjected into the dIPAG ( $p=0.0005$ , Fig 3B). In the same animals, the reflex increase in  $V_T$  was similar during sham or chemical DMH activation after intra-dIPAG vehicle ( $+1.42\pm 0.21$  and  $+1.21\pm 0.21$  ml, respectively,  $p=0.65$ ), and during chemical DMH activation after intra-dIPAG vehicle or muscimol ( $+1.2\pm 0.4$  and  $+1.4\pm 0.2$  ml, respectively,  $p=0.65$ ) (Fig 3B). Representative responses of RF and  $V_T$  to KCN administration during these different experimental conditions are shown in Fig 4 and a representative microinjection into the dIPAG is shown in Fig 5.

### **Experiment 2: Does low electrical (A) and chemical (B) dIPAG stimulation exert an inhibitory action on KCN-induced respiratory responses in rats?**

Experiment 2A: The magnitude of  $\Delta RF$  was smaller when KCN was administered simultaneously minimal suprathreshold activation (mild electrical stimulation) of the dIPAG vs no passing current ( $-7\pm 5$  and  $+34\pm 4$  cpm, respectively,  $p<0.001$ ,  $n=5$ , Fig 6A). In the same animals, the increase in  $V_T$  due to KCN administration was similar during both conditions ( $+1.32\pm 0.21$  and  $+1.34\pm 0.20$  ml, respectively,  $p=0.45$ , Fig 6A).

Experiment 2B: Reflex  $\Delta RF$  was smaller when KCN was administered 5 min after intra-dIPAG bicuculline (Fig 6B), than by vehicle ( $-2\pm 1$  and  $+34\pm 5$  cpm, respectively,  $p<0.001$ ,  $p<0.001$ ,  $n=7$ ). In the same animals, reflex  $\Delta V_T$  was similar whether KCN was administered after intra-dIPAG bicuculline or by vehicle ( $+0.94\pm 0.10$  and  $+1.07\pm 0.18$  ml, respectively,  $p=0.07$ , Fig 6B). Microinjections of bicuculline into the dIPAG didn't produce any modifications in baseline cardiorespiratory parameters (Table 2).

### **Experiment 3. Does NTS 5-HT<sub>3</sub> receptor inhibition reverse the negative influence exerted**

**by low electrical stimulation of the dIPAG on KCN-induced respiratory responses in rats?**

Microinjections of granisetron 125 and 250 pmol into the NTS were performed in the following experiments, and produced no modifications in baseline cardiorespiratory parameters (Table 2). Compared to sham, electrical dIPAG stimulation induced an inhibition of control KCN-induced  $\Delta$ RF after intra-NTS vehicle ( $+5\pm 8$  and  $+58\pm 8$  cpm, respectively,  $p=0.002$ ,  $n=6$ , Fig 7A) that was not reversed after intra-granisetron at the dose of 125 pmol (Fig 7A). The reflex increase in  $V_T$  was not statistically different between control and electrical dIPAG activation after intra-NTS vehicle ( $p=0.75$ ), as well as during electrical dIPAG activation after intra-dIPAG vehicle or granisetron 125 pmol ( $p=0.38$ ). In a second series of experiments, the inhibitory effect of chemoreflex  $\Delta$ RF response during dIPAG stimulation compared to sham after intra-NTS vehicle administration ( $+2\pm 3$  and  $+48\pm 10$  cpm, respectively,  $p<0.001$ ) was prevented when NTS 5-HT<sub>3</sub> receptors were blocked by granisetron at a dose of 250 pmol ( $p=0.70$ ,  $n=6$ , Fig 7B). The reflex increase in  $V_T$  was not statistically different between control and electrical dIPAG activation after intra-NTS vehicle ( $p=0.78$ ), and during electrical dIPAG activation after intra-NTS vehicle or granisetron 250 pmol ( $p=0.48$ ). Representative responses of RF and  $V_T$  to KCN administration after intra-NTS vehicle or granisetron 250 pmol are shown in Fig 8.

**Experiment 4. Does the 5-HT<sub>3a</sub> receptor exert a role in the effects of low electrical activation of the dIPAG on KCN-induced respiratory responses in mice?**

There was no statistical difference in basal RF ( $p=0.35$ ) and  $V_T$  ( $p=0.16$ ), nor in KCN respiratory responses (RF:  $p=0.75$  and  $V_T$ :  $p=0.32$ ), between WT and 5-HT<sub>3a</sub> KO mice. The magnitude of the reflex  $\Delta$ RF was smaller when KCN was administered during mild electrical dIPAG stimulation vs no passing current (sham) in WT (Experiment 4A,  $+10\pm 2$  and  $+30\pm 3$  cpm, respectively,  $p=0.008$ ,  $n=5$ ; Figs 9A and 10A), but not in 5-HT<sub>3a</sub> KO ( $+24\pm 2$  and  $+22\pm 3$  cpm, respectively,  $p=0.62$ ,  $n=5$ ; Figs 9B and 10B) mice. In the same animals, when KCN was administered,  $\Delta V_T$  was similar under both experimental conditions, either in WT ( $+0.50\pm 0.09$  and  $+0.35\pm 0.11$  ml, respectively,  $p=0.17$ ; Fig 9A and 10A) or KO ( $+0.37\pm 0.07$  and  $+0.48\pm 0.11$  ml, respectively,  $p=0.08$ , Fig 9B and 10B) mice.

**Experiment 5. Does NTS 5-HT<sub>3</sub> receptor activation exert an inhibitory effect on KCN-**

### induced respiratory responses in rats?

Experiment 5A: Microinjections into the NTS (Fig 3B) of vehicle (n=5) or different doses of CPBG (0.1 to 1.6 nmol, n=5 each) were performed in the following experiments. None of these treatments produced modifications in RF, VT and HR but induced an increase in ABP when the dose was equal or superior to 0.8 nmol (Table 2). Compared to vehicle, activation of NTS 5-HT<sub>3</sub> receptors by CPBG ( $\geq 0.4$  nmol) produced a dose-dependent inhibitory effect on KCN-induced  $\Delta$ RF (Fig 11), with a maximal effect at 0.4 nmol (Fig 11 and 12). In animals that received vehicle and CPBG (0.4 nmol), KCN-induced increase in  $V_T$  was comparable under both conditions ( $+1.22 \pm 0.11$  and  $+0.77 \pm 0.10$  ml, respectively,  $p=0.7$ ; Fig 12).

Experiment 5B: After prior intra-NTS microinjection of granisetron at a dose of 250 pmol but not 125 pmol, the KCN-induced increase in RF both after CPBG (0.4 nmol) and vehicle administration ( $+35 \pm 3$  and  $+38 \pm 8$  cpm,  $+30 \pm 3$  and  $+41 \pm 8$  cpm, respectively,  $p=0.34$ ,  $n=5$ ; respectively,  $p=0.78$ ,  $n=5$ ).

## 4. Discussion

The carotid body contains specialised type I cells that contain oxygen sensors. Type I cells depolarise in response to hypoxia, but also respond to hypercapnia, changes in pH, temperature, osmolality and glucose, and are important in promoting arousal (Izumizaki et al., 2004). Stimulation of these cells may also be obtained by intra-venous administration of KCN (Netzer et al., 2009). Cyanide (CN<sup>-</sup>) is toxic due to its chemical binding to cytochrome *c* oxidase, which blocks the mitochondrial electron transport chain and subsequently inhibits tissue aerobic respiration (Isom et al., 1982). Transient hypoxia and cyanide stimulation of the carotid bodies cause similar neural responses in cats and rats, including a profound activation of the minute ventilation and phrenic nerve discharge (Gonzalez et al., 1977; Izumizaki et al., 2004; Koshiya and Guyenet, 1994; Purves, 1966), and their excision causes chronic hypoventilation (Hayward, 2001; Olson et al., 1988).

Chemosensitive neurons in the medullary retrotrapezoid nucleus (RTN) are part of the ventral respiratory component (VRC) in the central pattern respiratory generator (CPG),

and are in contact with rythmogenic (pre-Bötzing complex) and pre-motor neurons (Smith et al., 2013). The RTN receives inputs from NTS second-order neurons in contact with peripheral chemosensitive afferents, and seems to be responsible for the production of the reflex increase in ventilation (Takakura et al., 2006). In addition to the RTN, neurons in the DMH seem to facilitate and/or participate, at least in part, to the increase in frequency respiratory chemoreflex response (Kinkead et al., 2005; Silva et al., 2015) (Fig 13). Some studies investigating the effects of total disinhibition of neurons within the DMH (activation of the defense reaction) concluded that this procedure induces an increase in respiratory rate (McDowall et al., 2007; Reynolds et al., 2008). In this respect, the region of the DMH and the closely related perifornical area (PeF), are essential for generation of respiratory (tachypnea) responses to both stressful and alerting stimuli (Bondarenko et al., 2015). A hypothalamo-ponto-medullary pathway (HPM) seems to be involved in this response (Fig 13). Kölliker-Fuse and the lateral parabrachial nuclei receive projections from the DMH (Johnson et al., 2012; Peyron et al., 1998; Yokota et al., 2016) and form the pontine respiratory group, the activation of which evokes tachypneic or apneic responses (Chamberlin and Saper, 1994) through efferents to the VRC, including the RTN (Feldman et al., 2003; Smith et al., 2013).

On the other hand, the DMH is also involved in the production of long-term bradypnea after social defeat (Brouillard et al., 2016). Considering that a reduction in chemoreflex tachypneic response induces a long-term hypoventilation (Hayward, 2001; Roux et al., 2000), we hypothesised that DMH may exert a long-term negative influence on the peripheral chemoreflex to reduce baseline respiratory values after stress condition. Accordingly, we previously showed that mild DMH activation reduces the tachypnea (but not the increase in  $V_T$ ) induced by KCN administration in male rats (Zafar et al., 2015). To note, . As the dIPAG intervenes downstream of the DMH to block the baroreflex cardiac response (Sévoz-Couche et al., 2003), we also hypothesised that dIPAG may be a relay in the DMH-induced negative effect on KCN-induced tachypnea (Lopes et al., 2014). Accordingly, chemical blockade of the dIPAG by muscimol in urethane-anesthetised rats prevented the negative modulation of the KCN-induced increase in RF exerted by DMH low activation. Our findings show that electrical and chemical minimal sub-threshold dIPAG activation almost completely abolished the KCN-induced increase in RF (but not the increase in  $V_T$ ).

Cardiovascular modifications induced by KCN were not analysed during mild DMH or dIPAG stimulation because higher intensity of stimulation is necessary to produce an effect on these responses (Netzer et al., 2009).

Social defeat induces long-term bradypnea via NTS 5-HT<sub>3</sub> receptor activation (Brouillard et al., 2016). This suggests that these receptors may play a crucial role in the inhibition of the chemoreflex tachypnea. In the present study, we showed that granisetron blocked the dIPAG-induced negative effect on chemoreflex tachypnea in rats. In addition, specific activation of NTS 5-HT<sub>3</sub> receptors by CPBG blocked the tachypnea induced by KCN administration in a dose-dependent manner. Moreover, this inhibitory effect of CPBG was prevented by a specific antagonist, granisetron. To confirm the key role of these receptors in the dIPAG inhibitory effect, we used genetically modified mice lacking 5-HT<sub>3a</sub> receptors. To our knowledge, there is no published report on ventilation in 5-HT<sub>3a</sub> KO mice. Both WT and 5-HT<sub>3a</sub> KO mice exhibited a significant lower V<sub>T</sub> than rats. The respiratory rate was higher in mice, albeit not significantly. WT and 5-HT<sub>3a</sub> KO mice had similar basal respiratory parameters, and KCN responses did not differ during baseline conditions. Inhibition of KCN-induced change in RF in WT, but not 5-HT<sub>3a</sub> KO mice, was triggered by mild dIPAG activation. These results demonstrate that 5-HT<sub>3a</sub> receptors are involved in dIPAG-induced reduction of KCN-induced respiratory responses, but are not tonically activated.

To explain these results, we propose the following neuronal pathway: during stress involving the DMH, two clusters of neurons (A and B, see Fig 13) are activated in the DMH. The cluster A is at the origin of the HPM circuit, responsible for the defense and alerting tachypnea. On the other hand, the cluster B is the origin of a hypothalamo-midbrain-medullary (HMM) pathway. Stimulation of the cluster B may activate the dIPAG. Direct projections have not been evidenced; the cuneiformis nucleus may be involved as an intermediary structure as it is reciprocally connected to both structures (Bernard et al., 2008). The B3 region, and in particular the raphe magnus, receives massive projections from the dIPAG (Fardin et al., 1984) that, in turn, provides a significant set of serotonergic projection to the NTS (Schaffar et al., 1988; Thor and Helke, 1987). Thus dIPAG activation may be at the origin of serotonin release into the NTS, to ultimately activate presynaptic 5-HT<sub>3a</sub> receptors (Hosford et al., 2014), as found previously (Bernard et al., 2008). The

activation of these receptors is at the origin of vagal presynaptic glutamatergic release into the NTS (Ashworth-Preece et al., 1995), and glutamate acts on GABAergic interneurons embedded with excitatory amino acid receptors (Callera et al., 1997; Sévoz et al., 1996). In turn, GABA inhibits second-order carotid chemoreflex neurons through activation of GABA<sub>A</sub> receptors, and may block tachypneic chemoreflex responses as it was found previously for bradycardic chemoreflex responses in awake and anesthetized rats (Sévoz et al., 1997). We hypothesized that, during stress, not only the HPM influence occurs to increase respiration, but the HMM pathway is activated to trigger a reduction in the chemoreflex RF produced from NTS second-order neurons by direct or indirect (activation of the HPM circuit) RTN cell activation. This inhibitory effect of the DMH and dPAG is observed only under low electrical and chemical activation. It may depend on a functional subset of neurons in the DMH that is: i) sensitive to low depolarisation, or ii) smaller than that responsible for the pathway at the origin of the defense tachypnea (Fig 13). To our point of view, the HMM inputs may contain increases in RF if the peripheral chemoreflex occurs punctually while an anxious state is already present, to avoid panick attacks for exemple.

#### *Conclusion and Clinical significance*

This study shows that DMH reduction of the tachypneic response to peripheral chemoreflex activation involves dPAG and NTS 5-HT<sub>3a</sub> receptor activation.

Failure to respond to hypoxia has been associated with early disturbances of respiratory control, including sudden infant death syndrome (SIDS) (Arata et al., 2013; McCulloch et al., 1982). In particular, decreased excitatory inputs from the peripheral arterial chemoreceptors to the second-order NTS neurons, result in reduced central drive to the muscles of respiration, predisposing to upper airway collapse during sleep (Gauda et al., 2007). A deficit in central chemoreception is commonly described in congenital central hypoventilation syndrome (CCHS) by a lack of Phox2B chemosensitive cells in the RTN (Bayliss et al., 2015; Guyenet et al., 2008). If it is generally accepted that CCHS patients have intact ventilatory responses to abrupt hypoxia, hypercapnia and hyperoxic challenges, indicating that peripheral chemoreceptor function is preserved in those who are able to sustain adequate ventilation during wakefulness (Gozal et al., 1993), some studies suggest that peripheral chemoreception may be affected for those who are not (Paton et al., 1989; Shea 1993). A dysfunctional peripheral chemoreception in CCHS was also mentioned by Shea

and collaborators (Shea et al., 1993). A pivotal role for NTS 5-HT<sub>3a</sub> receptors in the DMH-induced reduction of vagal activity was evidenced earlier (Sévoz-Couche et al., 2003).

As SIDS and CCHS patients have a low vagal activity (Trang et al., 2005; White et al., 1993) and a reduced peripheral chemoreception, we suggest that the HMB neurocircuitry and especially NTS 5-HT<sub>3a</sub> receptors may be a new target to prevent possible negative ventilatory and vagal outcomes in sub-groups of SIDS or CCHS.

#### Author contributions

The experimental team of Unit 1158 is involved in the automatic control of respiration. Caroline Sévoz-Couche is the principal coordinator of the projects on the modulatory effects of stress on reflex-induced modifications of respiration, and this study is part of these projects. Tabinda Zafar and Charly Brouillard performed the experiments, and Laurence Lanfumey is responsible for the mice breeding.

#### Acknowledgement

We would like to thank Brigitte Quenet for critical reading of the manuscript. This work was supported by Legs Poix (LEG1406).

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**Figure legends**

Figure 1.

Experimental design used to evaluate the role of the dIPAG (Experiments 1 and 2) on DMH-induced negative influence on KCN respiratory responses.

Elec: electrical activation; Bic: bicuculline; Musc: muscimol

Figure 2.

Experimental design used to evaluate the role of the NTS 5-HT<sub>3a</sub> receptor on dIPAG-induced negative influence on KCN respiratory responses (Experiments 3 to 5).

CPBG: m-chlorophenybiguanide; Grani: granisetron

Figure 3.

A and B. Box-Whisker graphs with min and max values (lines are the medians), showing the effect of intra-dIPAG vehicle (veh) or muscimol (musc) on the reduction of KCN-induced  $\Delta$ RF and  $\Delta$ V<sub>T</sub>, exerted by DMH electrical (A) and chemical (B) activation. Values are means $\pm$ sem.

\*\* $p$ <0.01 versus Sham DMH, ## $p$ <0.01 and ### $p$ <0.001 versus intra-dIPAG veh.

Figure 4.

Representative traces of respiratory responses induced by KCN administration (arrows) at baseline (i.e. during sham DMH, top) and during low chemical DMH activation (middle) by bicuculline (0.05 mM); the DMH-induced inhibitory effect on chemoreflex tachypnea was reversed by dIPAG blockade with bicuculline (5mM). HR: heart rate, ABP: differential arterial blood pressure.

Figure 5.

Photographs displaying the dIPAG (A) and NTS (B) sites (pontamine sky blue deposit at the very tip of the micropipette track, marked by the asterisks), at approximately -6.8 mm and -14.2 mm from bregma, respectively.

3N: oculomotor nucleus ; 10N: dorsal motor nucleus of vagus; 12N: hypoglossal nucleus; AP: area postrema; cc: central canal; CuR: cuneate nucleus (rotandus part); dIPAG: dorsolateral

column of the periaqueductal gray area; dmPAG: dorsomedial column of the periaqueductal gray area; Gr: gracile nucleus; IPAG: lateral column of the periaqueductal gray area; Sol: nucleus of the solitary tract;

Figure 6.

A and B. Box-Whisker graphs with min and max values (lines are the medians), showing the effect of sham (no passing current) or experimental (exp, passing current) electrical (A), and sham (vehicle) or experimental (bicuculline) chemical (B) dIPAG activation, on KCN-induced changes in tracheal breath frequency ( $\Delta RF$ ) and tidal volume ( $\Delta V_T$ ). Values are means  $\pm$  sem. \*\*\* $p < 0.001$  versus sham.

Figure 7.

A and B. Box-Whisker graphs with min and max values (lines are the medians), showing the effect of intra-NTS vehicle (veh) or granisetron (grani) at the dose of 125 (A) and 250 pmol (B) on the reduction of KCN-induced  $\Delta RF$  and  $\Delta V_T$ , exerted by DMH electrical activation. Values are means  $\pm$  sem. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus Sham dIPAG, # $p < 0.01$  and ### $p < 0.001$  versus intra-NTS veh.

Figure 8.

Representative traces of respiratory responses induced by KCN administration (arrows) at baseline (i.e. during sham dIPAG, top) and during low electrical dIPAG activation (middle); the dIPAG-induced inhibitory effect on chemoreflex tachypnea was reversed by NTS 5-HT<sub>3</sub> receptor blockade with granisetron (250 pmol).

Figure 9.

Box-Whisker graphs with min and max values (lines are the medians), showing the effect of sham (no passing current) or experimental (exp, passing current) dIPAG electrical activation on KCN-induced changes in RF and  $V_T$  in wild-type (WT, top) and 5-HT<sub>3a</sub> KO (bottom) mice. Values are means  $\pm$  sem. \*\* $p < 0.01$  versus sham.

Figure 10.

Representative responses of RF and  $V_T$  to KCN administration (arrows) during sham (no passing current) or experimental (exp, passing current) dIPAG electrical stimulation, in WT (A) or 5-HT<sub>3a</sub> KO (B) mice.

Figure 11.

Dose-dependent inhibition of peripheral chemoreflex tachypnea by various doses (0.1 to 1.6 nmol) of intra-NTS CPBG. Values are means $\pm$ sem. \* $p$ <0.05 and \*\* $p$ <0.01 vs intra-NTS veh; # $p$ <0.05 versus lower dose.

Figure 12.

Representative responses of RF and  $V_T$  to KCN administration (arrows), after intra-NTS vehicle (sham chemical stimulation) or CPBG (0.4 nmol, experimental chemical stimulation).

Figure 13.

Hypothetical schematic representation of the neurocircuitry involved in respiratory modulation by the DMH.

NTS second-order carotid chemoreflex neurons (grey cell) project to the DMH (Silva et al., 2015) and to the medullary ventral respiratory component (VRC), in particular to the retrotrapezoid nucleus (RTN) (Takakura et al., 2006). RTN neurons activate rhythmogenic neurons (to increase RF) and pre-motor neurons (to increase the tidal volume) (Takakura et al., 2006).

Firstly, DMH neurons ("A") are associated with the pontine formation (Hayward and Castellanos, 2003; Johnson et al., 2008; Krout et al., 1998) which, in turn, is in contact with the RTN (Damasceno et al., 2015; Song et al., 2011). During alerting stimuli, this Hypothalamo-Ponto-Medullary or HPM circuit (grey pathway) induces the typical defense respiratory response (tachypnea and increase in tidal volume). This pathway may also participate, at least in part, to the peripheral chemoreflex ventilatory response by direct projections from the NTS to the DMH.

Secondarily, DMH "B" cells are projecting to the cuneiform nucleus (CnF) and their activation

results in the stimulation dIPAG neurons . This circuit may be the origin of serotonin release into the NTS (presumably from the B3 region (Netzer et al., 2011)) to ultimately activate presynaptic 5-HT<sub>3a</sub> receptors located pre-synaptically on vagal afferents (white pathway). Activation of these receptors induces the presynaptic release of glutamate that stimulates GABAergic interneurons (black cell). Finally, GABA acts on GABA<sub>A</sub> receptors present on NTS second-order carotid chemoreflex neurons to block (black crosses) chemoreflex inputs to both DMH "A" and RTN cells. The activation of this Hypothalamo-Midbrain-Medullary circuit (HMM), ultimately reduces the chemoreflex respiratory response (black cross) presumably to prevent maximum RF levels during stress alert. Of note, RTN cells receiving chemoreflex inputs inhibited by the HMM circuit may be associated with rythmogenic cells, but not pre-motor cells in the VRC.

We suggest that during alerting stimuli, both HMM and HPM are activated in parallel; during a low stress state HMM alone is activated because only a low DMH activation occurs and may participate in the establishment of long-term stress-induced bradypnea.

**Table 1****Basal respiratory parameters in rats and mice**

	<b>Basal RF (cpm)</b>	<b>Basal V<sub>T</sub> (ml)</b>	<b>Basal HR (bpm)</b>	<b>Basal ABP (mmHg)</b>
<b>Rat (n=76)</b>	109±4	2.15±0.10	355±10	100±5
<b>wt (n=5)</b>	124±5	0.70±0.10	510±12	90±7
<b>ko (n=5)</b>	127±11	0.60±0.03	540±15	85±5

Values are the mean ±sem

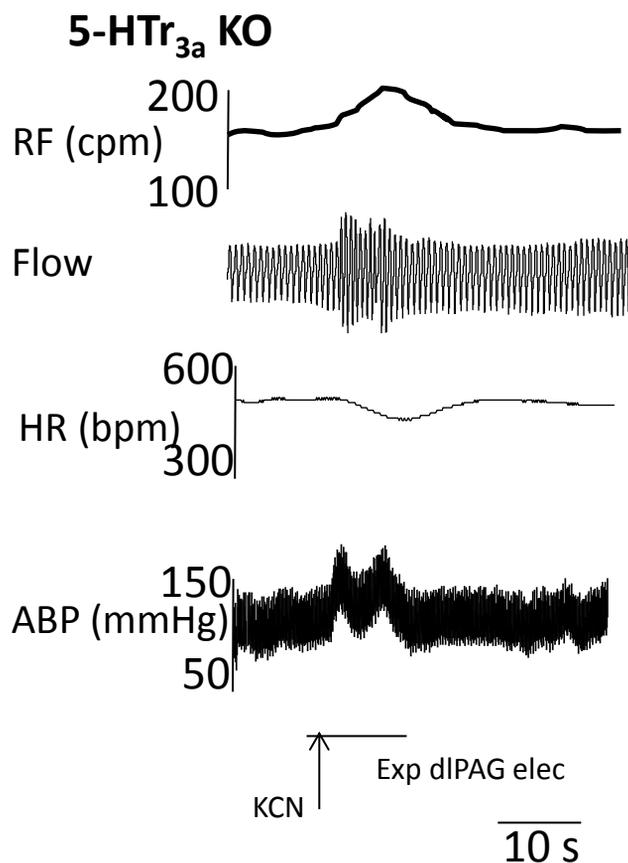
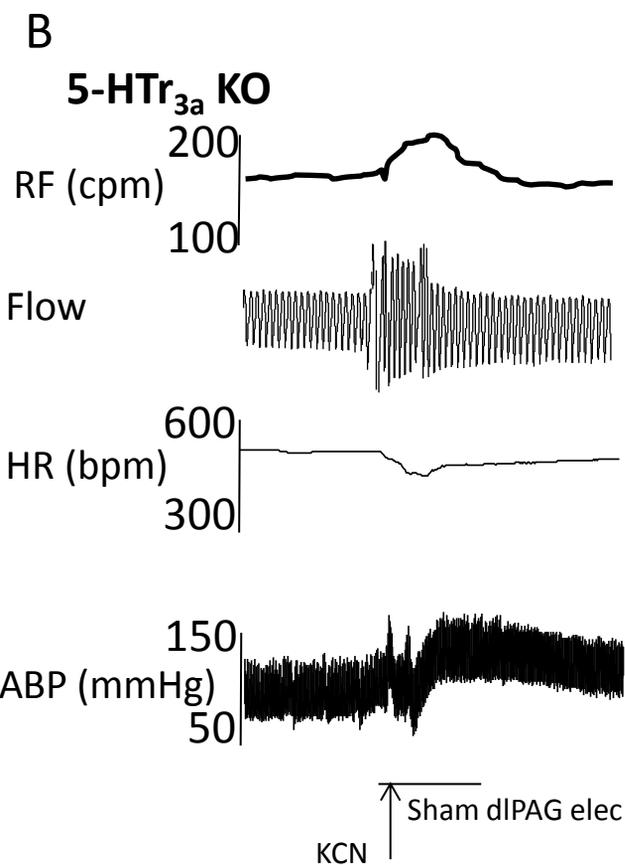
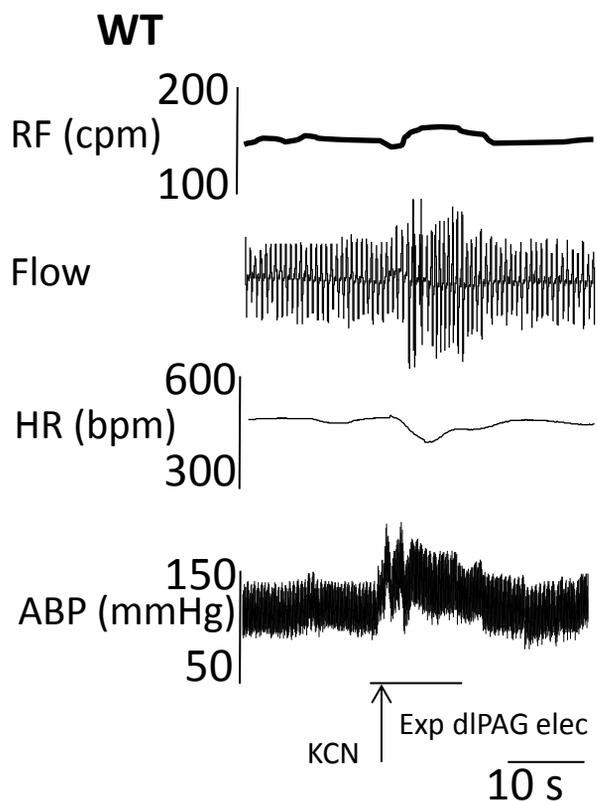
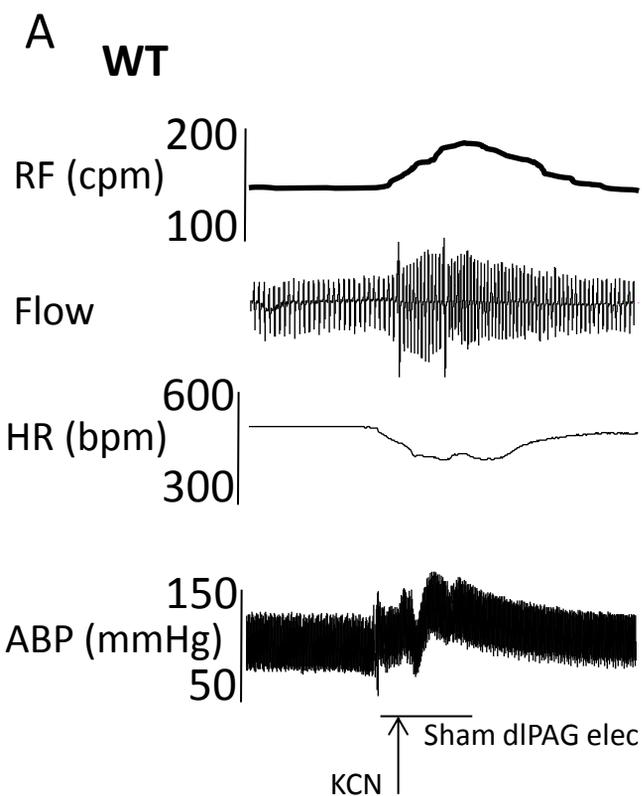
**Table 2**

Variations in RF and VT produced by microinjections in the rats of active substances in the DMH, dIPAG and NTS.

<b>Localisation</b>	<b>Treatment</b>	<b>ΔRF (cpm)</b>	<b>ΔVT (ml)</b>	<b>ΔHR (bpm)</b>	<b>ΔABP (mmHg)</b>
<b>DMH</b>	Vehicle (n=6)	+4±2	+0.1±0.2	+6±2	+4±2
	Bicuculline 5 pmol (n=6)	+4±3	+0.4±0.2	+15±8	+8±5
<b>dIPAG</b>	Vehicle (n=6)	+1±2	+0.2±0.2	+5±2	+4±3
	Bicuculline 5 pmol (n=19)	+6±3	+0.3±0.2	+10±5	+10±5
	Muscimol 500 pmol (n=12)	-1±3	-0.4±0.2	+7±5	+5±5
<b>NTS</b>	Vehicle (n=36)	+3±2	+0.2±0.2	+4±2	+3±2
	Granisetron 125 pmol (n=6)	+5±2	+0.2±0.1	+5±4	+8±5
	Granisetron 250 pmol (n=11)	+6±3	+0.3±0.1	+5±8	+8±5
	CPBG 0.1 nmol (n=5)	+3±2	+0.2±0.1	+8±2	+8±2
	CPBG 0.2 nmol (n=5)	+4±3	+0.4±0.1	+5±5	+9±3
	CPBG 0.4 nmol (n=5)	-3±3	+0.5±0.1	+10±8	+10±5
	CPBG 0.8 nmol (n=10)	-6±2	+0.5±0.1	+12±5	+21±5
	CPBG 1.6 nmol (n=5)	-5±3	+0.4±0.3	+5±4	+28±6

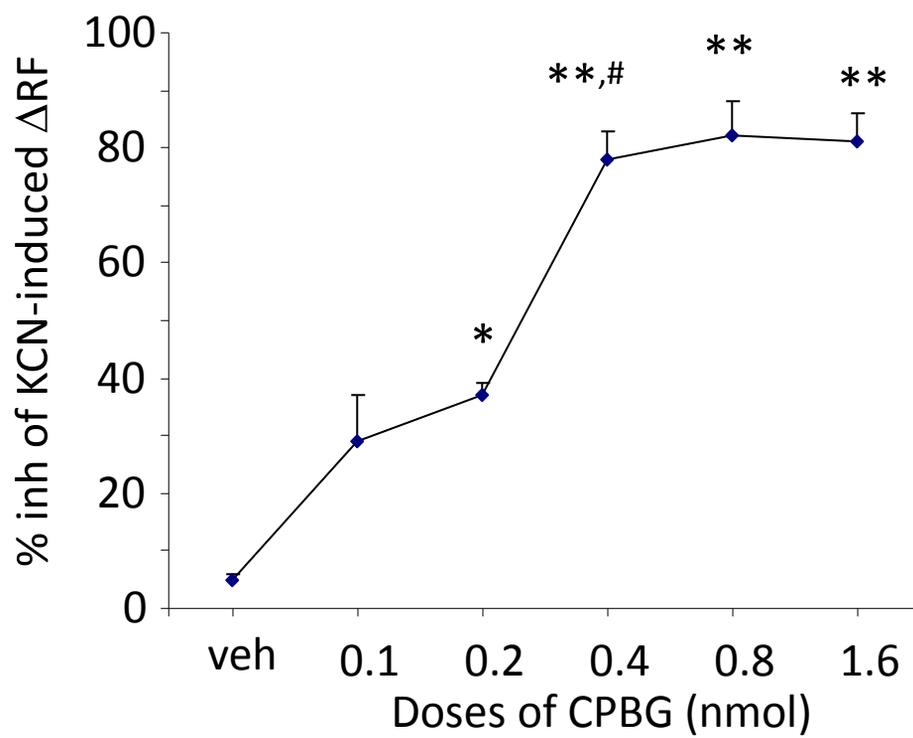
Values are the mean ±sem in n animals

# Experiment 4



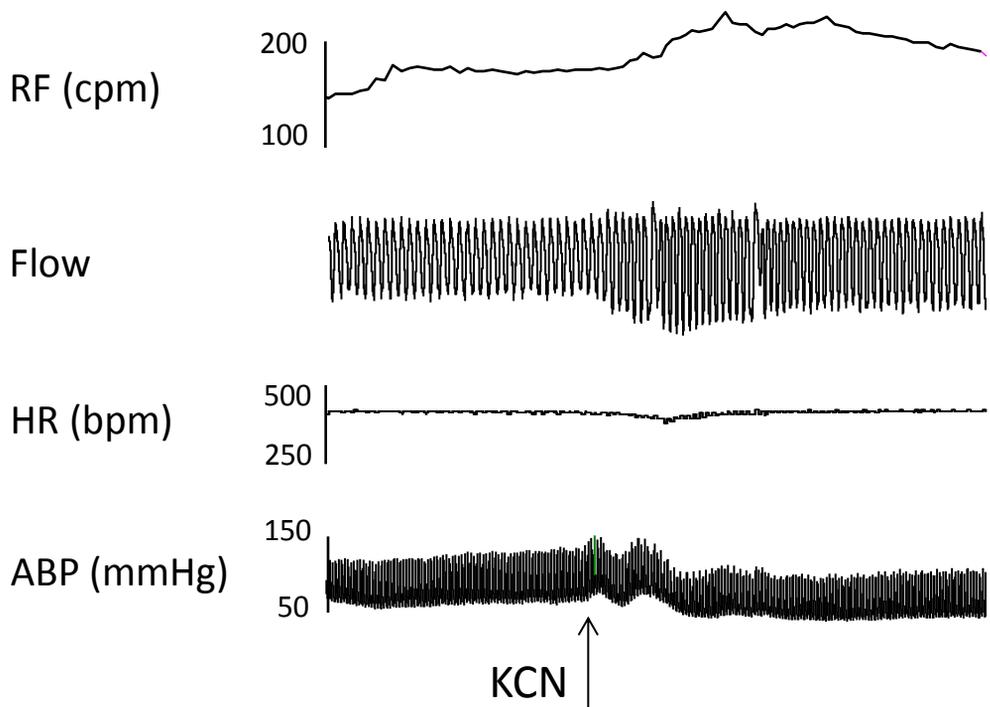
# Experiment 5

A

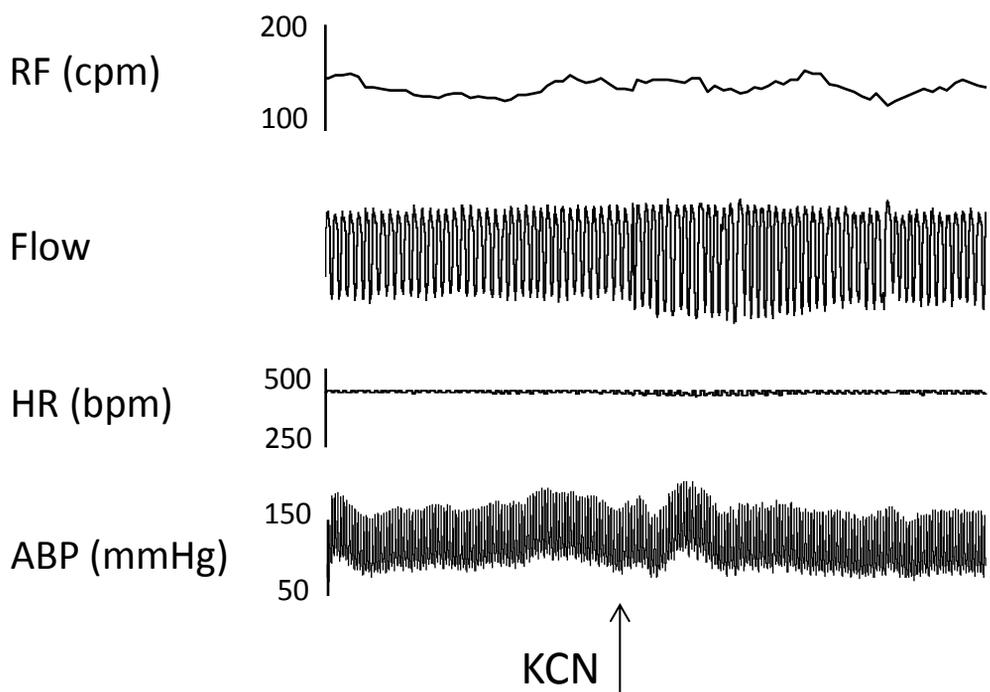


# Experiment 5

## *After intra-NTS vehicle*

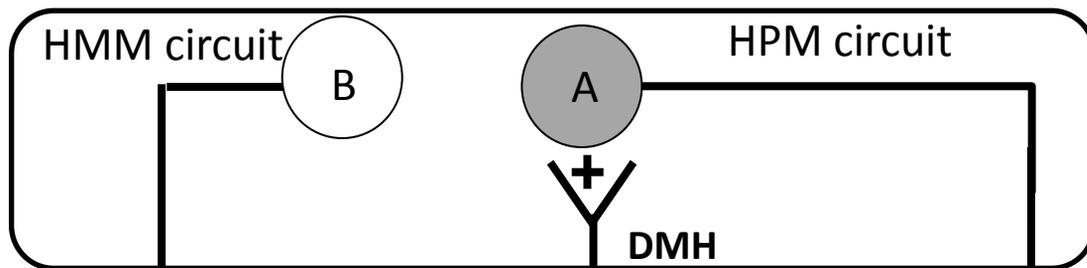


## *After intra-NTS CPBG (0.4 nmol)*

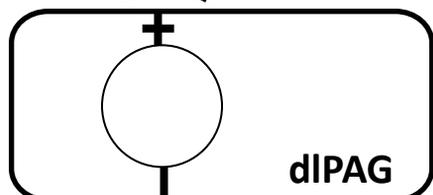
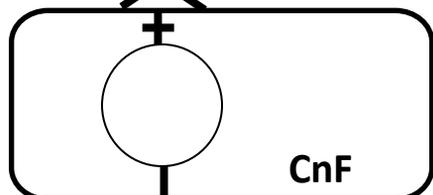


15 sec

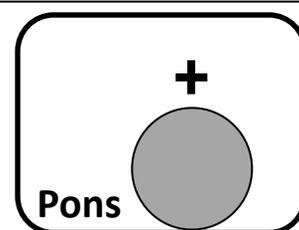
Hypothalamus



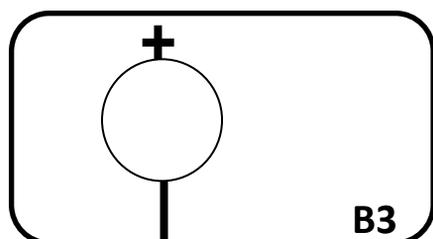
Midbrain



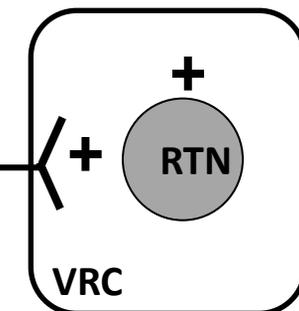
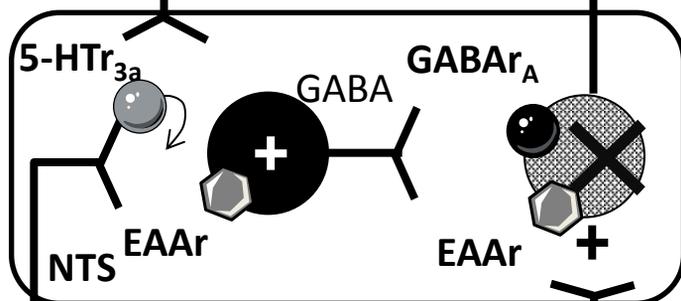
Pons



Medulla Oblongata



5-HT



Glu

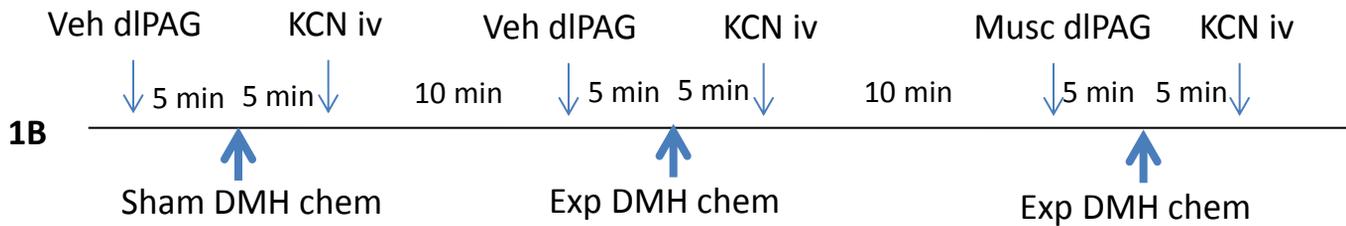
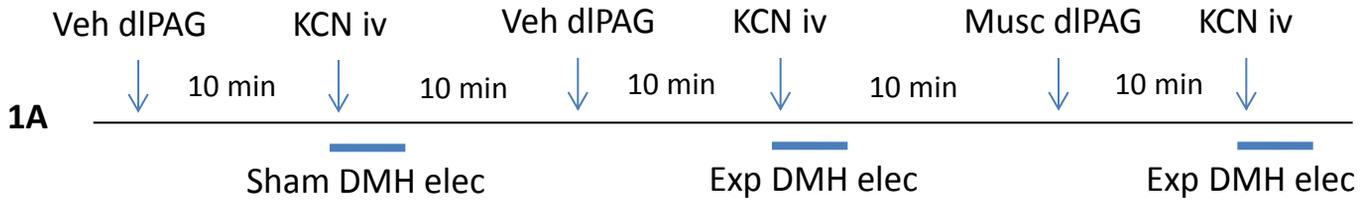
Glu

gastrointestinal  
vagal afferents

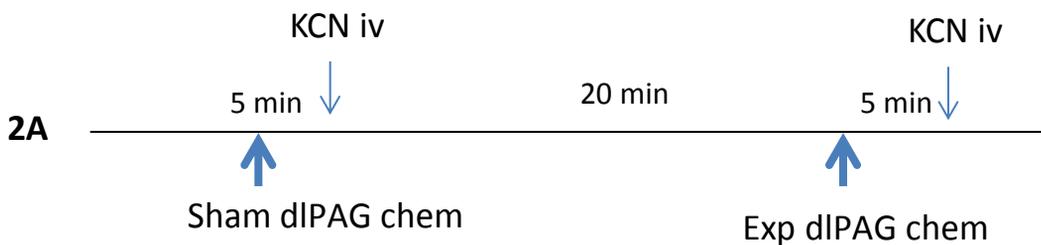
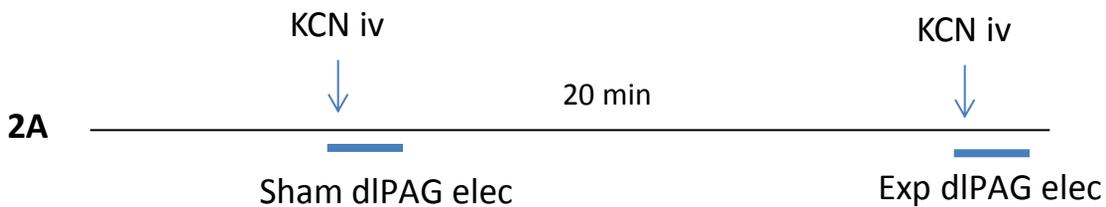
carotid chemoreflex  
afferents

Alerting and  
carotid chemoreflex  
hyperventilation

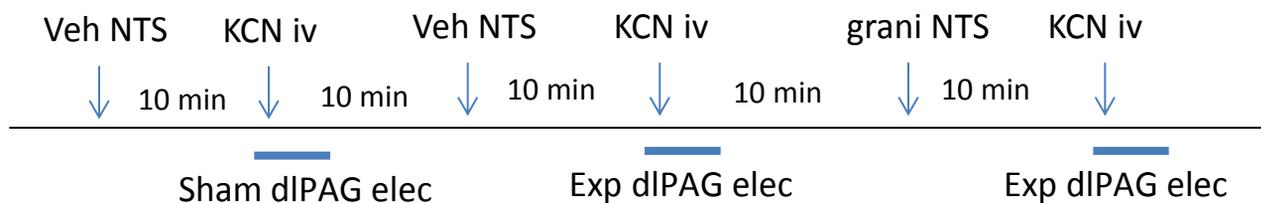
**-Experiment 1: Does dIPAG blockade reverse the inhibitory action of low electrical (A) and chemical (B) stimulation of the DMH on KCN-induced respiratory responses in rats?**



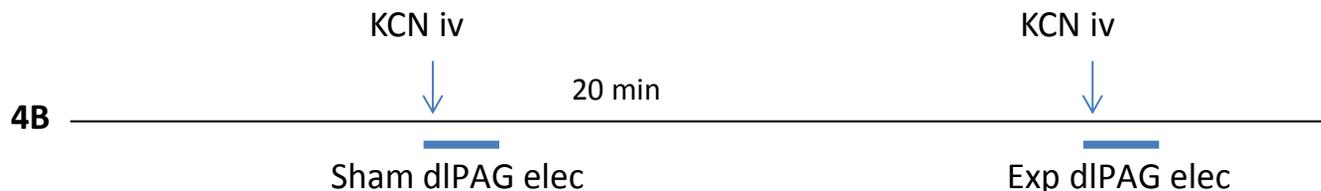
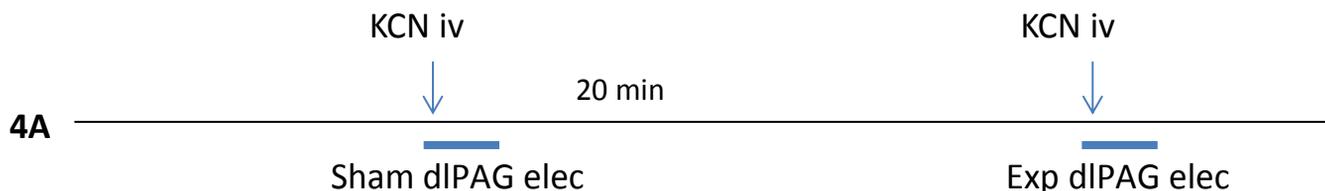
**-Experiment 2: Does low electrical (A) or chemical (B) dIPAG stimulation exert an inhibitory action on KCN-induced respiratory responses in rats?**



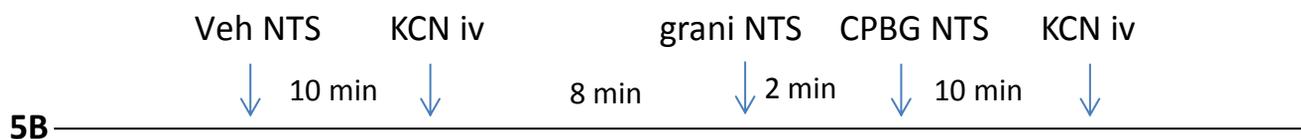
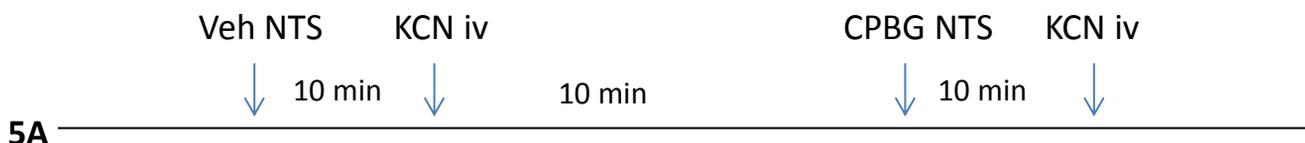
**-Experiment 3: Does NTS 5-HT<sub>3</sub> receptor inhibition reverse the negative influence exerted by low electrical stimulation of the dIPAG on KCN-induced respiratory responses in rats?**



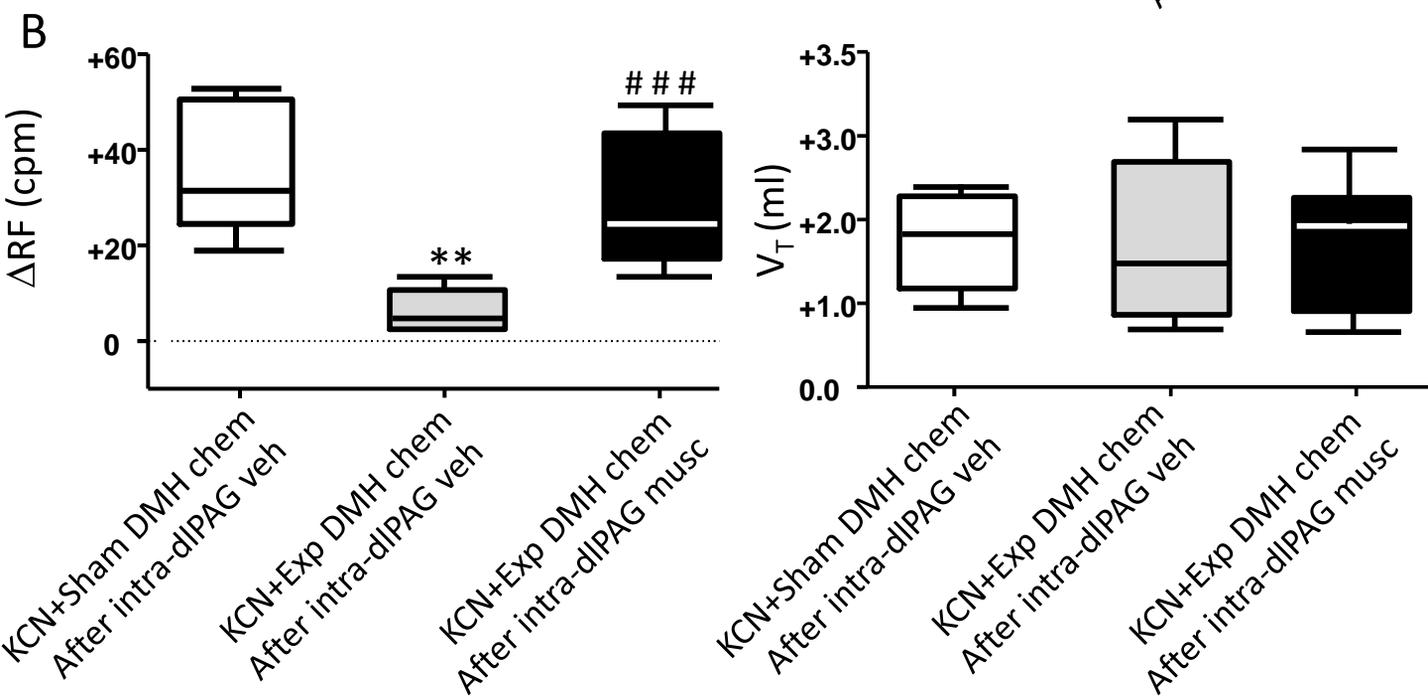
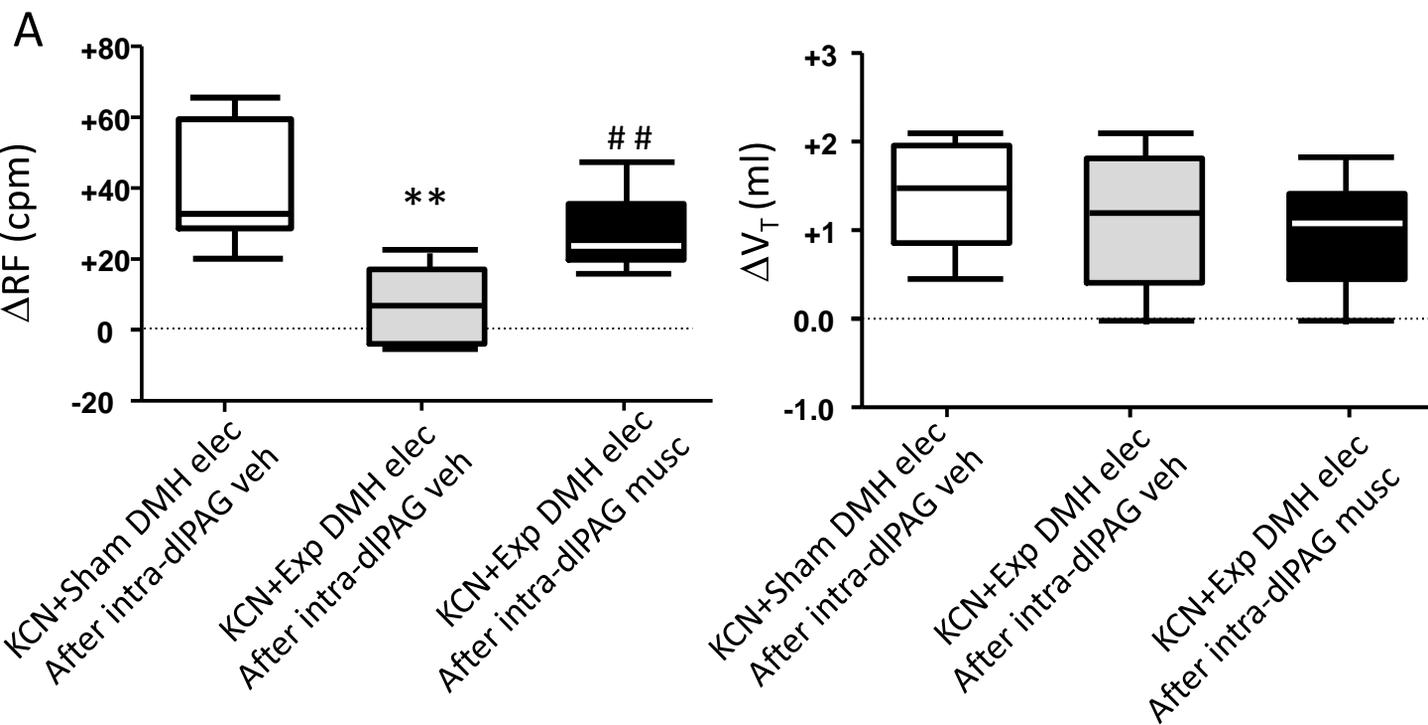
**-Experiment 4: Does the 5-HT<sub>3a</sub> receptor exert a role in the effects of low electrical activation of the dIPAG on KCN-induced respiratory responses in WT (A) and 5-HT<sub>3a</sub> KO (B) mice?**



**-Experiment 5: Does NTS 5-HT<sub>3</sub> receptor activation exert an inhibitory effect on KCN-induced respiratory responses (A), and can this inhibitory effect be prevented by prior injection of granisetron (B)?**

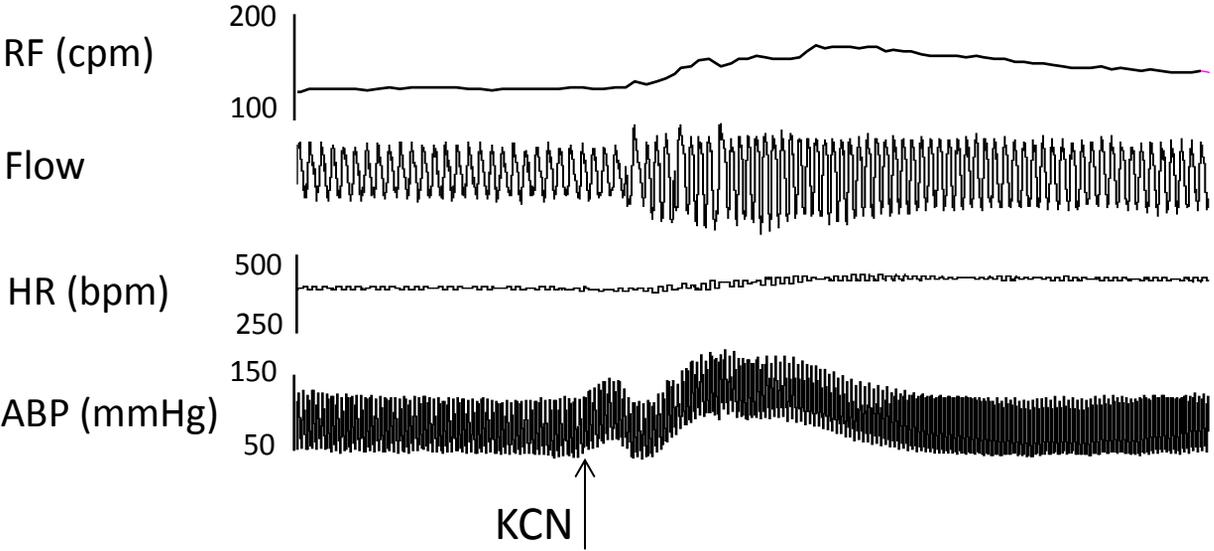


# Experiment 1

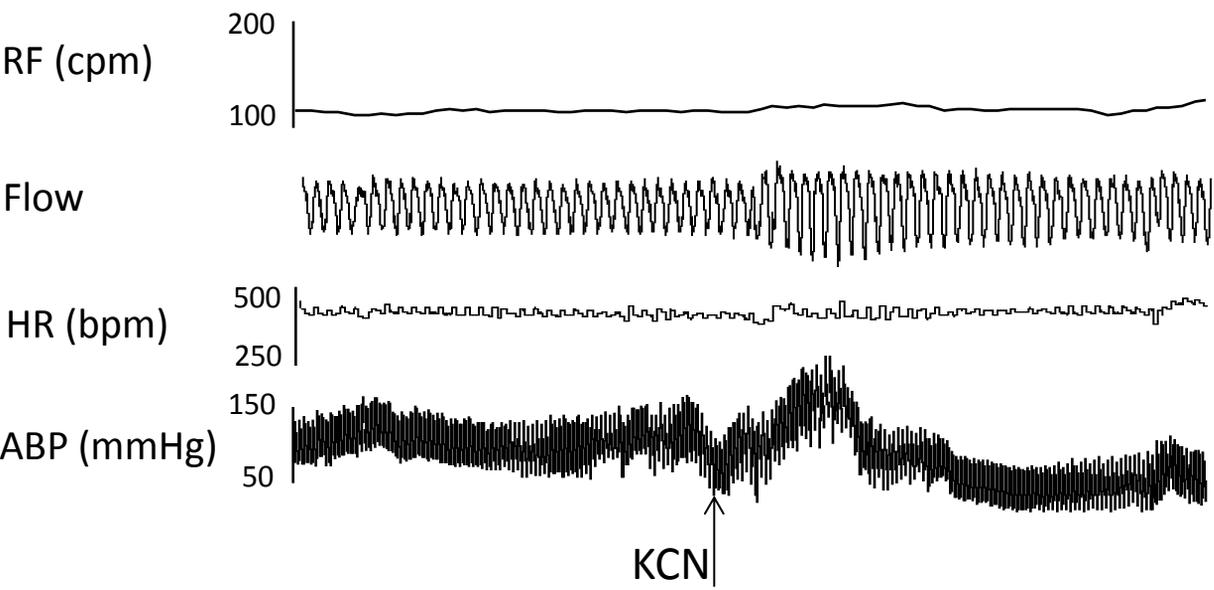


# Experiment 1

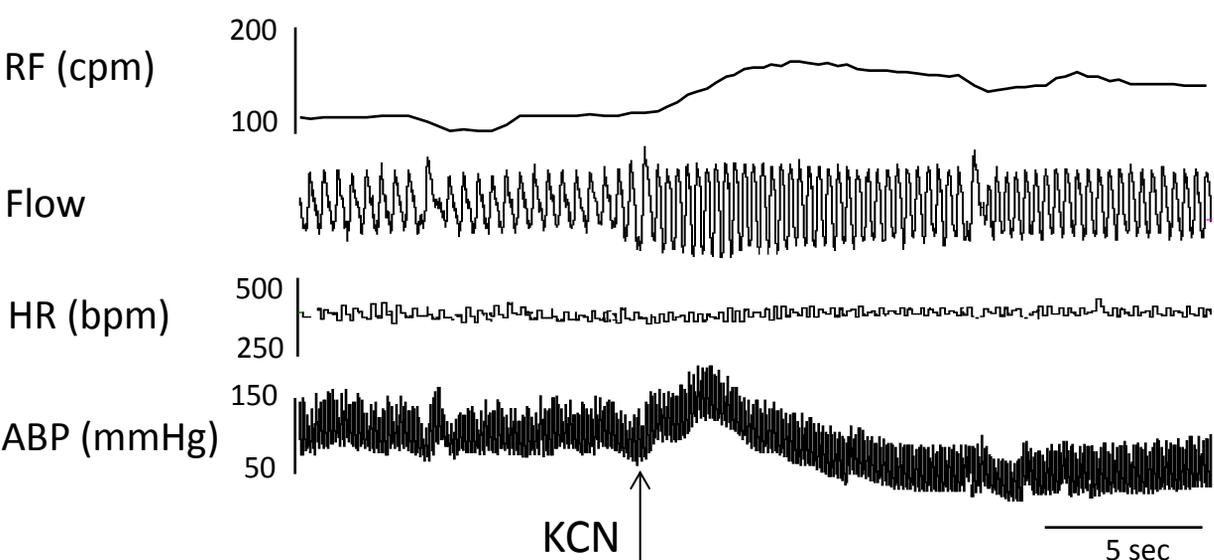
## After intra-DMH and intra-dIPAG vehicle



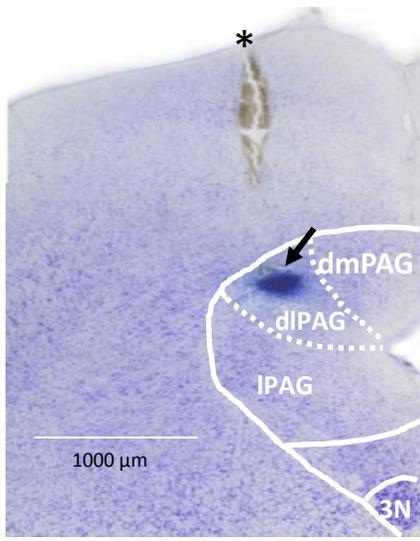
## After intra-dIPAG bicuculline and intra-dIPAG vehicle



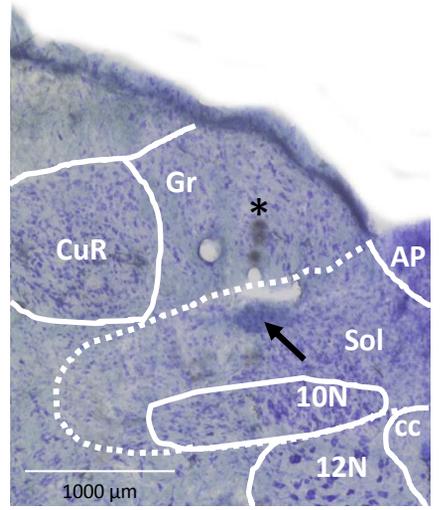
## After intra-dIPAG bicuculline and intra-dIPAG muscimol



A

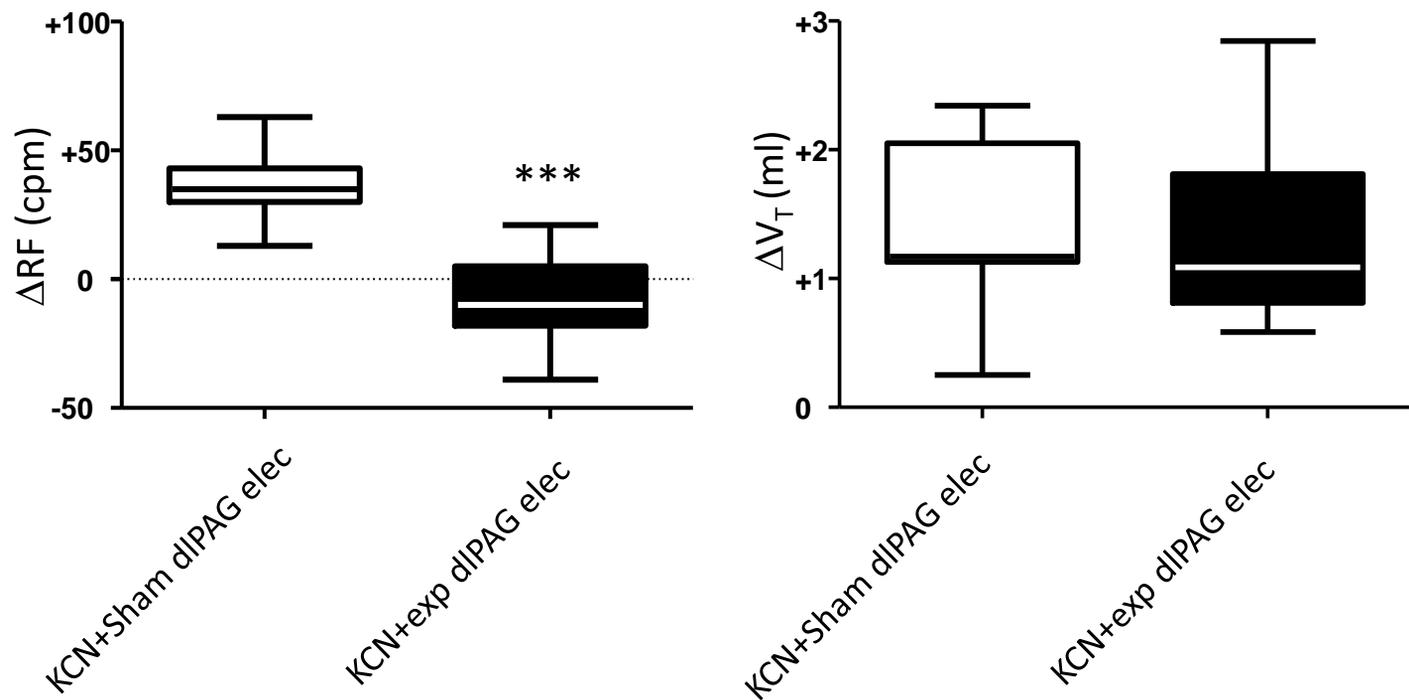


B

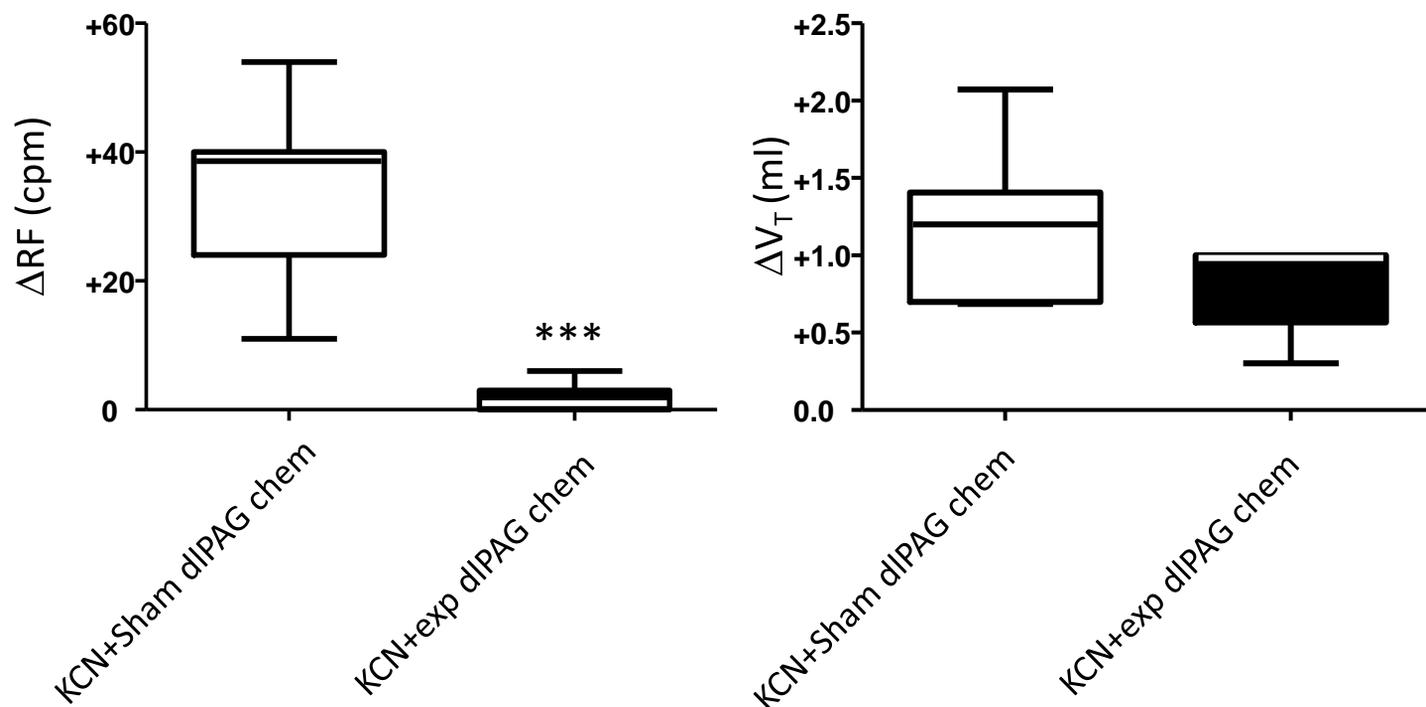


# Experiment 2

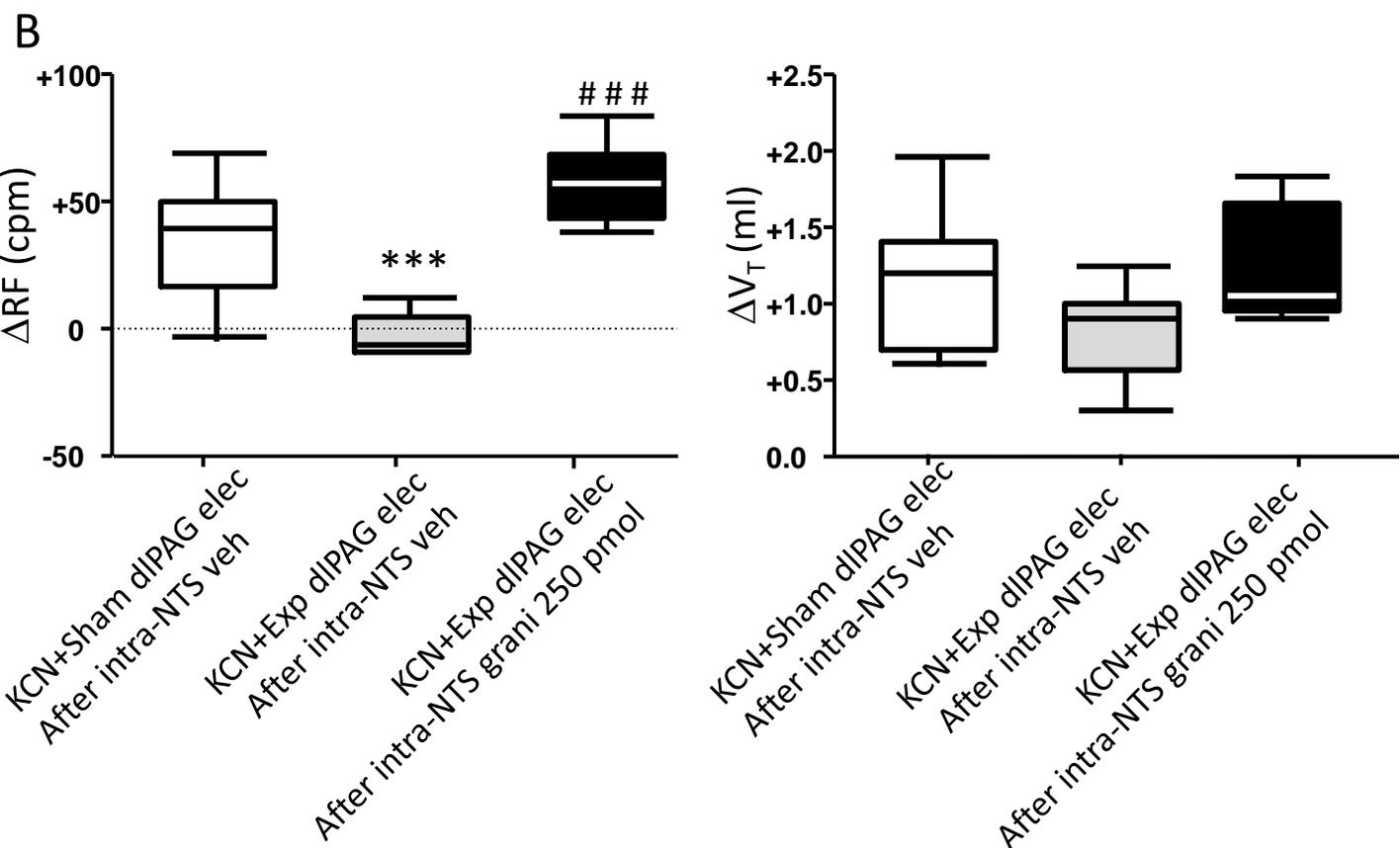
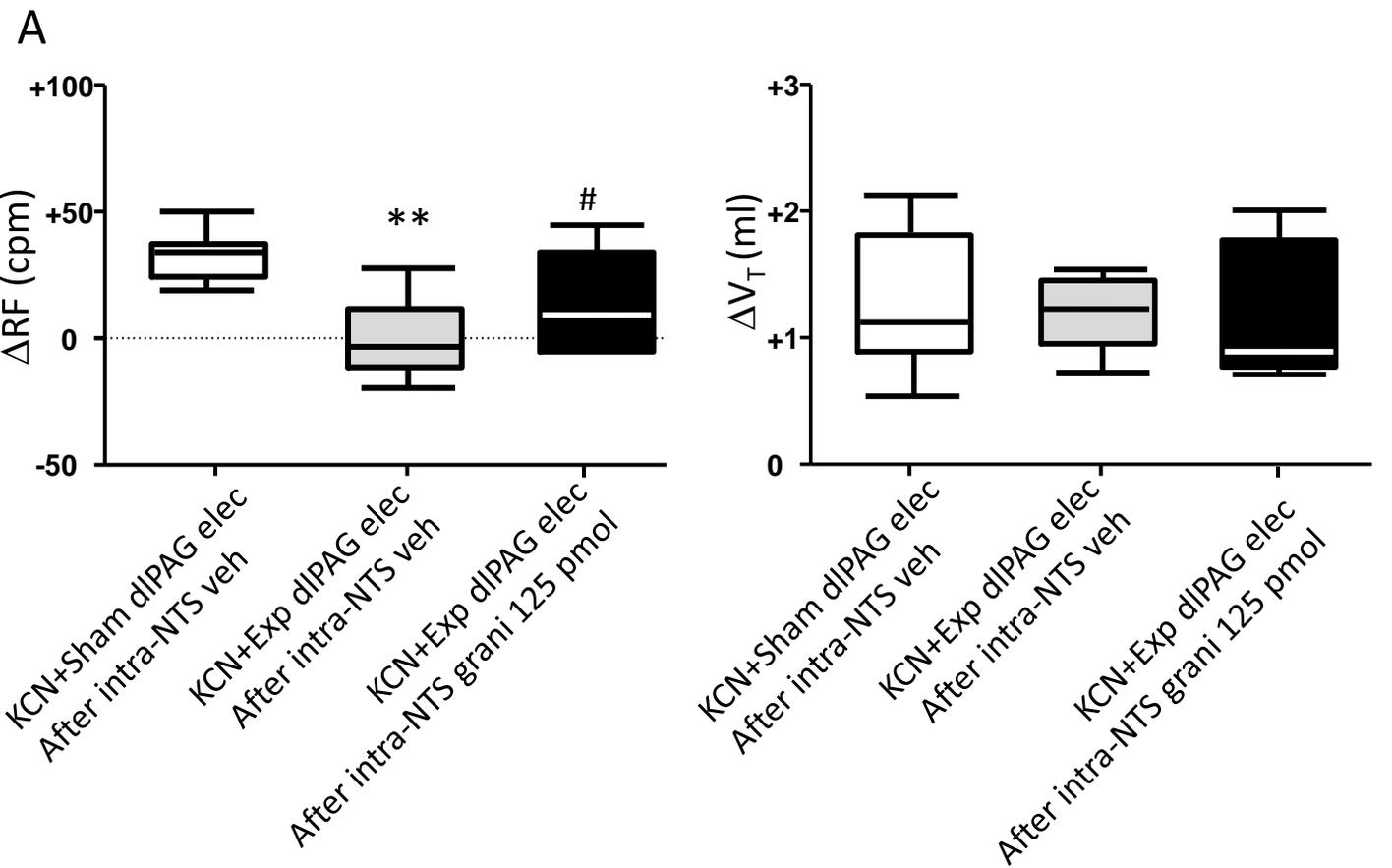
## A



## B

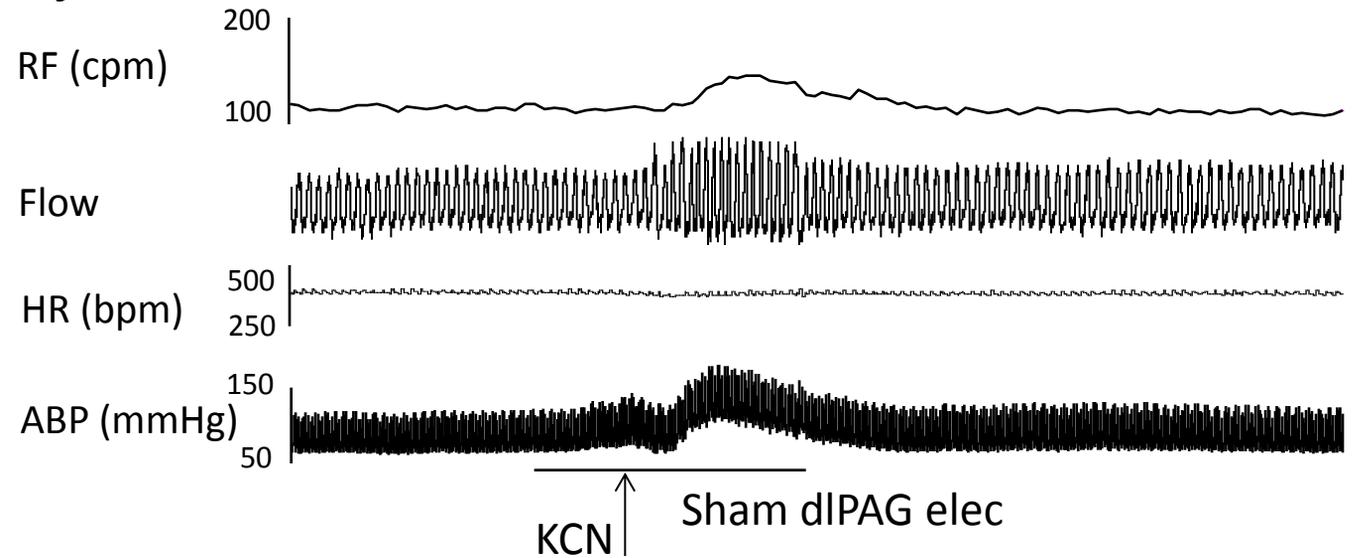


# Experiment 3

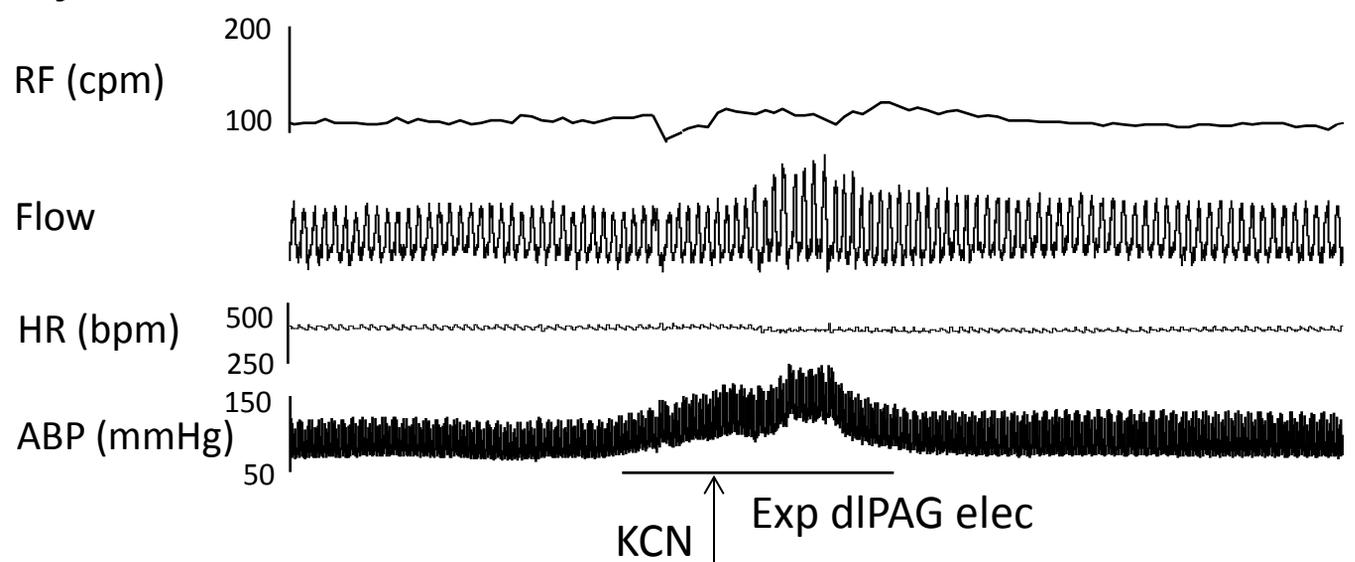


### Experiment 3

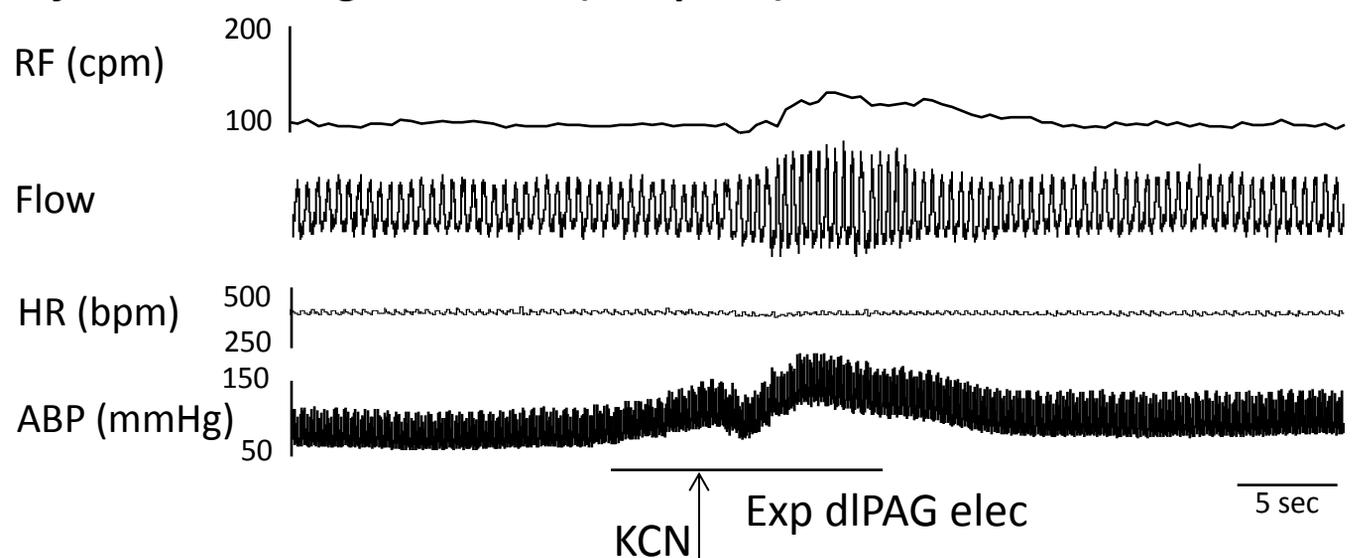
#### *After intra-NTS vehicle*



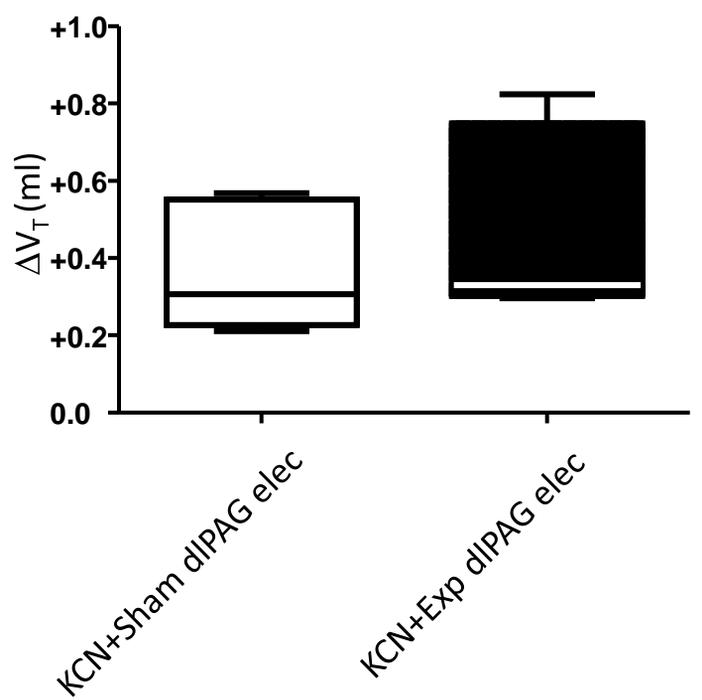
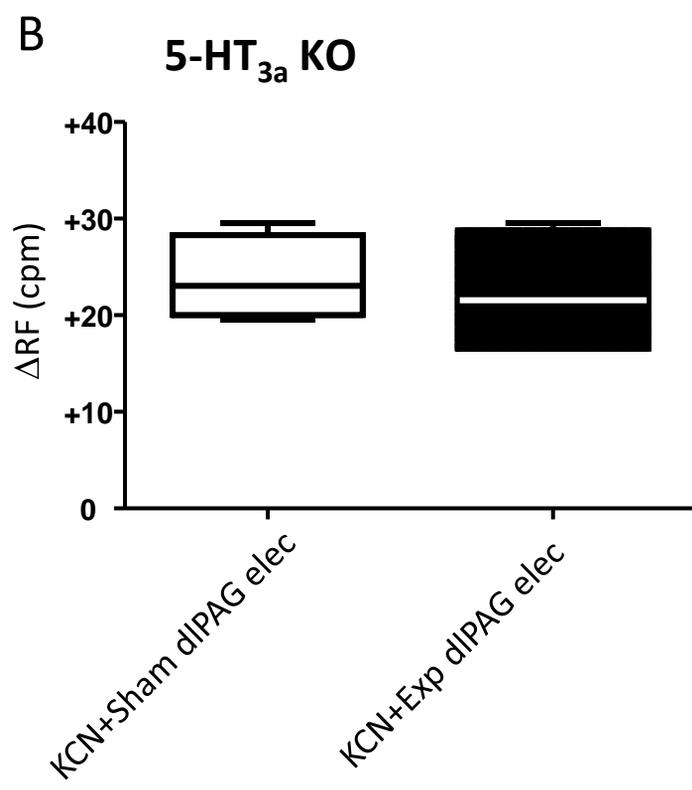
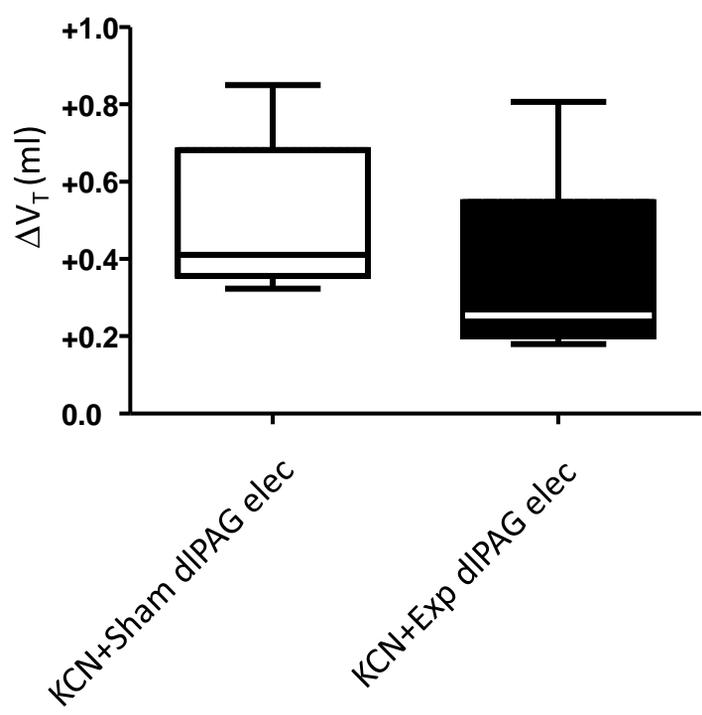
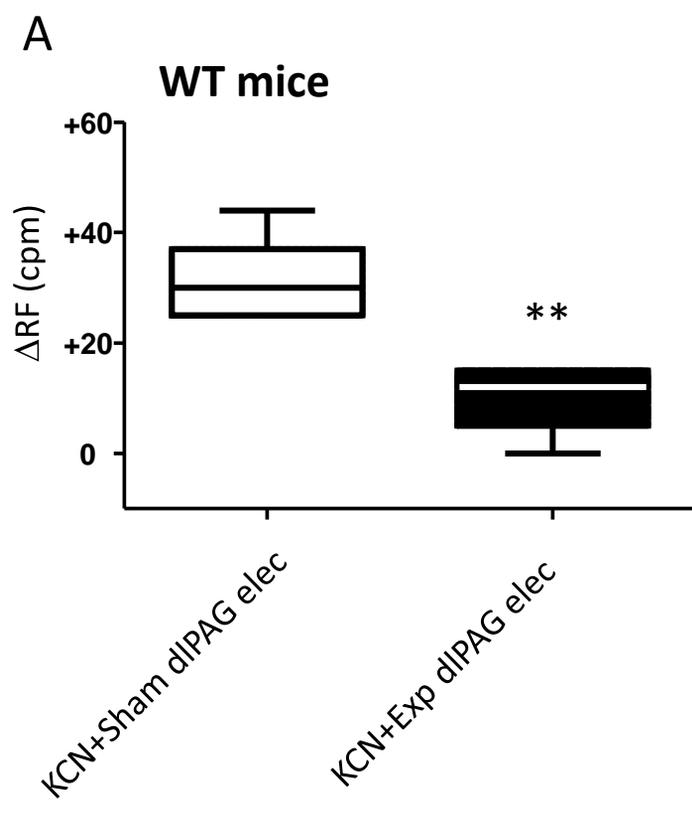
#### *After intra-NTS vehicle*



#### *After intra-NTS granisetron (250 pmol)*



# Experiment 4



- A hypothalamo-ponto-medullary (HPM) circuit produces hyperventilation and participates to the peripheral chemoreflex respiratory response.
- A new hypothalamo-midbrain-medullary (HMM) circuit observed with mild hypothalamic stimulation only, prevents the peripheral chemoreflex hyperventilatory response.
- The HMM circuit involves the dorsolateral periaqueductal grey matter and 5-HT<sub>3</sub> receptors in the nucleus tractus solitarius.
- The HMM circuit may be at the origin of the long-term bradypnea induced by emotional stress or observed in diseases characterised by a reduced chemoreflex response, such as the Congenital Central Hypoventilatory Syndrome.

Conflict of Interest: none

ACCEPTED MANUSCRIPT