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2 **Breathing stimulation mediated by 5-HT_{1A} and 5-HT₃ receptors within the**
3 **preBötzinger Complex of the adult rabbit**
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9 Ludovica Iovino, Donatella Mutolo, Elenia Cinelli, Massimo Contini, Tito Pantaleo and
10 Fulvia Bongianni
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Abstract

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4 Serotonin (5-HT) has been reported to play excitatory effects on respiration by acting on
5 preBötzing complex (preBötC) neurons in neonatal or juvenile rodents. However,
6 whether its action is circumscribed to the preBötC and present in other animal species,
7 particularly in adult preparations, is unknown. We investigated the respiratory role of 5-
8 HT within the preBötC and neighbouring respiration-related regions. Experiments were
9 performed on α -chloralose-urethane anesthetized, vagotomized, paralyzed and
10 artificially ventilated rabbits making use of bilateral microinjections (30-50 nl). 5-HT
11 caused excitatory effects on respiratory activity only when applied to the preBötC.
12 These effects were mediated by 5-HT_{1A} and 5-HT₃ receptors as shown by
13 microinjections of specific agonists of the different types of 5-HT receptors.
14 Unexpectedly, the blockade of 5-HT_{1A} receptors by methysergide or the specific
15 antagonist (S)-WAY 100135 induced excitatory respiratory effects. Microinjections of
16 the 5-HT₃ receptor antagonist ondansetron did not influence respiration, but prevented
17 (S)-WAY 100135-induced responses. The blockade of GABA_A receptors by bicuculline
18 within the preBötC prevented the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT.
19 The involvement of GABAergic inhibition and 5-HT_{1A} receptor-mediated disinhibition
20 is also corroborated by immunohistochemical data. The results show for the first time in
21 an adult animal preparation that 5-HT plays a pivotal role in the modulation of the
22 preBötC activity probably via both presynaptic and postsynaptic mechanisms and
23 highlight the importance of disinhibition phenomena. Present findings may be relevant
24 to some respiratory disorders in which an impairment of central 5-HT mechanisms has
25 been reported, such as sleep apnoea and sudden infant death syndrome.

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45 **Keywords:** control of breathing; preBötzing complex; serotonergic system

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49 **Abbreviations:** AU, arbitrary units; BötC, Bötzing complex; BSA, bovine serum
50 albumin; DLH, D,L-homocysteic acid; DMSO, dimethyl sulfoxide; 5-HT, serotonin;
51 iVRG, inspiratory portion of the VRG; 8-OH-DPAT, (R)-(+)-8-Hydroxy-DPAT
52 hydrobromide; PBG, 1-phenylbiguanide hydrochloride; PBS, phosphate-buffered
53 saline; PBS-TX, PBS containing 0.3% Triton X-100; preBötC, preBötzing complex;
54 SP, substance P; T_I, inspiratory time; T_E, expiratory time; VRG, ventral respiratory
55 group.
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1. Introduction

It is well known that serotonin (5-HT) and its ionotropic and metabotropic receptors are involved in the regulation of many brain functions, including breathing (for review, see Jacobs and Azmitia 1992; Hodges and Richerson 2008, 2010; Hilaire et al., 2010). Despite varied 5-HT effects on the respiratory motor output has been described, the bulk of evidence seems to support the conclusion that 5-HT neurons play an excitatory role on respiratory activity (Jacobs and Azmitia 1992; Hodges and Richerson 2008, 2010; Hilaire et al., 2010). Recent results (Ptak et al., 2009) have shown that excitatory amino acid-induced augmentation of the endogenous release of 5-HT and substance P (SP) from the raphe system in *in vitro* neonatal and in *in situ* juvenile rat preparations causes increases in the respiratory motor output in consequence of the activation of preBötzing complex (preBötC) neurons. More recently, an optogenetic study by DePuy and colleagues (2011) performed in anesthetized mice demonstrated that stimulation of raphe obscurus serotonergic neurons activates breathing and potentiates the central respiratory chemoreflex. However, no information is available on the role of 5-HT within the neighbouring respiration-related regions and in particular within important neural substrates possibly involved in respiratory rhythm generation (Smith et al., 2007, 2009; Mutolo et al., 2002, 2005; Bongiani et al., 2010), i.e. the Bötzing complex (BötC) and the inspiratory portion of the ventral respiratory group (iVRG). Impairments of central 5-HT mechanisms have been reported to be implicated in the pathophysiology of some respiratory disorders, such as sleep apnoea (Kubin et al., 1998; Kubin, 2016; Lipford et al., 2016 also for further Refs.) and sudden infant death syndrome (Paterson et al., 2006; Duncan et al., 2010; Machaalani and Waters 2014; Praveen and Praveen 2016 also for further Refs.). Interestingly, 5-HT has also been suggested to have a key role in autoresuscitation after anoxic stresses (e.g. Erickson and Sposato 2009; Chen et al., 2013; but see also Leiter, 2009) or opioid-induced respiratory depression (e.g. Sahibzada et al., 2000; Dutschmann et al., 2009; Manzke et al., 2009; Shevtsova et al., 2011; Ren et al., 2015). The 5-HT receptors are widely expressed in the VRG and 5-HT-induced excitatory effects on respiratory activity obviously depend on the combined activation of presynaptic and postsynaptic 5-HT receptors within neural structures involved in the respiratory control (e.g. Hodges and Richerson 2008, 2010; Nichols and Nichols 2008; Dutschmann et al., 2009; Hilaire et al., 2010). In

1 addition, disinhibition, i.e. 5-HT-mediated inhibition of inhibitory neurons, has been
2 proposed to contribute to the excitatory respiratory effects (e.g. Manzke et al., 2009;
3 Shevtsova et al., 2011; Corcoran et al., 2014). In this context, it is worth noting that
4 studies on the effects of 5-HT on respiration have hitherto been performed mostly in
5 neonatal or juvenile rodent preparations (e.g. Al Zubaidy et al., 1996; Pena and Ramirez
6 2002; Schwarzacher et al., 2002; Gunther et al., 2006; Ptak et al., 2009; Niebert et al.,
7 2011; Corcoran et al., 2014; for review, see Hodges and Richerson 2008, 2010; Hilaire
8 et al., 2010), thus studies in adult preparations of other animal species appear to be of
9 interest.

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16 The present research was undertaken to investigate the respiratory function of 5-
17 HT within the rostral VRG subregions, i.e. the preBötC and the adjacent BötC and
18 iVRG, making use of microinjections of 5-HT as well as of specific 5-HT receptor
19 agonists and antagonists in anesthetized, vagotomized, paralyzed and artificially
20 ventilated adult rabbits. Furthermore, the presence of inhibitory circuits possibly
21 subserving 5-HT-mediated excitatory effects was explored by means of the local
22 application of strychnine or bicuculline. Immunohistochemical studies were performed
23 to corroborate the results obtained with GABA_A receptor antagonism.
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2. Results

2.1 Excitatory respiratory effects of 5-HT

Bilateral microinjections of 5 mM 5-HT (150-250 pmol; $n = 6$) into the preBötC region induced excitatory effects on inspiratory activity (Fig. 1A) mainly consisting of increases in respiratory frequency from 44.1 ± 2.2 to 59.4 ± 2.0 breaths min^{-1} (35.9 ± 5.6 %; $t_{(5)} = 7.09$, $p = 0.0009$) due to reductions in T_I (from 0.49 ± 0.01 to 0.41 ± 0.01 s; $t_{(5)} = 7.81$, $p = 0.0006$) and T_E (from 0.89 ± 0.06 to 0.61 ± 0.03 s; $t_{(5)} = 5.83$, $p = 0.0021$). These effects were associated with small, but significant reductions in peak phrenic amplitude (8.3 ± 1.7 %; $t_{(5)} = 43.2$, $p < 0.0001$). Increases in both inspiratory rate of rise (11.5 ± 3.7 %; $t_{(5)} = 3.09$, $p = 0.027$) and phrenic minute output (24.7 ± 5.3 %; $t_{(5)} = 4.88$, $p = 0.0045$) were observed. The respiratory responses induced by 5-HT developed progressively, showing a maximum within 10 min while a complete recovery was observed within 60 min. Bilateral microinjections of 5 mM 5-HT into the BötC ($n = 4$) and the iVRG ($n = 4$) did not cause any obvious and consistent effects on respiratory activity. Histograms in Fig. 1B illustrate changes in some respiratory variables in response to 5 mM 5-HT microinjected into the preBötC, the BötC and the iVRG.

2.2 Respiratory effects of 5-HT receptor agonists microinjected into the preBötC

To ascertain the contribution of different types of receptors possibly involved in 5-HT-induced respiratory effects, microinjections of selective receptor agonists were performed into the preBötC. Bilateral microinjections of the 5-HT_{1A} receptor agonist 8-OH-DPAT at 1 mM (30-50 pmol; $n = 6$) induced mean increases in respiratory frequency of 46.4 ± 6.8 % (from 43.7 ± 0.6 to 63.9 ± 2.9 breaths min^{-1} ; $t_{(5)} = 6.09$, $p = 0.001$) due to reductions in both T_I (from 0.55 ± 0.01 to 0.41 ± 0.02 s; $t_{(5)} = 7.09$, $p = 0.0009$) and T_E (from 0.82 ± 0.01 to 0.54 ± 0.03 s; $t_{(5)} = 9.14$, $p = 0.0003$) accompanied by reductions in peak phrenic amplitude (12.1 ± 0.9 %; $t_{(5)} = 11.67$, $p < 0.0001$). Consistent increases in the inspiratory rate of rise (18.5 ± 5.9 %; $t_{(5)} = 3.43$, $p = 0.0187$) and the phrenic minute output (28.9 ± 6.8 %; $t_{(5)} = 5.17$, $p = 0.0035$) were also seen. Maximum effects and complete recovery occurred 10 and 90 min after the completion of the injections, respectively (Fig. 2A,C). Similar excitatory effects were induced by bilateral microinjections of 1 mM BP 554 (30-50 pmol; $n = 4$), another selective 5-HT_{1A} receptor agonist (Fig. 2C). Indeed, in the above mentioned experiments we used (R)-

(+)-8-Hydroxy-DPAT hydrobromide, i.e. the enantiomer form of this agonist, that has been described as selective for 5-HT_{1A} receptors (e.g. Wieronska et al., 2015). However, since it has been reported that this agonist, probably in the racemic form (±)-8-Hydroxy-DPAT (Sigma-Aldrich) can be a partial agonist at the 5-HT₇ receptors (Manzke et al., 2009; Corcoran et al., 2014), we applied 1 mM (R)-(+)-8-Hydroxy-DPAT hydrobromide in the presence of the 5-HT₇ receptor antagonist SB 258719 ($n = 4$). A repeated-measures ANOVA performed to evaluate this issue provided significant results ($F_{(2,6)} = 76.56$, $p = 0.0026$ for respiratory frequency; $F_{(2,6)} = 25.20$, $p = 0.0152$ for peak phrenic amplitude). *Post hoc* analysis showed that bilateral microinjections of 5 mM SB 258719 (150-250 pmol) did not change respiratory activity within 10 min, while microinjections of 1 mM 8-OH-DPAT performed at the same sites after this time interval caused increases in respiratory frequency (from 44.2 ± 2.1 to 62.2 ± 2.7 breaths min^{-1} ; 43.1 ± 5.1 %, $p = 0.006$) and reductions in peak phrenic amplitude (9.7 ± 1.6 %; $p = 0.0239$). All these outcomes indicate that 8-OH-DPAT effects were mediated only by 5-HT_{1A} receptors. Accordingly, local application to the preBötC of the 5-HT₇ receptor agonist LP 44 (1 mM, 30-50 pmol) in few trials ($n = 4$) did not cause any obvious change in respiratory variables (Fig. 2C).

Excitatory effects on inspiratory activity were obtained using a specific agonist of the excitatory 5-HT₃ receptors (Fig. 2B,C). Bilateral microinjections of 5 mM PBG (150-250 pmol; $n = 6$) caused increases in respiratory frequency of 15.8 ± 3.5 % (from 41.3 ± 1.3 to 47.7 ± 2.5 breaths min^{-1} ; $t_{(5)} = 4.03$, $p = 0.01$) due to reductions in both T_I (from 0.58 ± 0.04 to 0.47 ± 0.02 s; $t_{(5)} = 5.27$, $p = 0.0033$) and T_E (from 0.89 ± 0.03 to 0.80 ± 0.04 s; $t_{(5)} = 3.36$, $p = 0.0201$). Peak phrenic amplitude did not show significant changes, while the inspiratory rate of rise (18.1 ± 4.9 %; $t_{(5)} = 3.84$, $p = 0.0122$) and phrenic minute output (10.5 ± 3.4 %; $t_{(5)} = 2.97$, $p = 0.0310$) increased. Maximum effects were seen within 10 min, while the complete recovery occurred within 60 min after the completion of the injections. On the contrary, bilateral microinjections of the 5-HT_{1B} receptor agonist CP 93129 (1 mM, 30-50 pmol; $n = 4$), the 5-HT_{2A} receptor agonist TCB-2 (1 mM, 30-50 pmol; $n = 4$), the 5-HT_{2B} receptor agonist BW 723C86 (1 mM, 30-50 pmol; $n = 4$) and the 5-HT₄ receptor agonist BIMU 8 (1 mM, 30-50 pmol; $n = 4$) did not affect the ongoing pattern of breathing (Fig. 2C).

2.3 Respiratory effects of 5-HT receptor antagonists microinjected into the preBötC

Somewhat unexpectedly, bilateral microinjections of the 5-HT_{1/2} receptor antagonist methysergide at 5 mM (150-250 pmol; $n = 5$) increased respiratory frequency from 43.9 ± 0.5 to 58.0 ± 1.2 breaths min^{-1} (31.9 ± 1.7 %; $t_{(4)} = 16.46$, $p < 0.0001$) due to reductions in both T_I (from 0.62 ± 0.01 to 0.49 ± 0.01 s; $t_{(4)} = 9.44$, $p = 0.0007$) and T_E (from 0.74 ± 0.02 to 0.54 ± 0.02 s; $t_{(4)} = 36.51$, $p < 0.0001$). These effects were accompanied by reductions in peak phrenic amplitude (8.0 ± 1.6 %; $t_{(4)} = 16.06$, $p < 0.0001$) and increases in the inspiratory rate of rise (16.2 ± 3.1 %; $t_{(4)} = 4.88$, $p = 0.0081$) and phrenic minute output (21.4 ± 2.0 %; $t_{(4)} = 4.75$, $p = 0.0089$). Changes in some respiratory variables have been reported in Fig. 3A. On the basis of the results obtained with 5-HT receptor agonists, we inferred that methysergide-induced effects were caused by the blockade of 5-HT_{1A} receptors. As illustrated in Fig. 3, bilateral microinjections of the selective 5-HT_{1A} receptor antagonist (S)-WAY 100135 at 5 mM (150-250 pmol; $n = 6$) induced marked increases in respiratory frequency (from 43.5 ± 0.3 to 68.6 ± 1.2 breaths min^{-1} ; 57.5 ± 3.4 %; $t_{(5)} = 18.29$, $p < 0.0001$) due to decreases in both T_I (from 0.57 ± 0.01 to 0.39 ± 0.01 s; $t_{(5)} = 10.60$, $p = 0.0001$) and T_E (from 0.80 ± 0.01 to 0.48 ± 0.01 s; $t_{(5)} = 19.20$, $p < 0.0001$). Reductions in peak phrenic amplitude (17.0 ± 3.3 %; $t_{(5)} = 4.81$, $p = 0.0048$) and increases in both the inspiratory rate of rise (19.9 ± 2.02 %; $t_{(5)} = 10.56$, $p = 0.0001$) and phrenic minute output (30.6 ± 5.4 %; $t_{(5)} = 6.32$, $p = 0.0015$) were also observed (Fig. 3A,B). The excitatory effects of both methysergide and (S)-WAY 100135 developed progressively and reached a maximum within 10 min after the completion of the injections. A complete recovery was observed within 90 min. On the contrary, microinjections of the 5-HT₃ receptor antagonist ondansetron (5 mM, 150-250 pmol; $n = 4$) did not alter the breathing pattern (see Fig. 3A and Fig. 4A, middle panel), thus indicating that 5-HT₃ receptors do not exert a tonic influence on baseline respiratory activity. A repeated-measures ANOVA revealed that microinjections of 5 mM ondansetron performed in the same four preparations prevented the respiratory responses induced by 5 mM PBG microinjected into the same sites after an interval of ~5 min ($F_{(2,6)} = 0.04$, $p = 0.95$ for respiratory frequency; $F_{(2,6)} = 0.83$, $p = 0.4455$ for peak phrenic amplitude), thus showing that 5-HT₃ receptors were completely blocked (Fig. 4A, right panel).

We reasoned that the excitatory effects induced by the blockade of presynaptic 5-HT_{1A} receptors could be due to an increase in the release of 5-HT acting in turn on 5-HT₃ receptors. Consistently with this interpretation, an additional set of experiments was performed ($n = 4$). A repeated-measures ANOVA ($F_{(5,15)} = 41.04$, $p = 0.0044$ for

1 respiratory frequency; $F_{(5,15)} = 5.247$, $p = 0.0478$ for peak phrenic activity) showed (*post*
2 *hoc* analysis) that bilateral microinjections of 5 mM ondansetron prevented the
3 respiratory responses caused by microinjections of 5 mM (S)-WAY 100135 into the
4 same sites made after ~ 5 min and that microinjections of 5 mM 5-HT performed ~ 20
5 min after (S)-WAY 100135, i.e. during the blockade of both 5-HT₃ and 5-HT_{1A}
6 receptors, failed to induce any appreciable change in the respiratory motor output (Fig.
7 4B1). Of note, the blockade of both 5-HT₃ and 5-HT_{1A} receptors did not cause any
8 change in the control respiratory variables (Fig. 4B1, middle panel). *Post hoc* analysis
9 also proved that the characteristic excitatory responses to 5 mM 5-HT microinjections
10 were evoked again after > 90 min, i.e. after a time interval judged sufficient for the
11 complete washout of (S)-WAY 100135 ($p = 0.0241$ for respiratory frequency, $p =$
12 0.0457 for peak phrenic activity). An example of these findings is reported in Fig. 4B2.
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22 2.4 Role of disinhibition in the respiratory responses caused by 5-HT_{1A} receptor 23 activation 24 25

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27 The possibility that 8-OH-DPAT effects were mediated by disinhibition of inhibitory
28 neurons located within the preBötC (Manzke et al., 2009; Corcoran et al., 2014) was
29 addressed by investigating the responses induced by this agonist during the blockade of
30 glycine or GABA_A receptors. In the study of 8-OH-DPAT effects during glycine
31 receptor blockade ($n = 5$), a repeated-measures ANOVA showed significant changes in
32 the respiratory variables ($F_{(2,8)} = 105.2$, $p < 0.0001$ for respiratory frequency; $F_{(2,8)} =$
33 73.7 , $p = 0.0009$ for peak phrenic amplitude). In more detail (*post hoc* tests), bilateral
34 microinjections of 5 mM strychnine (150-250 pmol) induced within 5 min mild
35 increases in respiratory frequency from 43.5 ± 0.7 to 53.5 ± 1.6 breaths min⁻¹ ($23.1 \pm$
36 4.1% ; $p = 0.0132$) associated with decreases in peak phrenic amplitude ($21.2 \pm 3.4\%$; p
37 $= 0.0089$) in agreement with our previous results (Bongianni et al., 2010). The
38 subsequent (~ 10 min interval) microinjections of 1 mM 8-OH-DPAT at the same sites
39 caused within 10 min consistent further increases in respiratory frequency of $36.4 \pm$
40 5.4% (from 53.5 ± 1.6 to 72.7 ± 1.9 breaths min⁻¹; $p = 0.0054$) accompanied by further
41 decreases in peak phrenic amplitude ($11.2 \pm 0.4\%$; $p = 0.0003$). These outcomes are
42 illustrated in Fig. 5A.
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57 Our observation on the effects of 8-OH-DPAT during GABA_A receptor blockade
58 provided the following results (Fig. 5B). According to our previous findings (Bongianni
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1 et al., 2010), bilateral microinjections of 5 mM bicuculline (150-250 pmol; $n = 4$) into
2 the preBötC elicited within 3 min a pattern of breathing characterized by an overall
3 decrease in respiratory frequency and the presence of two alternating different levels of
4 peak phrenic activity. A repeated-measures ANOVA showed significant changes in the
5 respiratory variables ($F_{(2,6)} = 35.6$, $p = 0.0031$ for respiratory frequency; $F_{(4,12)} = 85.3$, p
6 $= 0.0007$ for peak phrenic amplitude). *Post hoc* tests showed that bilateral
7 microinjections of bicuculline induced decreases in respiratory frequency from $38.3 \pm$
8 2.0 to 28.7 ± 3.4 breaths min^{-1} ($26.2 \pm 5.1\%$; $p = 0.0263$) associated with a level of peak
9 phrenic activity higher than control ($21.8 \pm 1.5\%$; $p = 0.0009$) and a second level of
10 peak phrenic activity lower than control ($26.7 \pm 1.7\%$; $p = 0.0088$). The statistical
11 analysis also showed that the subsequent (~10 min interval) bilateral microinjections of
12 1 mM 8-OH-DPAT at the same sites did not induce significant changes in the already
13 disordered pattern of breathing, thus showing that bicuculline-induced disinhibition
14 actually prevented 8-OH-DPAT excitatory effects. In more detail, respiratory variables
15 maintained similar values, i.e., respiratory frequency was 29.0 ± 3.3 breaths min^{-1} (25.2
16 $\pm 5.3\%$ lower than control), peak phrenic amplitudes were $24.0 \pm 5.8\%$ higher than
17 control and $26.7 \pm 2.4\%$ lower than control, respectively. Respiratory activity recovered
18 control levels within 90 min.

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33 To corroborate these results, we sought for the presence of GABAergic neurons
34 and 5-HT_{1A} receptors within the preBötC by double-labelling experiments ($n = 3$).
35 GABA-immunoreactive structures were found (green signal) within the region
36 corresponding to the preBötC (Fig. 6). The nucleus ambiguus, along with stereotaxic
37 coordinates and data derived from the histological control of injection sites (see below),
38 were used to identify the location of the preBötC region, that resulted to be ventral and
39 slightly medial to the nucleus ambiguus. As illustrated in Fig. 6, photomicrographs at a
40 higher magnification of the portion of section (white rectangle in A) including part of
41 the preBötC show that GABAergic-immunoreactive structures (A1, green signal) are
42 present in this region and that 5-HT_{1A} receptors (A2, red signal) are widely expressed in
43 association with the soma of GABAergic-immunoreactive neurons (A3, merged image).

54 2.5 Controls

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57 The location of injection sites was confirmed by the histological control (Fig. 7). The
58 distribution of sites within the preBötC where 5 mM 5-HT was microinjected turned out
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1 to be ventral to the nucleus ambiguus and is diagrammatically illustrated in Fig. 7B. An
2 example of the localization of fluorescent beads is reported in Fig. 7C. The specificity
3 of drug-induced responses was confirmed by the results obtained with control
4 microinjections. According to previous studies (e.g. Mutolo et al., 2002, 2005;
5 Bongianni et al., 2010), bilateral microinjections of 5 mM 5-HT into medullary regions
6 sufficiently far (> 0.8 mm) from the responsive area did not induce any significant
7 change in the ongoing respiratory activity. These control microinjections were
8 performed both in the lateral direction (4 trials) and in the rostrocaudal direction, i.e. the
9 microinjections performed into the BötC and iVRG. In addition, similar control bilateral
10 microinjections (4 trials) were made into the nucleus tractus spinalis nervi trigemini or
11 the nucleus gigantocellularis of the same preparations already employed in the study of
12 5-HT-induced respiratory effects. Control microinjections of equal volumes of the
13 vehicle solutions performed in some preparations before 5-HT (3 trials) or (S)-WAY
14 100135 (3 trials) microinjections (0.9% NaCl solution and DMSO $<10\%$ in saline,
15 respectively) into the preBötC did not cause appreciable effects. Noticeably, all the
16 observed respiratory responses occurred without any significant concomitant change in
17 mean arterial blood pressure that ranged between 90 and 103 mmHg in the different
18 preparations. In the same preparation, small and inconsistent changes in mean arterial
19 blood pressure were observed during each experiment (as a rule $< 5\%$).

3. Discussion

This study is the first to provide evidence that 5-HT plays an excitatory role in the modulation of respiratory activity in the adult rabbit by acting on the preBötC, without affecting the neighbouring BötC and iVRG. We demonstrate that 5-HT microinjections performed into the preBötC have excitatory effects on respiration through the activation of 5-HT_{1A} and 5-HT₃ receptors. Unexpectedly, we also show that the 5-HT_{1A} receptor antagonist (S)-WAY 100135 microinjected into the preBötC causes excitatory respiratory effects, whereas the 5-HT₃ receptor antagonist ondansetron does not influence respiration. Interestingly, (S)-WAY 100135 is ineffective during 5-HT₃ receptor blockade, thus suggesting a presynaptic role of 5-HT_{1A} receptors. On the other hand, the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT are prevented by bicuculline microinjections into the preBötC. This latter finding indicates an involvement of a GABAergic inhibitory circuit and is corroborated by immunohistochemical data.

3.1 Methodological considerations

Microinjection procedures have been extensively discussed in previous reports (Lipski et al., 1988, Nicholson, 1985, Nicholson and Sykova 1998; see also e.g. Bongianni et al., 1997, 2002, 2008, 2010; Mutolo et al., 2002, 2005). In agreement with our previous findings in the rabbit (Bongianni et al., 1997, 2002, 2008, 2010; Mutolo et al., 2002, 2005), injection sites were selected by using stereotaxic coordinates and especially extracellular recordings of respiratory neurons from VRG subregions (see e.g. Bianchi et al., 1995, Von Euler, 1986). In particular, extracellular recordings allowed us to accurately define the preBötC region, where we encountered patterns of neuronal activities similar to those described in other animal species (see e.g. Connelly et al., 1992, Rekling and Feldman 1998, Schwarzacher et al., 1995). The localization of the injection sites was confirmed (see Methods) by DLH-induced tachypneic responses (see e.g. Solomon et al., 1999; Bongianni et al., 2002, 2010; Wang et al., 2002; Monnier et al., 2003; Mutolo et al., 2005; Fong and Potts 2006; Stucke et al., 2015). On the other hand, the effects of DLH microinjections into the preBötC region of the rabbit have already been described (Bongianni et al., 1997, 2008, 2010; Mutolo et al., 2002, 2005; Stucke et al., 2015). Excitatory respiratory effects induced by DLH microinjections into the preBötC have been compared with the responses induced in neighbouring regions

1 and have been taken as a functional marker for the preBötC in various animal models
2 (see e.g. Solomon et al., 1999; Krolo et al., 2005; Radocaj et al., 2015). Furthermore,
3 the localization of the injection sites was ascertained by the histological control of
4 pipette tracks and the localization of fluorescent beads microinjected into the preBötC.
5 The specificity of the observed responses is corroborated by the absence of respiratory
6 effects in response to control microinjections. In addition, respiratory responses were
7 not accompanied by appreciable changes in arterial blood pressure, thus ruling out any
8 role of baroreceptor reflexes in their development. On the other hand, in the rabbit
9 alterations in blood pressure were usually observed following drug microinjections into
10 the BötC, but not into the preBötC (see e.g. Bongianni et al., 1997, 2008, 2010).

11 Present findings, along with the results of some previous studies (Bongianni et
12 al., 2008, 2010; Mutolo et al., 2002, 2005), confirm that the rabbit can be a suitable
13 animal model for studies on the neural control of breathing and/or respiratory rhythm
14 generation. Although most of the basic knowledge on the functional organization of
15 respiratory neurons largely derives from past experiments on adult cats and more
16 recently on rodents, it should be recalled that rabbits have also been widely used in
17 recent and pioneering important studies on the control of breathing and on the
18 localization of respiration-related regions (Gromysz and Karczewski 1981; Yamamoto
19 and Lagercrantz 1985; Jiang and Shen 1991; Stucke et al., 2015; for reviews, see Von
20 Euler, 1986; Bianchi et al., 1995; Hilaire and Duron 1999). In addition, comparative
21 studies in different animal species are an important target of neuroscience.

22 *3.2 The preBötC as a target of 5-HT within the rostral VRG*

23 One of the main point of interest of the present study is the potent 5-HT-mediated
24 activation of the respiratory motor output through an action on the preBötC. This
25 finding appears to be in keeping with the vast majority of data obtained in neonatal and
26 juvenile *in situ* rodent preparations (e.g. Al Zubaidy et al., 1996; Pena and Ramirez
27 2002; Schwarzacher et al., 2002; Gunther et al., 2006; Ptak et al., 2009; Niebert et al.,
28 2011; Corcoran et al., 2014; for review, see Hilaire et al., 2010; Hodges and Richerson
29 2008, 2010). However, our results are at variance with those obtained in the dog by
30 Radocaj et al. (2015) who found that the local application of 8-OH-DPAT produced
31 dose-dependent decreases in the activity of preBötC neurons, while microinjections of
32 5-HT and 8-OH-DPAT along the whole extent of the ventral respiratory column did not
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1 produce any effect on respiration. They suggested that the tachypneic effects of
2 intravenous 8-OH-DPAT injections could be attributed to other brainstem areas.
3 However, species differences cannot be completely ruled out (see also Hilaire et al.,
4 2010).
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7 Indeed, medullary serotonergic neurons, mainly located in the brainstem raphe
8 nuclei and parapyramidal regions, project extensively to the brainstem respiratory
9 network including the preBötC and, in addition, to hypoglossal and phrenic
10 motoneurons (Ptak et al., 2009; see also Hodges and Richerson 2008; Paterson et al.,
11 2009; Hilaire et al., 2010; Ramirez et al., 2013). Interestingly, these 5-HT projections
12 are generally immunoreactive also for the neuropeptides SP and thyrotropin-releasing
13 hormone (for review, see Hodges and Richerson 2008). Accordingly, it has been shown
14 that raphe magnus stimulation increases respiratory frequency in mice due to the release
15 of 5-HT and, possibly, SP in the preBötC (Doi and Ramirez 2010). A recent study by
16 DePuy et al. (2011) in mice by using optogenetics demonstrated that the activation of
17 raphe obscurus serotonergic neurons stimulates breathing and that transfected
18 serotonergic neurons have multiple targets in both the lower brainstem and the spinal
19 cord. Their observations extend previous evidence that stimulation of the raphe
20 obscurus activates breathing and are consistent with the excitatory action of 5-HT on
21 phrenic motoneurons and many brainstem respiratory neurons, including those of the
22 preBötC and the retrotrapezoid nucleus.
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36 The mechanism by which raphe 5-HT neurons affect the respiratory network
37 (Richter et al., 2003; Hodges and Richerson 2008, 2010) and their role in the respiratory
38 rhythmogenesis (e.g. Pena and Ramirez 2002; Toppin et al., 2007) have been debated.
39 These issues have been recently clarified, at least to a great extent, by Ptak et al. (2009).
40 They showed in *in vitro* neonatal and in *in situ* juvenile rat preparations that the
41 stimulation of the raphe obscurus, that is reciprocally connected with the preBötC,
42 excites simultaneously both preBötC neurons and the respiratory motor output by means
43 of the release of 5-HT and SP. In accordance with these findings, our previous studies
44 have already shown that SP microinjections into the preBötC region, but not in the BötC
45 and iVRG, exert intense excitatory respiratory effects (see e.g. Bongianni et al., 2008
46 also for further Refs). In the light of the present results we can suggest that not only SP,
47 but also 5-HT microinjections into the preBötC can be additional tools for the
48 physiological identification of this VRG subregion. In conclusion, the preBötC proved
49 to be an important site of action of 5-HT, although we cannot exclude that other
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1 responsive brainstem respiration-related areas may exist. Indeed, 5-HT receptors have
2 been reported to be widely expressed in the VRG at least in some animal species
3 (Hodges and Richerson 2008, 2010; Dutschmann et al., 2009; Hilaire et al., 2010), but
4 unexpectedly we found that 5-HT microinjected into regions adjacent to the preBötC
5 was ineffective. On the other hand, to our knowledge, no extensive investigations exist
6 on the effects of microinjections of 5-HT into the BötC or the iVRG. We believe that
7 this topic deserves further immunohistochemical and neurophysiological studies.
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10 3.3 Excitatory role of 5-HT_{1A} and 5-HT₃ receptors within the preBötC

11 Somewhat at variance with previous studies on neonatal or juvenile rodents showing an
12 involvement of varied 5-HT receptors in the control and even in the generation or
13 maintenance of respiratory activity (Pena and Ramirez 2002; Schwarzacher et al., 2002;
14 Gunther et al., 2006; Ptak et al., 2009; Niebert et al., 2011; Corcoran et al., 2014; see
15 Hodges and Richerson 2008, 2010; Hilaire et al., 2010 for further Refs.), we found that
16 5-HT_{1A} and 5-HT₃ receptors are involved in 5-HT-induced excitatory effects on
17 respiration at the level of the preBötC in the adult rabbit. It seems too difficult to
18 propose an exhaustive explanation for such discrepancy. Probably it is mainly related to
19 differences in the animal species and developmental stage. Our outcomes have been
20 proven by several trials with 5-HT receptor agonists and antagonists and noticeably by
21 the lack of 5-HT effects during the blockade of both 5-HT_{1A} and 5-HT₃ receptors. To
22 our knowledge, this is the first report showing that 5-HT₃ receptors have an excitatory
23 role at the preBötC level. However, they do not seem to exert a tonic influence on
24 respiration under basal conditions since the 5-HT₃ receptor antagonist was ineffective.
25 Nevertheless, the presence of 5-HT₃ receptors allowed us to provide a tentative
26 explanation of the excitatory effects of the 5-HT_{1A} receptor antagonist (S)-WAY 100135
27 by suggesting an action of this drug at the presynaptic level. Indeed, 5-HT_{1A}
28 autoreceptors in the raphe nuclei have been extensively described only on
29 serotonergic neurons (Nichols and Nichols 2008). Although presynaptic 5-HT_{1A}
30 receptors have been found to be involved in the regulation of the release of GABA
31 and/or glutamate (e.g. Luttgen et al., 2005; Dergacheva et al., 2011; Ostrowski et al.,
32 2014; Stiedl et al., 2015), only suggestions have been advanced on the possible role of
33 5-HT_{1A} presynaptic receptors located on serotonergic terminals involved in the
34 release of 5-HT at sites far from serotonergic neurons (Wang and Ramage 2001).
35 Here, we provide only indirect evidence of the presence of such presynaptic receptors.
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1 A specifically designed immunohistochemical analysis should be performed to ascertain
2 our proposal as well as the presence of 5-HT₃ receptors on preBötC neurons. However,
3 this appears to be far beyond the scope of the present research.
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5 6 *3.4 Disinhibition phenomena underlying respiratory effects due to 5-HT_{1A} receptor* 7 *activation* 8 9

10 The interpretation of bicuculline-induced effects is very difficult, but it seems
11 conceivable that bicuculline microinjections do not affect a segregate inhibitory
12 pathway involved in the mediation of 5-HT-induced effects. Rather, they cause a
13 generalized blockade of the GABAergic inhibitory control within the preBötC that may
14 lead to a disruption of the ongoing pattern of breathing. For more details on this matter
15 we refer to our previous report (see also Kam et al., 2013). In any case, we found that
16 bicuculline prevented the excitatory effects of the 5-HT_{1A} receptor agonist, thus the
17 hypothesis was advanced that these effects were due to disinhibition of GABAergic
18 neurons located in the preBötC region (e.g. Kuwana et al., 2006; Shevtsova et al., 2011;
19 Koizumi et al., 2013) via postsynaptic 5-HT_{1A} inhibitory receptors, with a possible
20 additional regulatory role of presynaptic 5-HT_{1A} receptors. Thus, these findings imply
21 that GABAergic neurons exert a tonic inhibitory influence on preBötC neurons. These
22 conclusions are strongly corroborated by immunohistochemical results showing the
23 presence of 5-HT_{1A} immunoreactive dots in close apposition to the soma of GABAergic
24 immunoreactive neurons of the preBötC, consistently with the possibility of a
25 postsynaptic localization of 5-HT_{1A} receptor immunoreactive structures. The existence
26 of postsynaptic and presynaptic 5-HT_{1A} receptors within the preBötC region should be
27 ascertained in further studies. Present findings are consistent with the presence of 5-
28 HT_{1A} receptors on GABAergic neurons in other brain structures (e.g. Luttgen et al.,
29 2005; Ostrowski et al., 2014; Stiedl et al., 2015). Interestingly, a prominent role of
30 GABAergic inhibitory control at the level of the preBötC has already been described in
31 the rabbit (Bongianni et al., 2010). Furthermore, the presence of a disinhibitory
32 mechanism is in general agreement with the results of previous studies in rodents
33 (Manzke et al., 2009; Corcoran et al., 2014) in which, however, an involvement of
34 glycinergic neurons has been found. In conclusion, it seems that regardless of the
35 animal species disinhibition involving either glycinergic or GABAergic neurons can be
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1 a prominent mechanism subserving 5-HT excitatory effects on respiratory activity
2 (Manzke et al., 2009; Shevtsova et al., 2011; Corcoran et al., 2014).
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4 In conclusion, the main novel findings of the present study are that: 1) 5-HT
5 exerts its modulatory action only at the level of the preBötC through 5-HT_{1A} and 5-HT₃
6 receptors, without affecting the neighbouring respiration-related regions; 2) 5-HT_{1A}
7 receptors are endogenously activated, while 5-HT₃ receptors do not play any tonic role
8 on respiratory activity; 3) excitatory respiratory effects induced by the activation of 5-
9 HT_{1A} receptors involve preBötC GABAergic neurons. These outcomes are complex and
10 their interpretation is obscure. However, considering the novelty of present findings we
11 believe that would be of interest to advance a proposal on their functional significance.
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18 We propose the existence of two separate serotonergic mechanisms (Fig. 8)
19 that, according to the available literature, can reasonably be brought into action by
20 different stressors such as hypoxia (Erickson and Sposato 2009; Chen et al., 2013) or
21 hypercapnia (Phillipson et al., 1977; Berthon-Jones and Sullivan 1984; Buchanan et al.,
22 2015) and by opioid-induced respiratory depression (Sahibzada et al., 2000;
23 Dutschmann et al., 2009; Manzke et al., 2009; Ren et al., 2015). Under basal
24 conditions, 5-HT release should be minimum, if any, because the blockade of both 5-
25 HT₃ and 5-HT_{1A} receptors within the preBötC (see Fig. 4B1, middle panel) does not
26 alter the ongoing respiratory activity. However, it strongly increases when these 5-HT
27 mechanisms are activated. The first mechanism consists of the release of 5-HT tuned by
28 5-HT_{1A} receptor-mediated presynaptic inhibition and the activation of the ionotropic
29 excitatory 5-HT₃ receptors located on preBötC excitatory neurons. The second
30 mechanism, engaging the same or separate serotonergic afferent pathways, involves
31 the release of 5-HT (possibly controlled by presynaptic 5-HT_{1A} receptors?) causing the
32 activation of 5-HT_{1A} inhibitory receptors on a specific set of GABAergic cells
33 innervating the preBötC inspiratory neurons and consequent disinhibition phenomena
34 leading to the activation of inspiratory activity. These mechanisms should activate
35 simultaneously both the respiratory motor output and hypoglossal motoneurons
36 resulting in hyperventilation and increased airway patency (see Ptak et al., 2009).
37 Details on these mechanisms obviously require further investigations. Increases in
38 GABA_A-mediated inhibition have recently been suggested to be involved in the
39 correction of respiratory disorders (frequent apnoeas characterized by prolonged
40 postinspiratory activity in the cervical vagus nerve possibly associated with tonic
41 abdominal activity) in a mouse model of Rett syndrome (Abdala et al., 2010). However,
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in our experimental conditions disinhibition rather than increases in inhibition stimulates inspiratory activity. We did not record expiratory activity since our attention was focused on inspiratory rhythm generation and, according to our previous experience, anesthetized, vagotomized and paralyzed rabbits do not display either rhythmic or tonic expiratory activity.

Although neuromodulators are capable of modifying respiratory activity by targeting different types of preBötC neurons (Doi and Ramirez 2008), present results do not provide an overview of the neurochemical mechanisms operating within the preBötC microcircuits. Interestingly, it has been reported that optogenetic stimulation of the entire population of preBötC neurons in the rat potently drives inspiratory activity *in vivo* (Alsaħafī et al., 2015) consistently with its rhythmogenic role and with the presence of a core of excitatory neurons. The subpopulation of excitatory and rhythmogenic preBötC neurons has been shown to belong to the family of glutamatergic Dbx1⁺ neurons of the mouse that have preinspiratory or inspiratory firing patterns (Cui et al., 2016). We can speculate that preBötC neurons expressing 5-HT₃ receptors (first mechanism) pertain to a similar neuronal subpopulation of the rabbit. Both glycinergic (Sherman et al., 2015) and GABAergic (Cui et al., 2016) neurons represent important components of the preBötC neuronal population, although not essential for inspiratory rhythmogenesis in intact mammals (for review see Del Negro et al., 2018). Our findings show an important role of GABAergic inhibition (second mechanism) that can be hypothesized to correspond, at least in part, to the inhibitory mechanism activated by optogenetic stimulation of somatostatin-expressing neurons in the mouse (Cui et al., 2016). For a comprehensive review on the preBötC microcircuit and its interactions with additional breathing microcircuits see Del Negro et al. (2018).

It should be emphasized that in the last decades, rodents have been primarily employed in both *in vivo* and *in vitro* studies on the control of breathing (e.g. Feldman et al., 2013; Del Negro et al., 2018). We believe that the highly favourable experimental approach to the brainstem respiratory network in rodent and, particularly in murine models also supported by very modern techniques such as opto- and chemogenetics, may lead to substantial insights into the physiological characteristics of the neuronal circuits underlying breathing, that may be confirmed in larger animals. Since different animal species display similarities, but also differences (see e.g. Bongianni et al., 2008 also for further Refs.). As already mentioned, comparative studies on the control of breathing may also contribute to a better understanding of this matter.

1 Our results may be of interest for some respiratory disorders in which, as already
2 mentioned, an impairment of central 5-HT mechanisms has been found. Despite the fact
3 that therapeutic approaches based on drugs affecting the 5-HT system so far have been
4 proved to be unsuccessful (Oertel et al., 2007; Dempsey et al., 2010), it seems
5 conceivable that our results could provide hints for the development of novel
6 therapeutic strategies since they focus the attention on the preBötC, i.e. a region
7 characterized by the presence of multiple receptors that subserve the integration of
8 many neuromodulatory inputs to generate the breathing behaviour (Doi and Ramirez
9 2008; Del Negro et al., 2018).
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4. Experimental procedure

4.1 Ethical approval

Experiments were carried out on 85 male New Zealand white rabbits (2.8-3.4 kg) purchased from the Pampaloni Farm and Laboratory Animal Co. (Fauglia, Pisa, Italy). Rabbits were maintained on a 12-h light/12-h dark cycle with food and water *ad libitum*. All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Community Council on the use of laboratory animals (Decreto Legislativo 4/3/2014 no. 26 and directive 2010/63/UE). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize animal suffering and to reduce the number of animals used. At the end of the experiment the animal was euthanized with an overdose of anesthetic.

4.2 Animal preparation

Experimental procedures and details on the methods employed have previously been fully described (Bongianni et al., 1997, 2002, 2008, 2010; Mutolo et al., 2002, 2005; Pantaleo et al., 2011). The animals were anesthetized (ear marginal vein) with a mixture of α -chloralose (40 mg/kg i.v.; Sigma-Aldrich, St. Louis, MO, USA) and urethane (800 mg/kg i.v.; Sigma-Aldrich), supplemented (femoral vein) when necessary (4 mg/kg and 80 mg/kg, respectively). The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. In paralyzed animals (see below), the depth of anesthesia was assessed by ascertaining the presence of a stable and regular pattern of phrenic nerve activity as well as the absence of fluctuations in arterial blood pressure whether spontaneous or in response to somatic nociceptive stimulation. After cannulation of the trachea, polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and for drug administration, respectively. Both C5 phrenic roots were dissected free, cut distally and prepared for recordings. Both cervical vagus nerves were separated from the sympathetic trunks for subsequent vagotomy. The animal was placed in a prone position and fixed in a stereotaxic instrument by a stereotaxic head holder and vertebral clamps (Baltimore Instrument, Baltimore, MA, USA); the head was ventroflexed to facilitate recordings from the medulla. The dorsal surface of the medulla was widely exposed by

1 occipital craniotomy, and the dura and arachnoid membranes were removed. The
2 posterior part of the cerebellum was removed by gentle suction to provide access to the
3 rostral part of the medulla. All exposed tissues were covered with warm paraffin oil
4 (~38°C). Body temperature was maintained at 38.5-39°C by a heating blanket
5 controlled by a rectal thermistor probe. The animals were vagotomized, paralyzed
6 (gallamine triethiodide 4 mg/kg i.v., supplemented with 2 mg/kg every 30 min; Sigma-
7 Aldrich) and artificially ventilated with oxygen-enriched room air. The oxygen (80%)
8 was added to the inlet of the respirator. End-tidal CO₂ partial pressure was maintained
9 approximately at the level of spontaneous breathing (28.5-32 mmHg) by adjusting the
10 frequency and stroke volume of the respiratory pump.
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19 *4.3 Recording procedures*

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22 Efferent phrenic nerve activity was recorded with bipolar platinum electrodes from
23 desheathed C5 phrenic roots, amplified, full-wave rectified and passed through a leaky
24 integrator (low-pass RC filter, time constant 100 ms) to obtain a ‘moving average’ of
25 the activity, usually referred to in the literature as ‘integrated’ activity. Extracellular
26 recordings from medullary neurons were made with tungsten microelectrodes (5-10 MΩ
27 impedance as tested at 1 kHz). The most rostral extent of the area postrema on the
28 midline was defined as the obex and used as a standard point of anatomic reference.
29 Neuronal activity was recorded from rostral expiratory neurons of the BötC (3.0-4.5
30 mm rostral to the obex, 2.4-3.2 mm lateral to the midline and 3.5-4.6 mm below the
31 dorsal medullary surface), from the iVRG (from 0.7 caudal to 2.0 mm rostral to the
32 obex, 2.3-3.2 mm lateral to the midline and 3.0-3.5 mm below the dorsal medullary
33 face) and from the transition zone between the BötC and the iVRG where a mix of
34 inspiratory and expiratory neurons is present (2.1-2.9 mm rostral to the obex, 2.4-3.2
35 mm lateral to the midline and 3.5-4.2 mm below the dorsal medullary surface). The
36 latter region has already been extensively investigated in the rabbit with lesion and
37 neuropharmacological approaches (Bongianni et al., 2008, 2010; Mutolo et al., 2002,
38 2005; Pantaleo et al., 2011, Stucke et al., 2015) and corresponds to the preBötC
39 described in adult cats and rats (see e.g. Connelly et al., 1992; Schwarzacher et al.,
40 1995; Rekling and Feldman 1998; Solomon 1999; Feldman and Del Negro 2006;
41 Feldman et al., 2013; Del Negro et al., 2018). A strain-gauge manometer was used for
42 monitoring arterial blood pressure. End-tidal CO₂ partial pressure was monitored by an
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1 infrared CO₂ analyzer (Capnostream Plus, Smiths Medical PM, Waukesha, WI, USA).
2 Cardiorespiratory variables were acquired and analyzed using a personal computer
3 equipped with an analogue-to-digital interface (Digidata 1440, Molecular Devices,
4 Sunnyvale, CA, USA) and appropriate software (Axoscope, Molecular Devices).
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7 8 *4.4 Microinjection procedures and experimental protocol* 9

10 Bilateral microinjections were performed into the BötC, preBötC and iVRG regions as
11 defined by neuronal recordings. In each experiment, recordings of neuronal activity
12 preceded drug microinjections. The patterns of neuronal discharges recorded in the
13 BötC and the preBötC have been already illustrated in our previous reports (e.g. Mutolo
14 et al., 2002; Bongianini et al., 2008, 2010). In addition, the localization of the preBötC
15 was confirmed by evaluating the excitatory respiratory responses induced by bilateral
16 microinjections (30-50 nl) of 20 mM D,L-homocysteic acid (DLH; Sigma-Aldrich), a
17 broad-spectrum excitatory amino acid agonist, into this region (e.g. Solomon et al.,
18 1999; Wang et al., 2002; Monnier et al., 2003; Mutolo et al., 2005; Bongianini et al.,
19 2008, 2010; Stucke et al., 2015).
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22 The following drugs were used: 5 mM 5-HT (endogenous agonist at 5-HT
23 receptors; Tocris Bioscience, Bristol, UK), 1 mM (*R*)-(+)-8-Hydroxy-DPAT
24 hydrobromide (8-OH-DPAT; full 5-HT_{1A} receptor agonist, more active enantiomer;
25 Tocris Bioscience), 1 mM BP 554 maleate (selective 5-HT_{1A} receptor agonist; Tocris
26 Bioscience), 1 mM CP 93129 dihydrochloride (potent and highly selective 5-HT_{1B}
27 receptor agonist; Tocris Bioscience), 1 mM TCB-2 (high affinity 5-HT_{2A} receptor
28 agonist; Tocris Bioscience), 1 mM BW 723C86 hydrochloride (selective 5-HT_{2B}
29 receptor agonist; Tocris Bioscience), 5 mM 1-phenylbiguanide hydrochloride (PBG,
30 selective 5-HT₃ receptor agonist; Sigma-Aldrich), 1 mM BIMU 8 (potent 5-HT₄
31 receptor full agonist; Tocris Bioscience), 1 mM LP 44 (high affinity 5-HT₇ receptor
32 agonist; Tocris Bioscience), 5 mM methysergide maleate (mixed 5-HT₁/5-HT₂ receptor
33 antagonist; Tocris Bioscience), 5 mM (*S*)-WAY 100135 dihydrochloride (potent and
34 selective 5-HT_{1A} receptor antagonist; Tocris Bioscience), 5 mM ondansetron
35 hydrochloride (selective 5-HT₃ receptor antagonist; Tocris Bioscience), 5 mM SB
36 258719 hydrochloride (selective 5-HT₇ receptor antagonist; Tocris Bioscience), 5 mM
37 strychnine hydrochloride (glycine receptor antagonist; Sigma-Aldrich), 5 mM
38 bicuculline methiodide (GABA_A receptor antagonist; Sigma-Aldrich). Drugs were
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dissolved in 0.9% NaCl solution except for LP 44, BP 554, BW 723C86 and (S)-WAY 100135 that were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted to volume with 0.9% NaCl solution. In the final solution, the concentration of DMSO was < 10%. Only one drug was tested in each preparation, unless otherwise stated. Drug concentrations were selected in preliminary trials. They were in the same range as those previously used in *in vivo* preparations (Merahi and Laguzzi 1995; Sevoz et al., 1996; Comet et al., 2007; Monti et al., 2008; Rauch et al., 2008; Bongiani et al., 2010; Valic et al., 2010; Ostrowski et al., 2014; Zhang et al., 2014; Sardari et al., 2015).

Bilateral microinjections (30-50 nl) were performed in succession using a single glass micropipette (tip diameter 10-25 μm) by applying pressure using an air-filled syringe connected to the micropipette by polyethylene tubing. The volume of the injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a dissecting microscope equipped with a fine reticule. The duration of each injection ranged from 5 to 10 s. Control injections of equal volumes of the vehicle solution at the responsive sites were also made. In a few experiments (2 with 5-HT and 1 for 8-OH-DPAT), preBötC injection sites were confirmed by injecting green fluorescent latex microspheres (LumaFluor, New City, NY, USA) added to the drug solution. In an attempt to ensure an accurate representation of the preBötC location, these microinjections were performed only unilaterally according to the stereotaxic coordinates derived from the results of neuronal activity recordings and DLH microinjections on the contralateral side.

4.5 Histology

At the end of the experiment, the brain was perfused via a carotid artery with 0.9% NaCl solution and then with 10% formalin solution. After at least a 48-h immersion in 10% formalin solution, the brain was placed in a hypertonic sucrose solution. Frozen 20- μm coronal sections stained with Cresyl violet were used for the histological control of pipette tracks and injection sites, as already shown in our previous reports (e.g. Bongiani et al., 2008, 2010, Mutolo et al., 2002, 2005), as well as of fluorescent microsphere spots. Coronal sections of the medulla in which injection sites were marked by fluorescent microspheres were examined by a light and epifluorescence microscope (Eclipse E400, Nikon, Japan) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. A Nikon DS-Fi1 digital camera was used to take photomicrographs.

Illustrations were prepared in Adobe Photoshop CS3 (Adobe Systems Incorporated). The atlas of Shek et al. (1986) was used for comparison.

4.6 Immunohistochemistry

Three rabbits were deeply anesthetized with an overdose of pentobarbitone sodium (100 mg/kg i.v.; Sigma-Aldrich). The brains were perfused via a carotid artery with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) and subsequently with 500 ml of ice-cold 4% paraformaldehyde in PBS. The brains were postfixed for 48 h at 4°C in 4% paraformaldehyde and cryoprotected in 20% sucrose in PBS solution for at least 1 week. Then, 30 µm-thick serial coronal sections of the medulla were cut with a cryostat, placed in an anti-freeze solution and stored at -20°C until use. Free-floating sections were rinsed (3 x 10 min) in PBS containing 0.3% Triton X-100 (PBS-TX), blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature, and washed again three times as above. Sections were then incubated for 3 days at 4°C with a rabbit polyclonal antibody anti-GABA dissolved in BSA (1:1000; NBP1-78346; Novus Biologicals, Cambridge, UK) and a mouse monoclonal antibody against 5-HT_{1A} receptor (clone 19A9.2) dissolved in BSA (1:500; MAB11041; Millipore Corporation, Billerica, MA, USA). After washing in PBS-TX (3 x 10 min), slices were subsequently incubated for 2 h in the dark at room temperature with a mixture of Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen, Life Technologies, Carlsband, CA, USA) and Alexa Fluor 568 goat anti-mouse IgG (1:200; Invitrogen) dissolved in BSA. After washing in PBS-TX (3 x 10 min), tissue sections were mounted on gelatin-coated slides and coverslipped with glycerol containing 2.5% diazabicyclooctane (Sigma-Aldrich). Coronal sections of the medulla were examined by a epifluorescence microscope (Eclipse E400) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. Photomicrographs of key results were taken using a Nikon DS-Fi1 digital camera. Illustrations were prepared in Adobe Photoshop CS3 (Adobe Systems Incorporated). Images were only adjusted for brightness and contrast.

Although our rabbit polyclonal antibody anti-GABA has not been previously used in the rabbit, we are confident that it is specific for GABA. In fact, the pattern of distribution of GABAergic neurons produced by this antibody is similar to that obtained with different anti-GABA antibodies or with anti-GAD antibodies in the ventrolateral medulla of rabbits and rats (Blessing, 1990; Ellenberger, 1999), and in particular within

1 the preBötC of the rat (Koizumi et al., 2013). The specificity of the primary antibody
2 against GABA cannot be validated by using Western blot analysis. In fact, owing to the
3 very small size of GABA molecule, this technique cannot be applied (Novus
4 Biologicals and other suppliers producing antibodies against GABA). Also the mouse
5 monoclonal antibody against 5-HT_{1A} receptors has not been previously used in the
6 rabbit. However, the 5-HT_{1A} receptor expression pattern observed in the present study
7 in the preBötC of the rabbit closely recalls that observed with a different antibody in the
8 preBötC of the rat (Dutschmann et al., 2009; Manzke et al., 2009). Admittedly, Western
9 blot validation of the specificity of the primary antibodies in the rabbit brain tissue
10 would be appropriate. On the other hand, protein structures appear to be highly
11 conserved throughout evolution and this antibody demonstrated cross-reaction with rats
12 and humans (Millipore Corporation). This makes us confident that this antibody is
13 specific also for rabbit brain tissue.
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24 *4.7 Data collection and statistical analysis*

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27 We measured the respiratory frequency (breaths/min), the inspiratory (T_I) and
28 expiratory (T_E) times, as well as the peak amplitude of the integrated phrenic nerve
29 activity that was normalized relative to mean control values (baseline) and expressed in
30 arbitrary units (AU). The phrenic minute output (neural minute ventilation) was
31 calculated as the product of phrenic tidal activity and respiratory frequency. The slope
32 of the straight line drawn from the onset to 90% of the maximum level of the phrenic
33 ramp was considered a reliable estimate of the inspiratory rate of rise (e.g. Bongianni et
34 al., 2002, 2008, 2010; Mutolo et al., 2002, 2005). Respiratory variables were measured
35 for an average of five consecutive breaths in the period immediately preceding each trial
36 and at the time when the maximum response to drug microinjections occurred. In the
37 same periods, systolic and diastolic blood pressure were measured at 2-s intervals.
38 Mean arterial pressure was calculated as the diastolic pressure plus one-third of the
39 pulse pressure. Owing to the small variations in respiratory and cardiovascular variables
40 within a measurement period, average values for each period were taken as single
41 measurements for the purpose of statistical analysis (GraphPad Prism 7, GraphPad
42 Software, Inc., La Jolla, CA, USA). The recovery of control values was followed for a
43 maximum of two hours. In each preparation, we considered respiratory activity fully
44 recovered when respiratory frequency and peak phrenic amplitude were within $\pm 3\%$ of
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1 control values. Paired *t*-tests were employed to evaluate changes in cardiorespiratory
2 variables induced by each drug and by vehicle solutions. Unpaired *t*-tests were
3 employed to compare drug-induced cardiorespiratory changes in different preparations.
4 One-way repeated-measures ANOVA followed by Bonferroni's multiple comparisons
5 test was used to assess the effects of two or more treatments on cardiorespiratory
6 variables. For simplicity, only respiratory frequency and peak phrenic amplitude were
7 considered in this latter type of statistical analysis. Changes in respiratory variables
8 were also expressed as percentage variations of control values. All values are presented
9 as means \pm SEM; $p < 0.05$ was considered as significant. In the legends, the level of
10 significance is indicated as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
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Disclosure statement

The authors have no conflicts of interest to disclose.

Authors' contributions

L.I., D.M., F.B., T.P., E.C. conceived, designed and performed the experiments. L.I., D.M., F.B., M.C., T.P., E.C. analysed data and interpreted the results. L.I., D.M., F.B., T.P., E.C. drafted the article and revised it critically for important intellectual content. D.M., F.B., T.P. wrote the manuscript in interactions with all authors. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work. Experiments were performed at the Dipartimento di Medicina Sperimentale e Clinica, Sezione Scienze Fisiologiche, Università degli Studi di Firenze. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure legends

Fig. 1. Respiratory responses caused by bilateral microinjections of 5 mM 5-HT into different subregions of the ventral respiratory group. (A) Only 5-HT applied to the preBötC region caused marked excitatory effects on the respiratory motor output. Traces are integrated phrenic nerve activity (IPA) and raw phrenic nerve activity (PA) under control conditions, ~10 and 60 min (recovery) after the completion of 5-HT microinjections. AU, arbitrary units. (B) Histograms illustrating changes in respiratory frequency, peak phrenic activity, inspiratory rate of rise and phrenic minute output elicited by 5 mM 5-HT microinjected into the preBötC ($n = 6$), the BötC ($n = 4$) and the iVRG ($n = 4$). Values are means \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Respiratory responses elicited by 5-HT receptor agonists microinjected into the preBötC. (A) Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT_{1A} receptor agonist 8-OH-DPAT. Recovery 90 min after 8-OH-DPAT. (B) Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT₃ receptor agonist PBG. Recovery 60 min after PBG. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity. (C) Histograms illustrating changes in respiratory frequency, peak phrenic activity and inspiratory rate of rise elicited by 8-OH-DPAT ($n = 6$), BP 554 ($n = 4$), CP 93129 ($n = 4$), TCB-2 ($n = 4$), BW 723C86 ($n = 4$), PBG ($n = 6$), BIMU 8 ($n = 4$) and LP 44 ($n = 4$). Values are means \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3. Respiratory effects of some 5-HT receptor antagonists microinjected into the preBötC. (A) Histograms illustrating changes in respiratory frequency, peak phrenic activity, inspiratory rate of rise and phrenic minute output induced by methysergide ($n = 5$), (S)-WAY 100135 ($n = 6$) and ondansetron ($n = 4$). Values are means \pm SEM. ** $p < 0.01$; *** $p < 0.001$. (B) Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT_{1A} receptor antagonist (S)-WAY 100135. Recovery ~ 80 min after (S)-WAY 100135. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity.

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Fig. 4. Role of 5-HT₃ receptor antagonist in counteracting the effects mediated by 5-HT₃ and 5-HT_{1A} receptors. (A) Microinjections of ondansetron did not change the ongoing pattern of breathing, but prevented the excitatory responses of PBG injected into the same sites after an interval of 5 min (as observed 15 min after PBG). (B1) During the blockade of 5-HT₃ receptors, microinjections of (S)-WAY 100135 performed ~5 min after the completion of ondansetron microinjections did not cause obvious respiratory changes. After an interval of ~20 min (i.e. during the blockade of both 5-HT₃ and 5-HT_{1A} receptors), microinjections of 5-HT were ineffective (observation made at ~ 15 min after 5-HT). (B2) After an interval > 90 min after (S)-WAY 100135 scheduled to allow a complete washout of the employed drugs, 5-HT induced again the characteristic excitatory responses (observation made ~ 10 min after the injections). The recovery ~ 60 min after 5-HT has also been reported. AU, arbitrary units; IPA, integrated phrenic nerve activity.

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Fig. 5. Glycine and GABA_A receptors in the mediation of 8-OH-DPAT respiratory effects. (A) Increases in respiratory frequency and decreases in peak phrenic amplitude observed ~ 5 min following strychnine microinjections into the preBötC. Additional changes in respiratory activity ~ 10 min after the subsequent 8-OH-DPAT microinjections performed into the same sites. Recovery ~ 90 min after 5-HT_{1A} receptor agonist microinjections. (B) A breathing pattern characterized by the presence of two alternating different levels of peak phrenic activity ~ 5 min after bicuculline microinjections into the preBötC. Absence of changes in this altered pattern of breathing ~ 15 min following the subsequent 8-OH-DPAT microinjections into the same sites. Recovery taken ~ 70 min after 5-HT_{1A} receptor agonist microinjections. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity.

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Fig. 6. Double immunostaining of GABAergic structures and 5-HT_{1A} receptors in the preBötC. (A) Photomicrograph of a coronal section of the medulla oblongata at the level of the preBötC (~ 2.6 mm rostral to the obex) showing the distribution of GABA-immunoreactive structures (green signal). NA, nucleus ambiguus. The white box ventral and slightly medial to the NA delineates a portion of the preBötC region. (A1-A3) Photomicrographs at a higher magnification of the portion of the transverse section indicated by the white box in (A) showing GABAergic immunoreactive structures (A1,

green signal), 5-HT_{1A} receptor binding sites (A2, red signal) and merged image (A3). Arrowheads point to the localization of 5-HT_{1A}-immunoreactive structures located in close apposition to GABAergic neurons. The insets (higher magnification) indicate the location of some 5-HT_{1A}-immunoreactive dots in close proximity to a single GABAergic neuron. Scale bars: (A), 200 μ m; (A1-A3), 50 μ m; insets, 10 μ m.

Fig. 7. Localization of injection sites. (A) Diagrammatic representation of a dorsal view of the medulla oblongata of the rabbit illustrating some of the main components of the respiratory network. AP, area postrema; BötC, Bötzinger complex; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group; iVRG, inspiratory ventral respiratory group; preBötC, preBötzinger complex. (B) Diagram of a coronal section of the medulla oblongata at the level (\sim 2.5 mm rostral to the obex) indicated in (A) (dashed line) showing the distribution of sites (\blacktriangle) where microinjections of 5 mM 5-HT were performed. Outlines of the map derive from a selected section of one histological preparation. The atlas of Shek et al. (1986) was used for comparison. (C) Photomicrograph of a coronal section of the medulla oblongata at the same level as in (B) showing an example of the location of fluorescent beads microinjected into the preBötC. The histological section is counterstained with Cresyl violet. Light-field and fluorescent photomicrographs have been superimposed. The preBötC region is approximately indicated by dashed lines in (B). NA, nucleus ambiguus; NOI, nucleus olivaris inferior; NTS, nucleus tractus solitarii; NV, nucleus tractus spinalis nervi trigemini; P, tractus pyramidalis.

Fig. 8. Schematic drawing, partially inferred from the present results, representing two hypothetical 5-HT mechanisms in the rabbit medulla oblongata responsible for the activation of preBötC excitatory neurons. Under basal conditions only presynaptic 5-HT_{1A} receptors are endogenously activated, as suggested by the differential effects of specific 5-HT₃ and 5-HT_{1A} receptor antagonists, and minimize or even annihilate 5-HT release at the level of the synaptic knobs on preBötC cells. In any case, the release of 5-HT should be minimum or even absent, since the blockade of both 5-HT_{1A} and 5-HT₃ receptors does not alter baseline respiratory activity. Conceivably, different stressors, such as hypoxia, hypercapnia or opioid-induced respiratory depression, have been reported to activate serotonergic mechanisms. The augmented release of 5-HT overcomes presynaptic inhibition and stimulates postsynaptic 5-HT₃ receptors on

1 preBötC cells (A). Simultaneously, the increased 5-HT release also activates the
2 inhibitory 5-HT_{1A} receptors on a specific set of GABAergic neurons that exert an
3 inhibitory control on preBötC cells through GABA_A receptors. Present results do not
4 allow us to advance any hypothesis on the respiratory or non-respiratory nature of these
5 neurons. This induces disinhibition of preBötC neurons and the subsequent excitatory
6 effects on respiratory activity (B). Presynaptic 5-HT_{1A} receptors might be present also at
7 the level of synaptic terminals on GABAergic neurons.
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Figure 1
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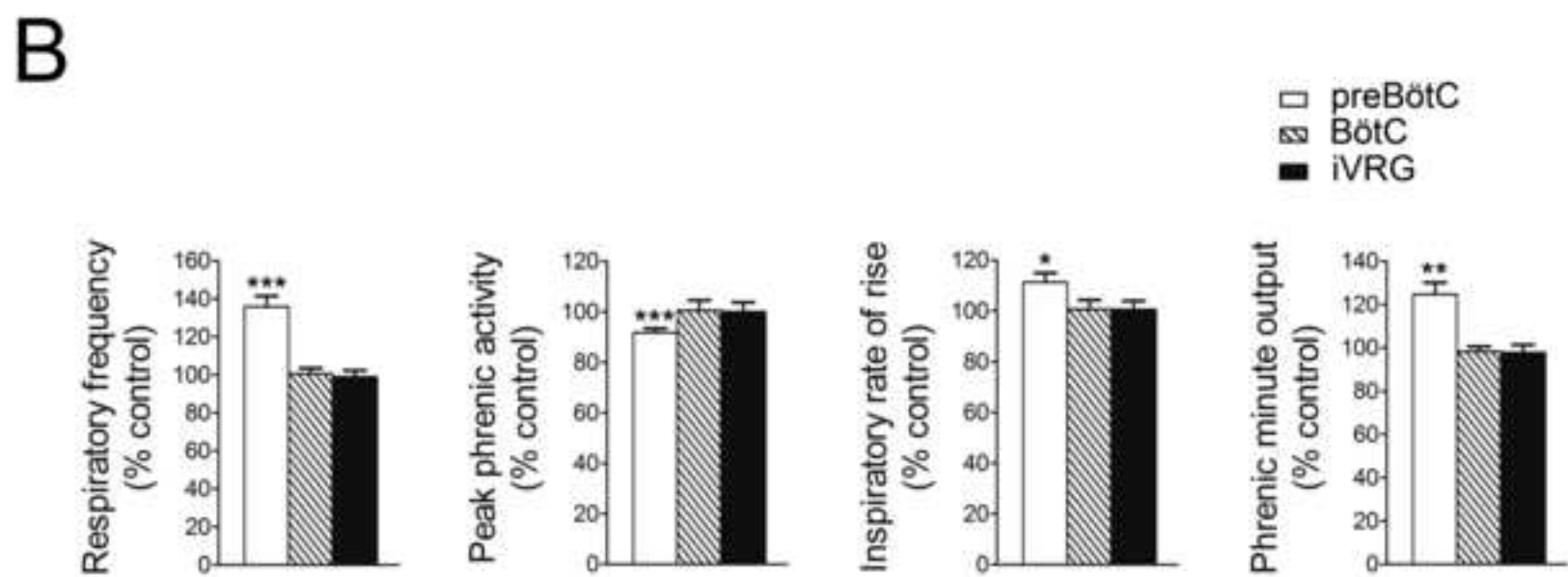
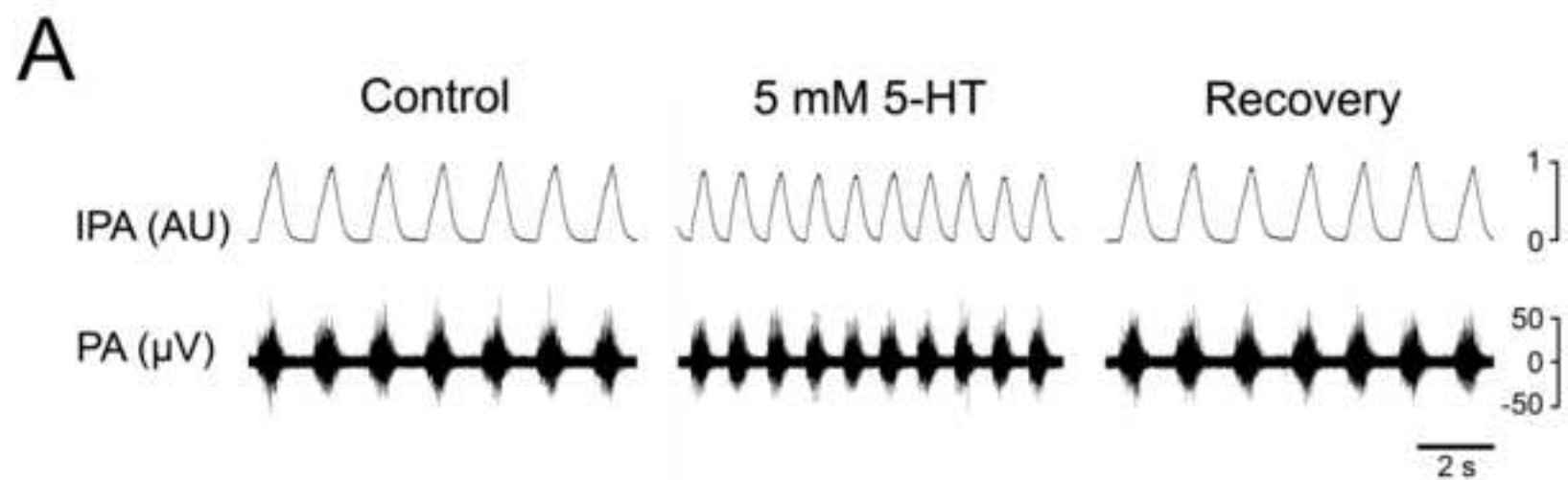


Figure 2
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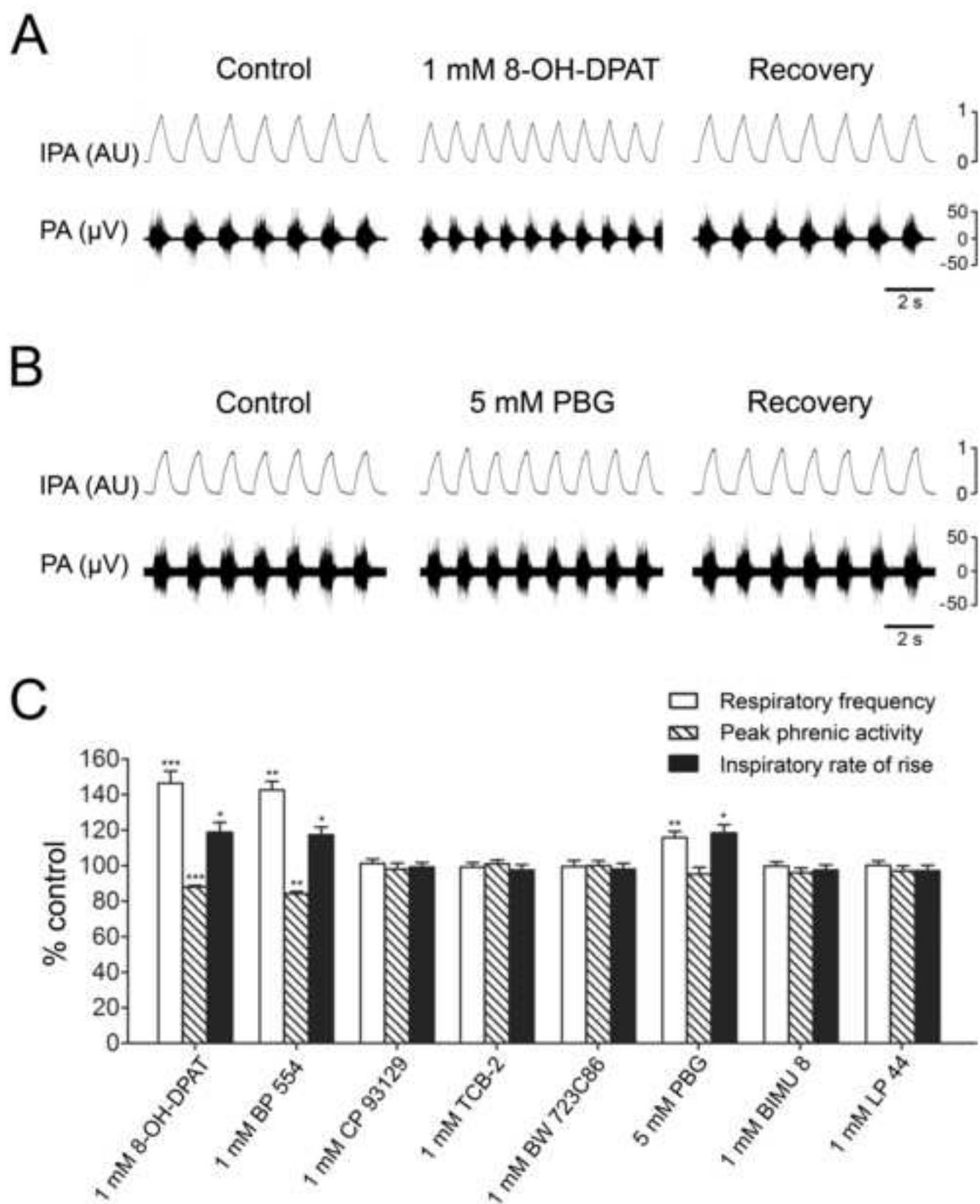
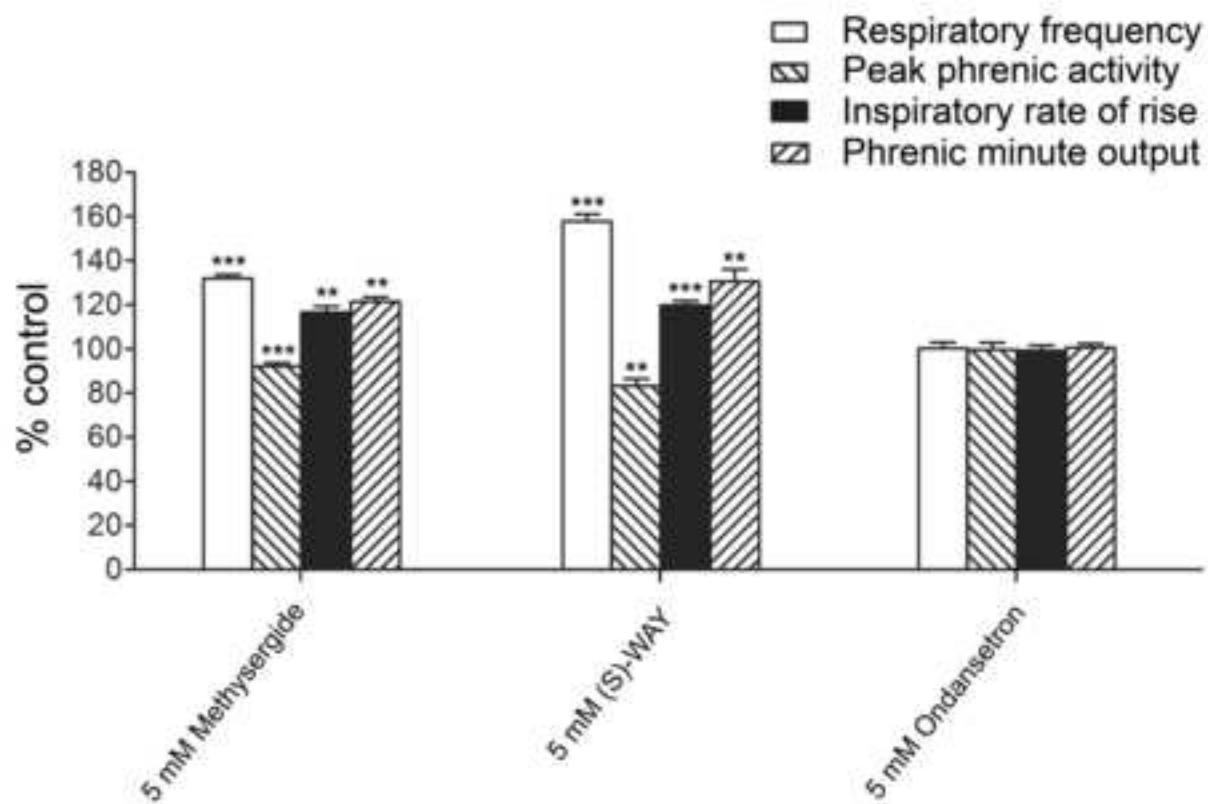


Figure 3
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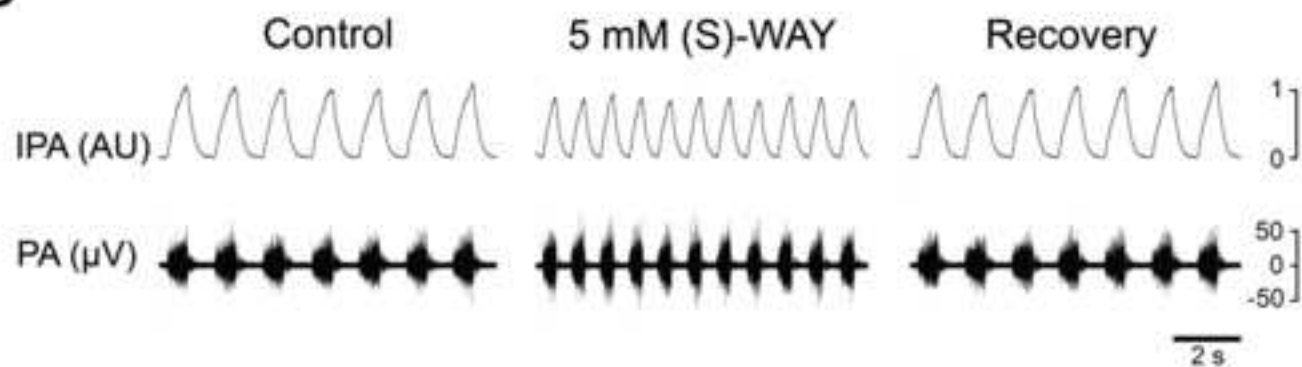


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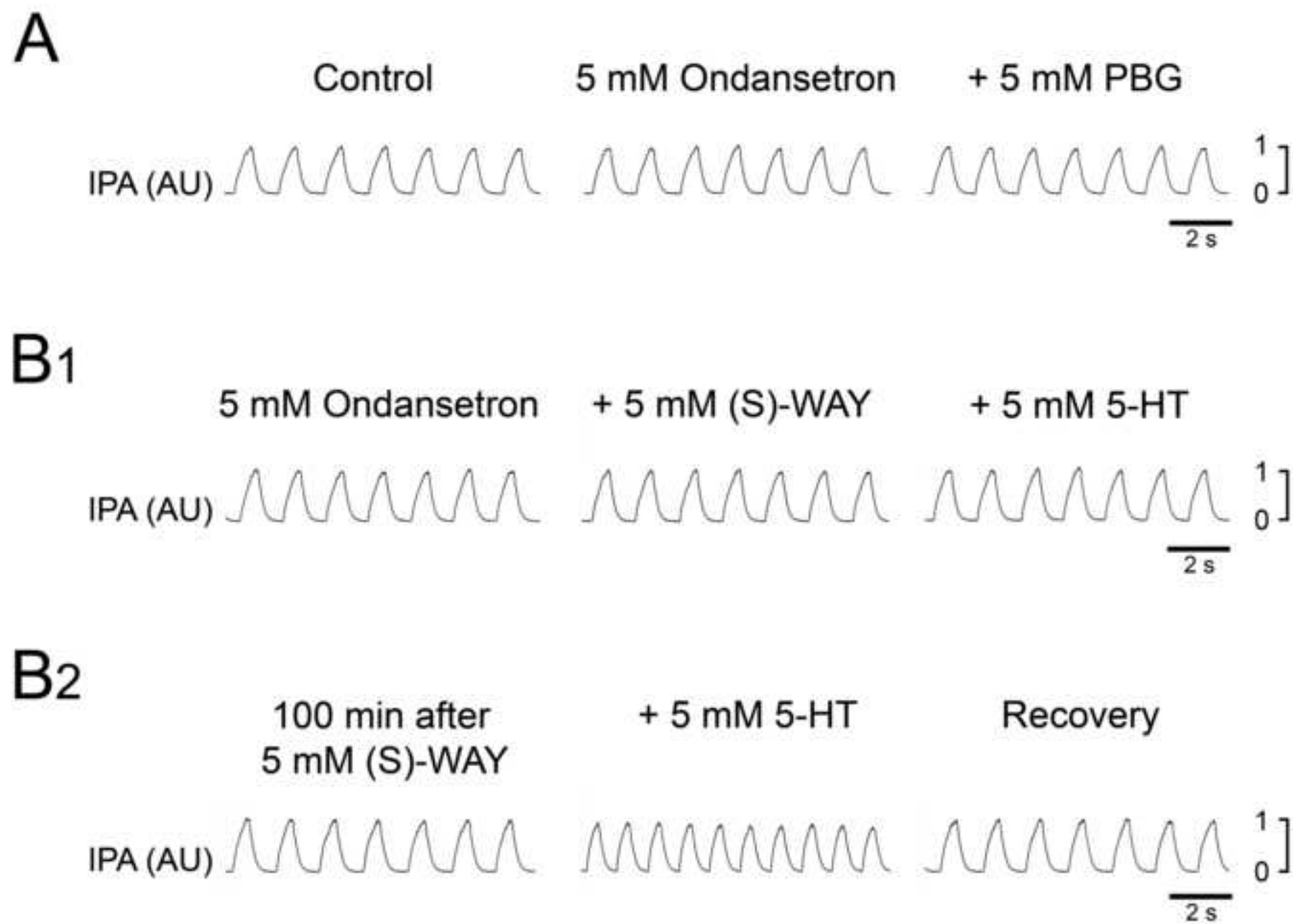


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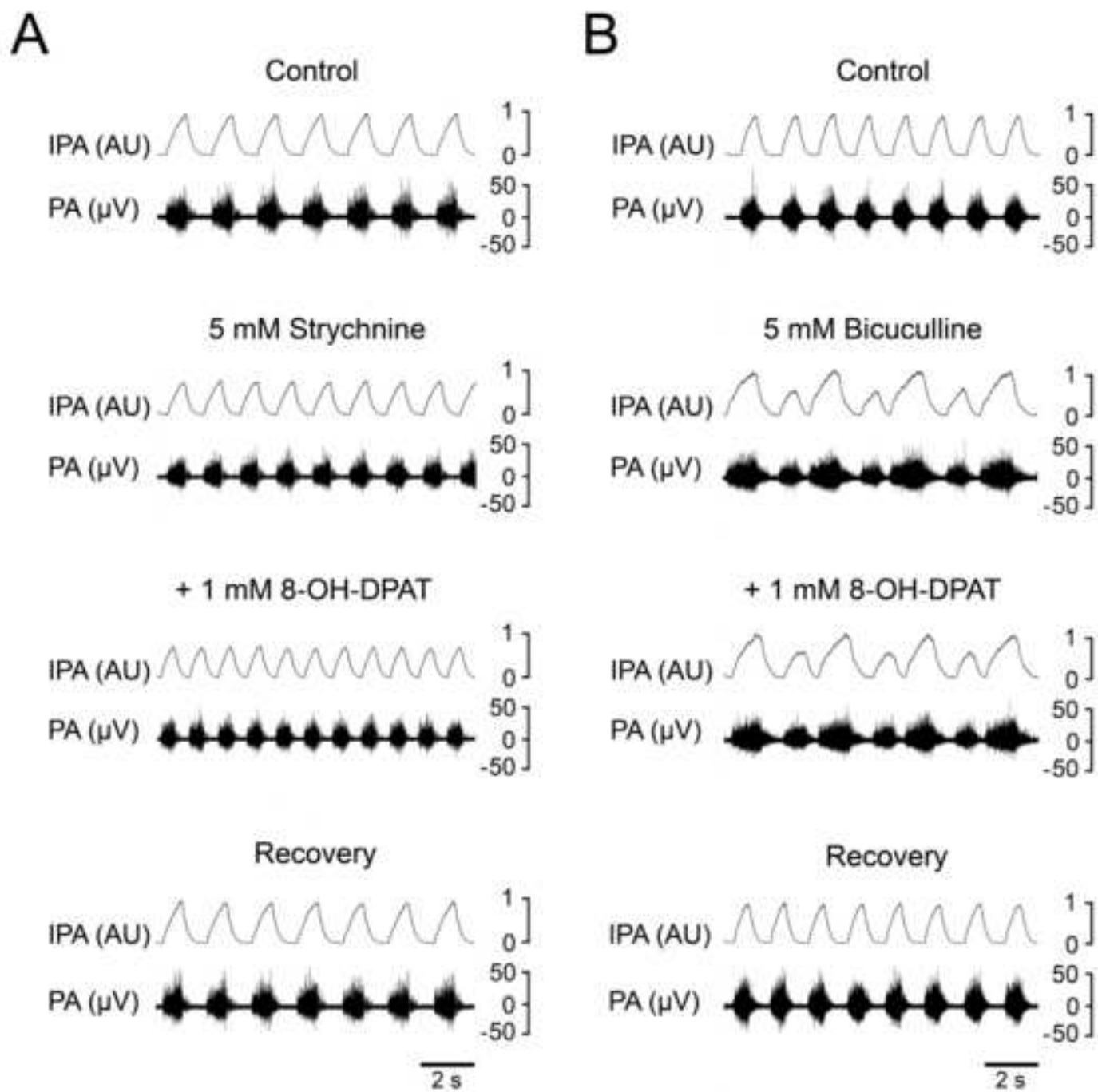


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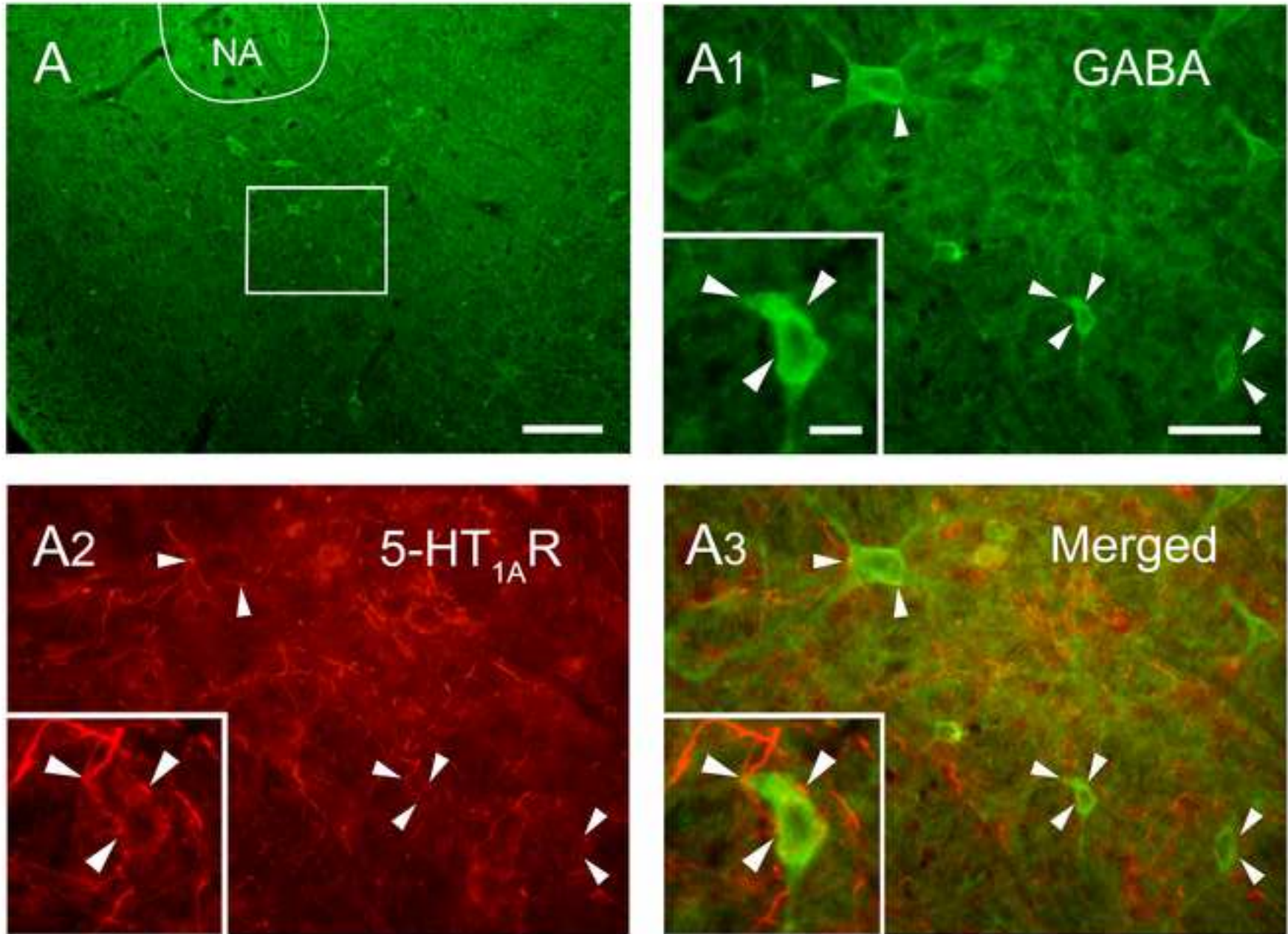


Figure 7

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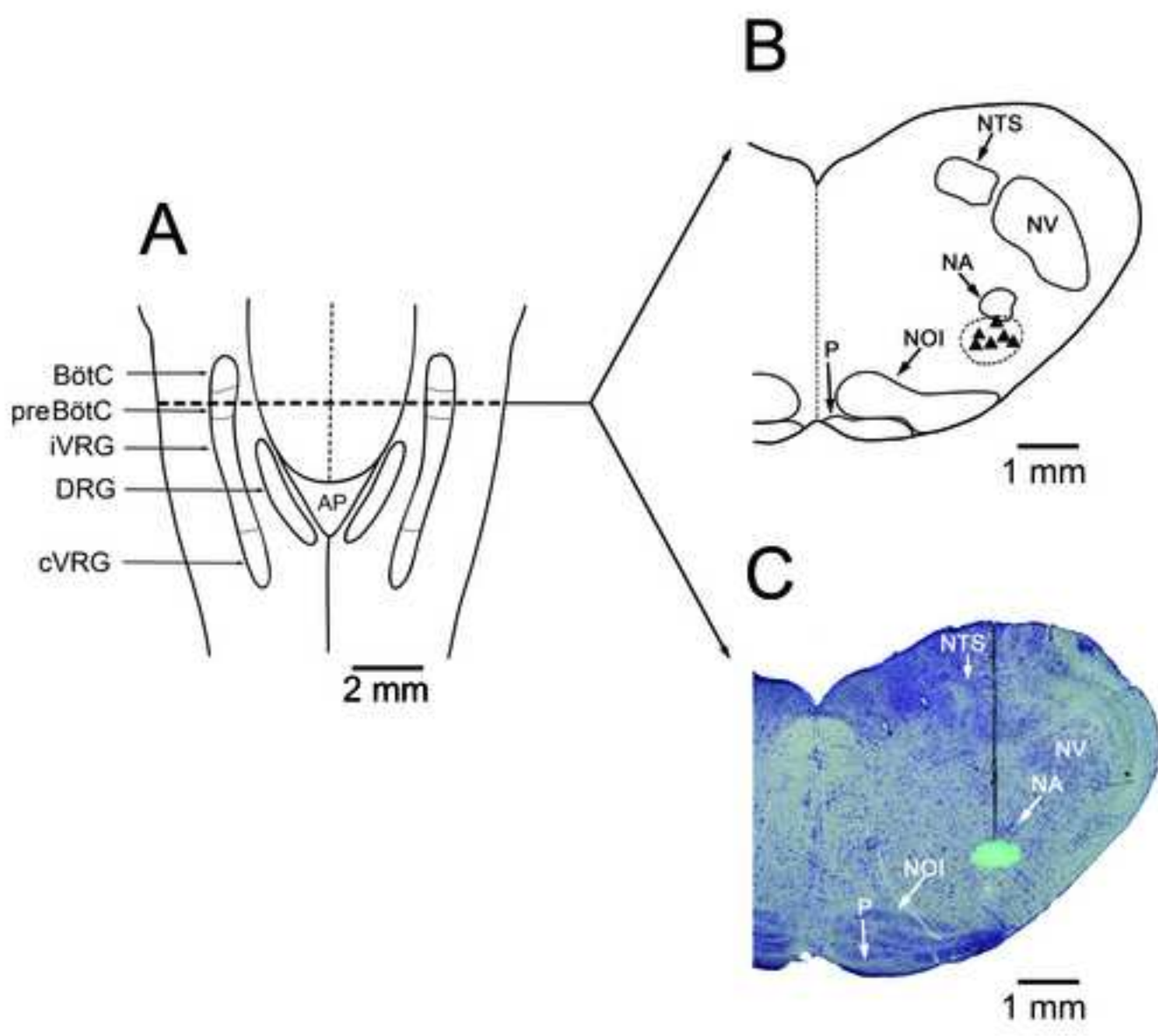


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