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Abstract

Serotonin (5-HT) has been reported to play excitatory effects on respiration by acting on preBötzinger complex (preBötC) neurons in neonatal or juvenile rodents. However, whether its action is circumscribed to the preBötC and present in other animal species, particularly in adult preparations, is unknown. We investigated the respiratory role of 5-HT within the preBötC and neighbouring respiration-related regions. Experiments were performed on α-chloralose-urethane anesthetized, vagotomized, paralyzed and artificially ventilated rabbits making use of bilateral microinjections (30-50 nl). 5-HT caused excitatory effects on respiratory activity only when applied to the preBötC. These effects were mediated by 5-HT_{1A} and 5-HT_3 receptors as shown by microinjections of specific agonists of the different types of 5-HT receptors. Unexpectedly, the blockade of 5-HT_{1A} receptors by methysergide or the specific antagonist (S)-WAY 100135 induced excitatory respiratory effects. Microinjections of the 5-HT_3 receptor antagonist ondansetron did not influence respiration, but prevented (S)-WAY 100135-induced responses. The blockade of $GABA_A$ receptors by bicuculline within the preBötC prevented the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT. The involvement of GABAergic inhibition and 5-HT_{1A} receptor-mediated disinhibition is also corroborated by immunohistochemical data. The results show for the first time in an adult animal preparation that 5-HT plays a pivotal role in the modulation of the preBötC activity probably via both presynaptic and postsynaptic mechanisms and highlight the importance of disinhibition phenomena. Present findings may be relevant to some respiratory disorders in which an impairment of central 5-HT mechanisms has been reported, such as sleep apnoea and sudden infant death syndrome.

Keywords: control of breathing; preBötzinger complex; serotoninergic system

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

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 preBötzinger complex (preBötC) neurons. More recently, an optogenetic study by DePuy and colleagues (2011) performed in anesthetized mice demonstrated that stimulation of raphe obscurus serotoninergic 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; Bongianni et al., 2010), i.e. the Bötzinger 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

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

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

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 $5\text{-}HT_{1A}$ 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 $5\text{-}HT_{1A}$ 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-HT_7 receptors (Manzke et al., 2009; Corcoran et al., 2014), we applied 1 mM (*R*)-(+)-8-Hydroxy-DPAT hydrobromide in the presence of the 5-HT_7 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-HT_7 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-HT_3 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-HT_4 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-HT_3 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-HT_3 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-HT_3 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-HT_3 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

respiratory frequency; $F_{(5,15)} = 5.247$, $p = 0.0478$ for peak phrenic activity) showed (*post hoc* analysis) that bilateral microinjections of 5 mM ondansetron prevented the respiratory responses caused by microinjections of 5 mM (S)-WAY 100135 into the same sites made after ~ 5 min and that microinjections of 5 mM 5-HT performed ~ 20 min after (S)-WAY 100135, i.e. during the blockade of both 5-HT_3 and 5-HT_{1A} receptors, failed to induce any appreciable change in the respiratory motor output (Fig. 4B1). Of note, the blockade of both 5-HT_3 and 5-HT_{1A} receptors did not cause any change in the control respiratory variables (Fig. 4B1, middle panel). *Post hoc* analysis also proved that the characteristic excitatory responses to 5 mM 5-HT microinjections were evoked again after > 90 min, i.e. after a time interval judged sufficient for the complete washout of (S)-WAY 100135 ($p = 0.0241$ for respiratory frequency, $p = 0.0457$ for peak phrenic activity). An example of these findings is reported in Fig. 4B2.

2.4 Role of disinhibition in the respiratory responses caused by 5-HT_{1A} receptor activation

The possibility that 8-OH-DPAT effects were mediated by disinhibition of inhibitory neurons located within the preBötC (Manzke et al., 2009; Corcoran et al., 2014) was addressed by investigating the responses induced by this agonist during the blockade of glycine or $GABA_A$ receptors. In the study of 8-OH-DPAT effects during glycine receptor blockade ($n = 5$), a repeated-measures ANOVA showed significant changes in the respiratory variables ($F_{(2,8)} = 105.2$, $p < 0.0001$ for respiratory frequency; $F_{(2,8)} = 73.7$, $p = 0.0009$ for peak phrenic amplitude). In more detail (*post hoc* tests), bilateral microinjections of 5 mM strychnine (150-250 pmol) induced within 5 min mild increases in respiratory frequency from 43.5 ± 0.7 to 53.5 ± 1.6 breaths min^{-1} (23.1 ± 4.1%; $p = 0.0132$) associated with decreases in peak phrenic amplitude (21.2 ± 3.4%; $p = 0.0089$) in agreement with our previous results (Bongianni et al., 2010). The subsequent (~ 10 min interval) microinjections of 1 mM 8-OH-DPAT at the same sites caused within 10 min consistent further increases in respiratory frequency of 36.4 ± 5.4% (from 53.5 ± 1.6 to 72.7 ± 1.9 breaths min^{-1}; $p = 0.0054$) accompanied by further decreases in peak phrenic amplitude (11.2 ± 0.4%; $p = 0.0003$). These outcomes are illustrated in Fig. 5A.

Our observation on the effects of 8-OH-DPAT during $GABA_A$ receptor blockade provided the following results (Fig. 5B). According to our previous findings (Bongianni

et al., 2010), bilateral microinjections of 5 mM bicuculline (150-250 pmol; n = 4) into the preBötC elicited within 3 min a pattern of breathing characterized by an overall decrease in respiratory frequency and the presence of two alternating different levels of peak phrenic activity. A repeated-measures ANOVA showed significant changes in the respiratory variables ($F_{(2,6)}$ = 35.6, p = 0.0031 for respiratory frequency; $F_{(4,12)}$ = 85.3, p = 0.0007 for peak phrenic amplitude). *Post hoc* tests showed that bilateral microinjections of bicuculline induced decreases in respiratory frequency from 38.3 ± 2.0 to 28.7 ± 3.4 breaths min^{-1} (26.2 ± 5.1%; p = 0.0263) associated with a level of peak phrenic activity higher than control (21.8 ± 1.5 %; p = 0.0009) and a second level of peak phrenic activity lower than control (26.7 ± 1.7%; p = 0.0088). The statistical analysis also showed that the subsequent (~10 min interval) bilateral microinjections of 1 mM 8-OH-DPAT at the same sites did not induce significant changes in the already disordered pattern of breathing, thus showing that bicuculline-induced disinhibition actually prevented 8-OH-DPAT excitatory effects. In more detail, respiratory variables maintained similar values, i.e., respiratory frequency was 29.0 ± 3.3 breaths min^{-1} (25.2 ± 5.3 % lower than control), peak phrenic amplitudes were 24.0 ± 5.8 % higher than control and 26.7 ± 2.4 % lower than control, respectively. Respiratory activity recovered control levels within 90 min.

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

2.5 Controls

The location of injection sites was confirmed by the histological control (Fig. 7). The distribution of sites within the preBötC where 5 mM 5-HT was microinjected turned out

to be ventral to the nucleus ambiguus and is diagrammatically illustrated in Fig. 7B. An example of the localization of fluorescent beads is reported in Fig. 7C. The specificity of drug-induced responses was confirmed by the results obtained with control microinjections. According to previous studies (e.g. Mutolo et al., 2002, 2005; Bongianni et al., 2010), bilateral microinjections of 5 mM 5-HT into medullary regions sufficiently far (> 0.8 mm) from the responsive area did not induce any significant change in the ongoing respiratory activity. These control microinjections were performed both in the lateral direction (4 trials) and in the rostrocaudal direction, i.e. the microinjections performed into the BötC and iVRG. In addition, similar control bilateral microinjections (4 trials) were made into the nucleus tractus spinalis nervi trigemini or the nucleus gigantocellularis of the same preparations already employed in the study of 5-HT-induced respiratory effects. Control microinjections of equal volumes of the vehicle solutions performed in some preparations before 5-HT (3 trials) or (S)-WAY 100135 (3 trials) microinjections (0.9% NaCl solution and DMSO <10% in saline, respectively) into the preBötC did not cause appreciable effects. Noticeably, all the observed respiratory responses occurred without any significant concomitant change in mean arterial blood pressure that ranged between 90 and 103 mmHg in the different preparations. In the same preparation, small and inconsistent changes in mean arterial 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-HT_3 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-HT_3 receptor antagonist ondansetron does not influence respiration. Interestingly, (S)-WAY 100135 is ineffective during 5-HT_3 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

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

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

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

One of the main point of interest of the present study is the potent 5-HT-mediated activation of the respiratory motor output through an action on the preBötC. This finding appears to be in keeping with the vast majority of data obtained in neonatal and juvenile *in situ* rodent preparations (e.g. Al Zubaidy et al., 1996; Pena and Ramirez 2002; Schwarzacher et al., 2002; Gunther et al., 2006; Ptak et al., 2009; Niebert et al., 2011; Corcoran et al., 2014; for review, see Hilaire et al., 2010; Hodges and Richerson 2008, 2010). However, our results are at variance with those obtained in the dog by Radocaj et al. (2015) who found that the local application of 8-OH-DPAT produced dose-dependent decreases in the activity of preBötC neurons, while microinjections of 5-HT and 8-OH-DPAT along the whole extent of the ventral respiratory column did not

produce any effect on respiration. They suggested that the tachypneic effects of intravenous 8-OH-DPAT injections could be attributed to other brainstem areas. However, species differences cannot be completely ruled out (see also Hilaire et al., 2010).

Indeed, medullary serotoninergic neurons, mainly located in the brainstem raphe nuclei and parapyramidal regions, project extensively to the brainstem respiratory network including the preBötC and, in addition, to hypoglossal and phrenic motoneurons (Ptak et al., 2009; see also Hodges and Richerson 2008; Paterson et al., 2009; Hilaire et al., 2010; Ramirez et al., 2013). Interestingly, these 5-HT projections are generally immunoreactive also for the neuropeptides SP and thyrotropin-releasing hormone (for review, see Hodges and Richerson 2008). Accordingly, it has been shown that raphe magnus stimulation increases respiratory frequency in mice due to the release of 5-HT and, possibly, SP in the preBötC (Doi and Ramirez 2010). A recent study by DePuy et al. (2011) in mice by using optogenetics demonstrated that the activation of raphe obscurus serotoninergic neurons stimulates breathing and that transfected serotoninergic neurons have multiple targets in both the lower brainstem and the spinal cord. Their observations extend previous evidence that stimulation of the raphe obscurus activates breathing and are consistent with the excitatory action of 5-HT on phrenic motoneurons and many brainstem respiratory neurons, including those of the preBötC and the retrotrapezoid nucleus.

The mechanism by which raphe 5-HT neurons affect the respiratory network (Richter et al., 2003; Hodges and Richerson 2008, 2010) and their role in the respiratory rhythmogenesis (e.g. Pena and Ramirez 2002; Toppin et al., 2007) have been debated. These issues have been recently clarified, at least to a great extent, by Ptak et al. (2009). They showed in *in vitro* neonatal and in *in situ* juvenile rat preparations that the stimulation of the raphe obscurus, that is reciprocally connected with the preBötC, excites simultaneously both preBötC neurons and the respiratory motor output by means of the release of 5-HT and SP. In accordance with these findings, our previous studies have already shown that SP microinjections into the preBötC region, but not in the BötC and iVRG, exert intense excitatory respiratory effects (see e.g. Bongianni et al., 2008 also for further Refs). In the light of the present results we can suggest that not only SP, but also 5-HT microinjections into the preBötC can be additional tools for the physiological identification of this VRG subregion. In conclusion, the preBötC proved to be an important site of action of 5-HT, although we cannot exclude that other

responsive brainstem respiration-related areas may exist. Indeed, 5-HT receptors have been reported to be widely expressed in the VRG at least in some animal species (Hodges and Richerson 2008, 2010; Dutschmann et al., 2009; Hilaire et al., 2010), but unexpectedly we found that 5-HT microinjected into regions adjacent to the preBötC was ineffective. On the other hand, to our knowledge, no extensive investigations exist on the effects of microinjections of 5-HT into the BötC or the iVRG. We believe that this topic deserves further immunohistochemical and neurophysiological studies.

3.3 Excitatory role of 5-HT$_{1A}$ and 5-HT$_3$ receptors within the preBötC

Somewhat at variance with previous studies on neonatal or juvenile rodents showing an involvement of varied 5-HT receptors in the control and even in the generation or maintenance of respiratory activity (Pena and Ramirez 2002; Schwarzacher et al., 2002; Gunther et al., 2006; Ptak et al., 2009; Niebert et al., 2011; Corcoran et al., 2014; see Hodges and Richerson 2008, 2010; Hilaire et al., 2010 for further Refs.), we found that 5-HT$_{1A}$ and 5-HT$_3$ receptors are involved in 5-HT-induced excitatory effects on respiration at the level of the preBötC in the adult rabbit. It seems too difficult to propose an exhaustive explanation for such discrepancy. Probably it is mainly related to differences in the animal species and developmental stage. Our outcomes have been proven by several trials with 5-HT receptor agonists and antagonists and noticeably by the lack of 5-HT effects during the blockade of both 5-HT$_{1A}$ and 5-HT$_3$ receptors. To our knowledge, this is the first report showing that 5-HT$_3$ receptors have an excitatory role at the preBötC level. However, they do not seem to exert a tonic influence on respiration under basal conditions since the 5-HT$_3$ receptor antagonist was ineffective. Nevertheless, the presence of 5-HT$_3$ receptors allowed us to provide a tentative explanation of the excitatory effects of the 5-HT$_{1A}$ receptor antagonist (S)-WAY 100135 by suggesting an action of this drug at the presynaptic level. Indeed, 5-HT$_{1A}$ autoreceptors in the raphe nuclei have been extensively described only on serotoninergic neurons (Nichols and Nichols 2008). Although presynaptic 5-HT$_{1A}$ receptors have been found to be involved in the regulation of the release of GABA and/or glutamate (e.g. Luttgen et al., 2005; Dergacheva et al., 2011; Ostrowski et al., 2014; Stiedl et al., 2015), only suggestions have been advanced on the possible role of 5-HT$_{1A}$ presynaptic receptors located on serotoninergic terminals involved in the release of 5-HT at sites far from serotoninergic neurons (Wang and Ramage 2001). Here, we provide only indirect evidence of the presence of such presynaptic receptors.

A specifically designed immunohistochemical analysis should be performed to ascertain our proposal as well as the presence of 5-HT_3 receptors on preBötC neurons. However, this appears to be far beyond the scope of the present research.

3.4 Disinhibition phenomena underlying respiratory effects due to 5-HT_{1A} receptor activation

The interpretation of bicuculline-induced effects is very difficult, but it seems conceivable that bicuculline microinjections do not affect a segregate inhibitory pathway involved in the mediation of 5-HT-induced effects. Rather, they cause a generalized blockade of the GABAergic inhibitory control within the preBötC that may lead to a disruption of the ongoing pattern of breathing. For more details on this matter we refer to our previous report (see also Kam et al., 2013). In any case, we found that bicuculline prevented the excitatory effects of the 5-HT_{1A} receptor agonist, thus the hypothesis was advanced that these effects were due to disinhibition of GABAergic neurons located in the preBötC region (e.g. Kuwana et al., 2006; Shevtsova et al., 2011; Koizumi et al., 2013) via postsynaptic 5-HT_{1A} inhibitory receptors, with a possible additional regulatory role of presynaptic 5-HT_{1A} receptors. Thus, these findings imply that GABAergic neurons exert a tonic inhibitory influence on preBötC neurons. These conclusions are strongly corroborated by immunohistochemical results showing the presence of 5-HT_{1A} immunoreactive dots in close apposition to the soma of GABAergic immunoreactive neurons of the preBötC, consistently with the possibility of a postsynaptic localization of 5-HT_{1A} receptor immunoreactive structures. The existence of postsynaptic and presynaptic 5-HT_{1A} receptors within the preBötC region should be ascertained in further studies. Present findings are consistent with the presence of 5-HT_{1A} receptors on GABAergic neurons in other brain structures (e.g. Luttgen et al., 2005; Ostrowski et al., 2014; Stiedl et al., 2015). Interestingly, a prominent role of GABAergic inhibitory control at the level of the preBötC has already been described in the rabbit (Bongianni et al., 2010). Furthermore, the presence of a disinhibitory mechanism is in general agreement with the results of previous studies in rodents (Manzke et al., 2009; Corcoran et al., 2014) in which, however, an involvement of glycinergic neurons has been found. In conclusion, it seems that regardless of the animal species disinhibition involving either glycinergic or GABAergic neurons can be

a prominent mechanism subserving 5-HT excitatory effects on respiratory activity (Manzke et al., 2009; Shevtsova et al., 2011; Corcoran et al., 2014).

In conclusion, the main novel findings of the present study are that: 1) 5-HT exerts its modulatory action only at the level of the preBötC through 5-HT_{1A} and 5-HT_3 receptors, without affecting the neighbouring respiration-related regions; 2) 5-HT_{1A} receptors are endogenously activated, while 5-HT_3 receptors do not play any tonic role on respiratory activity; 3) excitatory respiratory effects induced by the activation of 5-HT_{1A} receptors involve preBötC GABAergic neurons. These outcomes are complex and their interpretation is obscure. However, considering the novelty of present findings we believe that would be of interest to advance a proposal on their functional significance.

We propose the existence of two separate serotoninergic mechanisms (Fig. 8) that, according to the available literature, can reasonably be brought into action by different stressors such as hypoxia (Erickson and Sposato 2009; Chen et al., 2013) or hypercapnia (Phillipson et al., 1977; Berthon-Jones and Sullivan 1984; Buchanan et al., 2015) and by opioid-induced respiratory depression (Sahibzada et al., 2000; Dutschmann et al., 2009; Manzke et al., 2009; Ren et al., 2015). Under basal conditions, 5-HT release should be minimum, if any, because the blockade of both 5-HT_3 and 5-HT_{1A} receptors within the preBötC (see Fig. 4B1, middle panel) does not alter the ongoing respiratory activity. However, it strongly increases when these 5-HT mechanisms are activated. The first mechanism consists of the release of 5-HT tuned by 5-HT_{1A} receptor-mediated presynaptic inhibition and the activation of the ionotropic excitatory 5-HT_3 receptors located on preBötC excitatory neurons. The second mechanism, engaging the same or separate serotoninergic afferent pathways, involves the release of 5-HT (possibly controlled by presynaptic 5-HT_{1A} receptors?) causing the activation of 5-HT_{1A} inhibitory receptors on a specific set of GABAergic cells innervating the preBötC inspiratory neurons and consequent disinhibition phenomena leading to the activation of inspiratory activity. These mechanisms should activate simultaneously both the respiratory motor output and hypoglossal motoneurons resulting in hyperventilation and increased airway patency (see Ptak et al., 2009). Details on these mechanisms obviously require further investigations. Increases in $GABA_A$-mediated inhibition have recently been suggested to be involved in the correction of respiratory disorders (frequent apnoeas characterized by prolonged postinspiratory activity in the cervical vagus nerve possibly associated with tonic abdominal activity) in a mouse model of Rett syndrome (Abdala et al., 2010). However,

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* (Alsahafi 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-HT_3 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.

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

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

occipital craniotomy, and the dura and arachnoid membranes were removed. The posterior part of the cerebellum was removed by gentle suction to provide access to the rostral part of the medulla. All exposed tissues were covered with warm paraffin oil (~38°C). Body temperature was maintained at 38.5-39°C by a heating blanket controlled by a rectal thermistor probe. The animals were vagotomized, paralyzed (gallamine triethiodide 4 mg/kg i.v., supplemented with 2 mg/kg every 30 min; Sigma-Aldrich) and artificially ventilated with oxygen-enriched room air. The oxygen (80%) was added to the inlet of the respirator. End-tidal CO_2 partial pressure was maintained approximately at the level of spontaneous breathing (28.5-32 mmHg) by adjusting the frequency and stroke volume of the respiratory pump.

4.3 Recording procedures

Efferent phrenic nerve activity was recorded with bipolar platinum electrodes from desheathed C5 phrenic roots, amplified, full-wave rectified and passed through a leaky integrator (low-pass RC filter, time constant 100 ms) to obtain a 'moving average' of the activity, usually referred to in the literature as 'integrated' activity. Extracellular recordings from medullary neurons were made with tungsten microelectrodes (5-10 MΩ impedance as tested at 1 kHz). The most rostral extent of the area postrema on the midline was defined as the obex and used as a standard point of anatomic reference. Neuronal activity was recorded from rostral expiratory neurons of the BötC (3.0-4.5 mm rostral to the obex, 2.4-3.2 mm lateral to the midline and 3.5-4.6 mm below the dorsal medullary surface), from the iVRG (from 0.7 caudal to 2.0 mm rostral to the obex, 2.3-3.2 mm lateral to the midline and 3.0-3.5 mm below the dorsal medullary face) and from the transition zone between the BötC and the iVRG where a mix of inspiratory and expiratory neurons is present (2.1-2.9 mm rostral to the obex, 2.4-3.2 mm lateral to the midline and 3.5-4.2 mm below the dorsal medullary surface). The latter region has already been extensively investigated in the rabbit with lesion and neuropharmacological approaches (Bongianni et al., 2008, 2010; Mutolo et al., 2002, 2005; Pantaleo et al., 2011, Stucke et al., 2015) and corresponds to the preBötC described in adult cats and rats (see e.g. Connelly et al., 1992; Schwarzacher et al., 1995; Rekling and Feldman 1998; Solomon 1999; Feldman and Del Negro 2006; Feldman et al., 2013; Del Negro et al., 2018). A strain-gauge manometer was used for monitoring arterial blood pressure. End-tidal CO_2 partial pressure was monitored by an

infrared CO_2 analyzer (Capnocheck Plus, Smiths Medical PM, Waukesha, WI, USA). Cardiorespiratory variables were acquired and analyzed using a personal computer equipped with an analogue-to-digital interface (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) and appropriate software (Axoscope, Molecular Devices).

4.4 Microinjection procedures and experimental protocol

Bilateral microinjections were performed into the BötC, preBötC and iVRG regions as defined by neuronal recordings. In each experiment, recordings of neuronal activity preceded drug microinjections. The patterns of neuronal discharges recorded in the BötC and the preBötC have been already illustrated in our previous reports (e.g. Mutolo et al., 2002; Bongianni et al., 2008, 2010). In addition, the localization of the preBötC was confirmed by evaluating the excitatory respiratory responses induced by bilateral microinjections (30-50 nl) of 20 mM D,L-homocysteic acid (DLH; Sigma-Aldrich), a broad-spectrum excitatory amino acid agonist, into this region (e.g. Solomon et al., 1999; Wang et al., 2002; Monnier et al., 2003; Mutolo et al., 2005; Bongianni et al., 2008, 2010; Stucke et al., 2015).

The following drugs were used: 5 mM 5-HT (endogenous agonist at 5-HT receptors; Tocris Bioscience, Bristol, UK), 1 mM (*R*)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; full 5-HT_{1A} receptor agonist, more active enantiomer; Tocris Bioscience), 1 mM BP 554 maleate (selective 5-HT_{1A} receptor agonist; Tocris Bioscience), 1 mM CP 93129 dihydrochloride (potent and highly selective 5-HT_{1B} receptor agonist; Tocris Bioscience), 1 mM TCB-2 (high affinity 5-HT_{2A} receptor agonist; Tocris Bioscience), 1 mM BW 723C86 hydrochloride (selective 5-HT_{2B} receptor agonist; Tocris Bioscience), 5 mM 1-phenylbiguanide hydrochloride (PBG, selective 5-HT_3 receptor agonist; Sigma-Aldrich), 1 mM BIMU 8 (potent 5-HT_4 receptor full agonist; Tocris Bioscience), 1 mM LP 44 (high affinity 5-HT_7 receptor agonist; Tocris Bioscience), 5 mM methysergide maleate (mixed 5-HT_1/5-HT_2 receptor antagonist; Tocris Bioscience), 5 mM (S)-WAY 100135 dihydrochloride (potent and selective 5-HT_{1A} receptor antagonist; Tocris Bioscience), 5 mM ondansetron hydrochloride (selective 5-HT_3 receptor antagonist; Tocris Bioscience), 5 mM SB 258719 hydrochloride (selective 5-HT_7 receptor antagonist; Tocris Bioscience), 5 mM strychnine hydrochloride (glycine receptor antagonist; Sigma-Aldrich), 5 mM bicuculline methiodide ($GABA_A$ receptor antagonist; Sigma-Aldrich). Drugs were

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; Bongianni 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. Bongianni 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.

Illustration 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

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

4.7 Data collection and statistical analysis

We measured the respiratory frequency (breaths/min), the inspiratory (T_I) and expiratory (T_E) times, as well as the peak amplitude of the integrated phrenic nerve activity that was normalized relative to mean control values (baseline) and expressed in arbitrary units (AU). The phrenic minute output (neural minute ventilation) was calculated as the product of phrenic tidal activity and respiratory frequency. The slope of the straight line drawn from the onset to 90% of the maximum level of the phrenic ramp was considered a reliable estimate of the inspiratory rate of rise (e.g. Bongianni et al., 2002, 2008, 2010; Mutolo et al., 2002, 2005). Respiratory variables were measured for an average of five consecutive breaths in the period immediately preceding each trial and at the time when the maximum response to drug microinjections occurred. In the same periods, systolic and diastolic blood pressure were measured at 2-s intervals. Mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. Owing to the small variations in respiratory and cardiovascular variables within a measurement period, average values for each period were taken as single measurements for the purpose of statistical analysis (GraphPad Prism 7, GraphPad Software, Inc., La Jolla, CA, USA). The recovery of control values was followed for a maximum of two hours. In each preparation, we considered respiratory activity fully recovered when respiratory frequency and peak phrenic amplitude were within ± 3% of

control values. Paired *t*-tests were employed to evaluate changes in cardiorespiratory variables induced by each drug and by vehicle solutions. Unpaired *t*-tests were employed to compare drug-induced cardiorespiratory changes in different preparations. One-way repeated-measures ANOVA followed by Bonferroni's multiple comparisons test was used to assess the effects of two or more treatments on cardiorespiratory variables. For simplicity, only respiratory frequency and peak phrenic amplitude were considered in this latter type of statistical analysis. Changes in respiratory variables were also expressed as percentage variations of control values. All values are presented as means ± SEM; $p < 0.05$ was considered as significant. In the legends, the level of significance is indicated as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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 ± 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-HT_3 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 ± 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 ± SEM. **p < 0.01; ***p < 0.001. (B) Respiratory effects ~ 10 min after bilateral microinjections of the 5-$HT1_A$ 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.

Fig. 4. Role of 5-HT_3 receptor antagonist in counteracting the effects mediated by 5-HT_3 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-HT_3 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-HT_3 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.

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.

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 µm; (A1-A3), 50 µm; insets, 10 µm.

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 (~ 2.5 mm rostral to the obex) indicated in (A) (dashed line) showing the distribution of sites (▲) 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-HT_3 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-HT_3 receptors does not alter baseline respiratory activity. Conceivably, different stressors, such as hypoxia, hypercapnia or opioid-induced respiratory depression, have been reported to activate serotoninergic mechanisms. The augmented release of 5-HT overcomes presynaptic inhibition and stimulates postsynaptic 5-HT_3 receptors on

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

Figure 1

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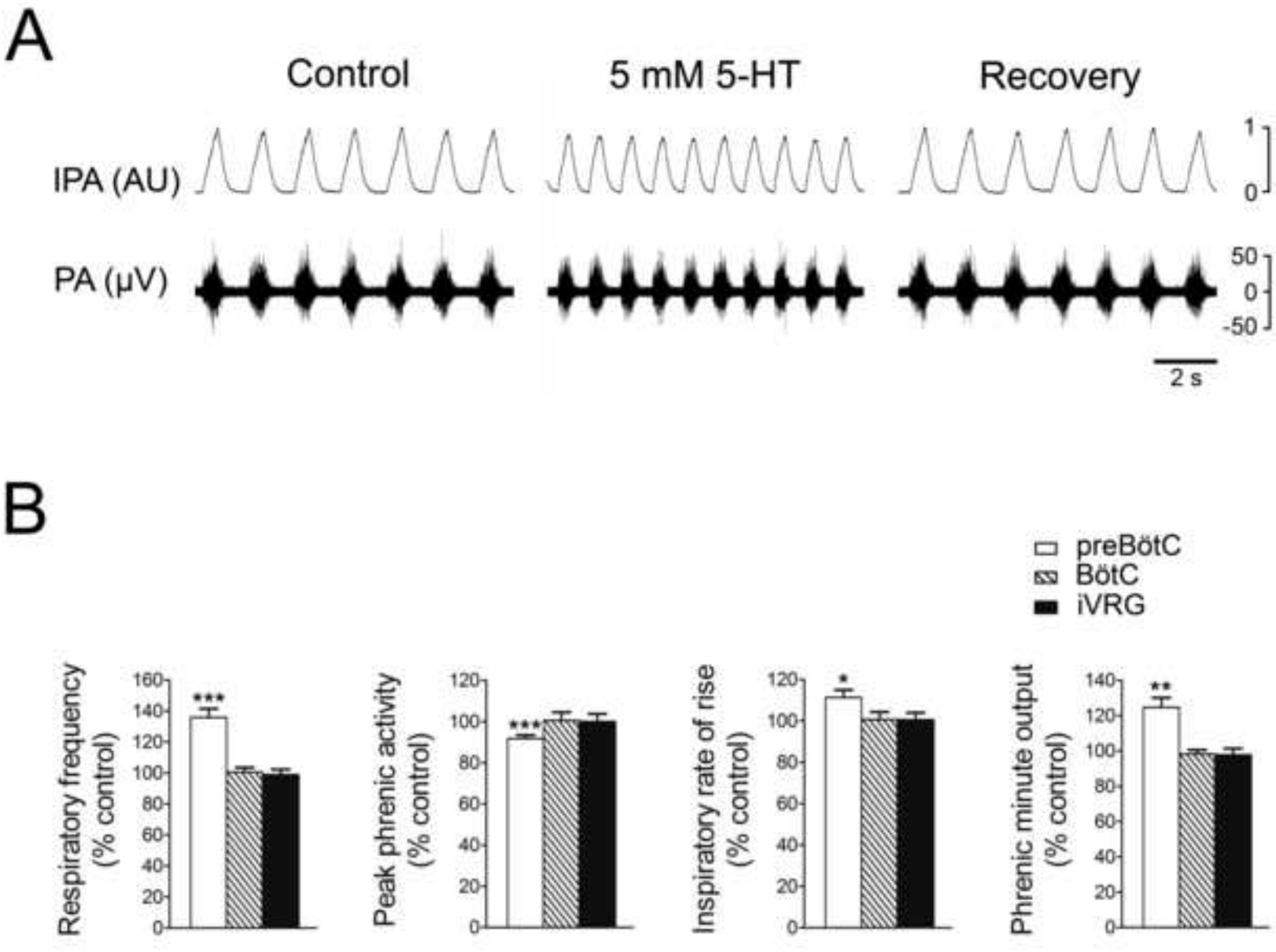

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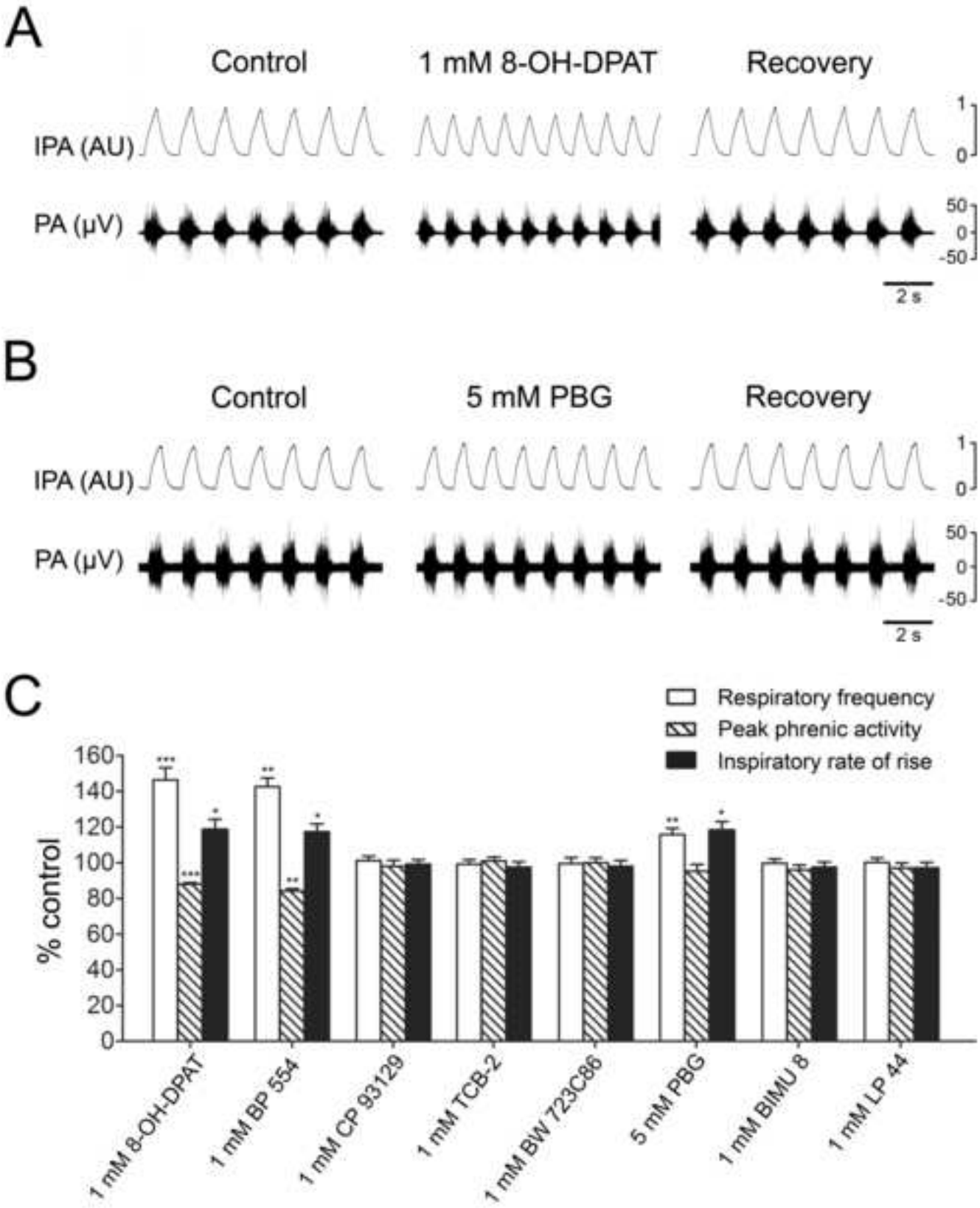

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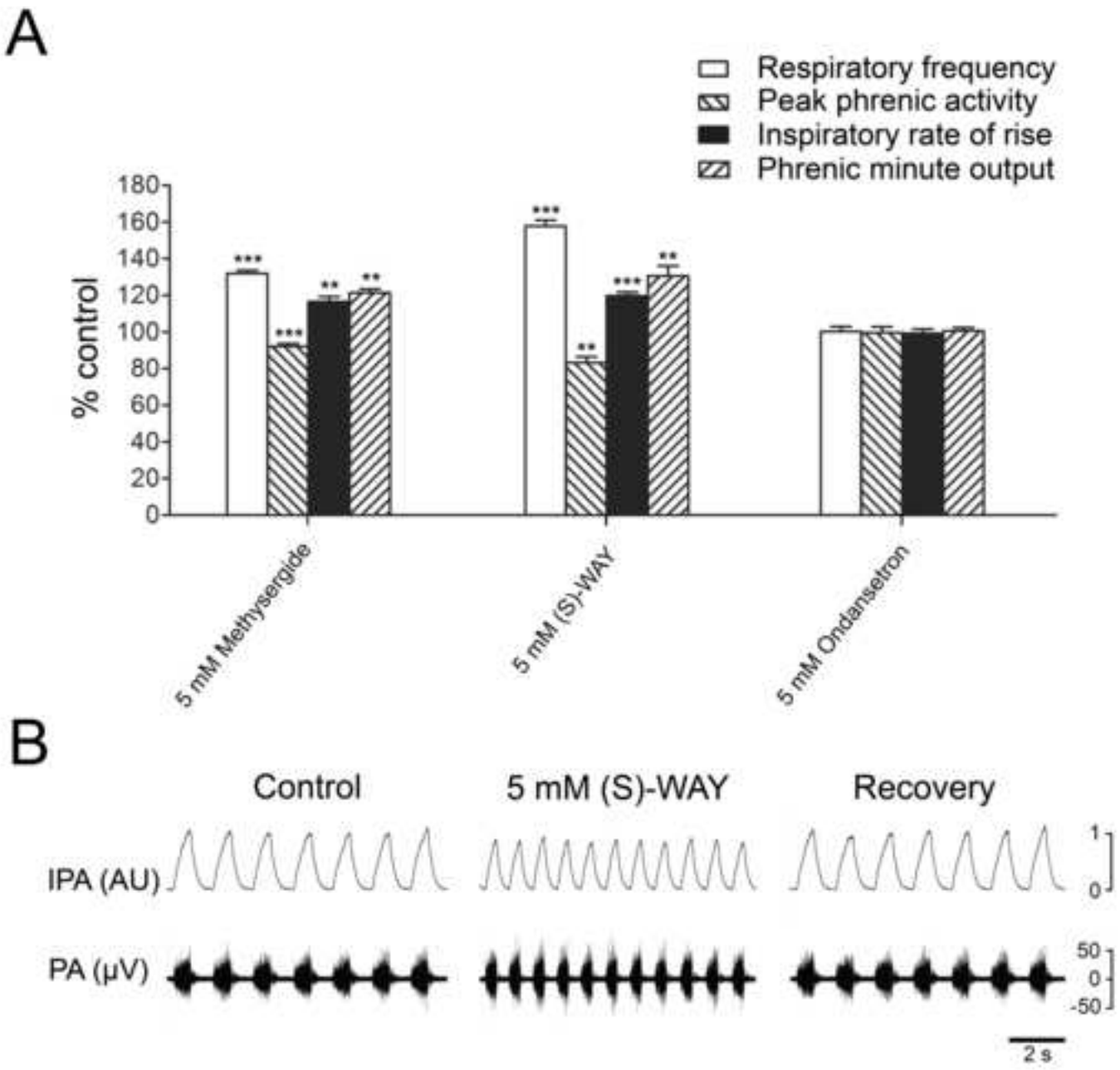

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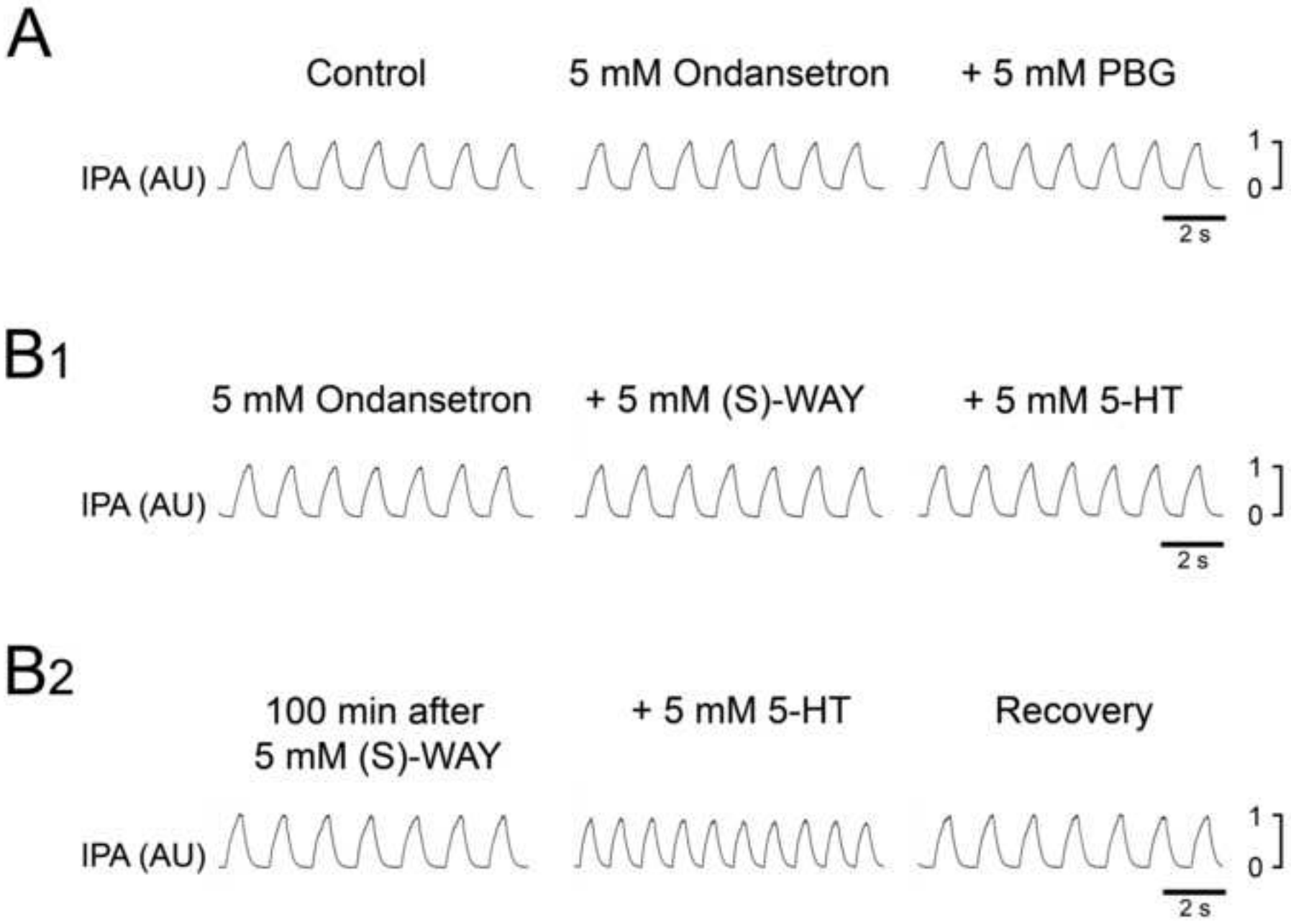

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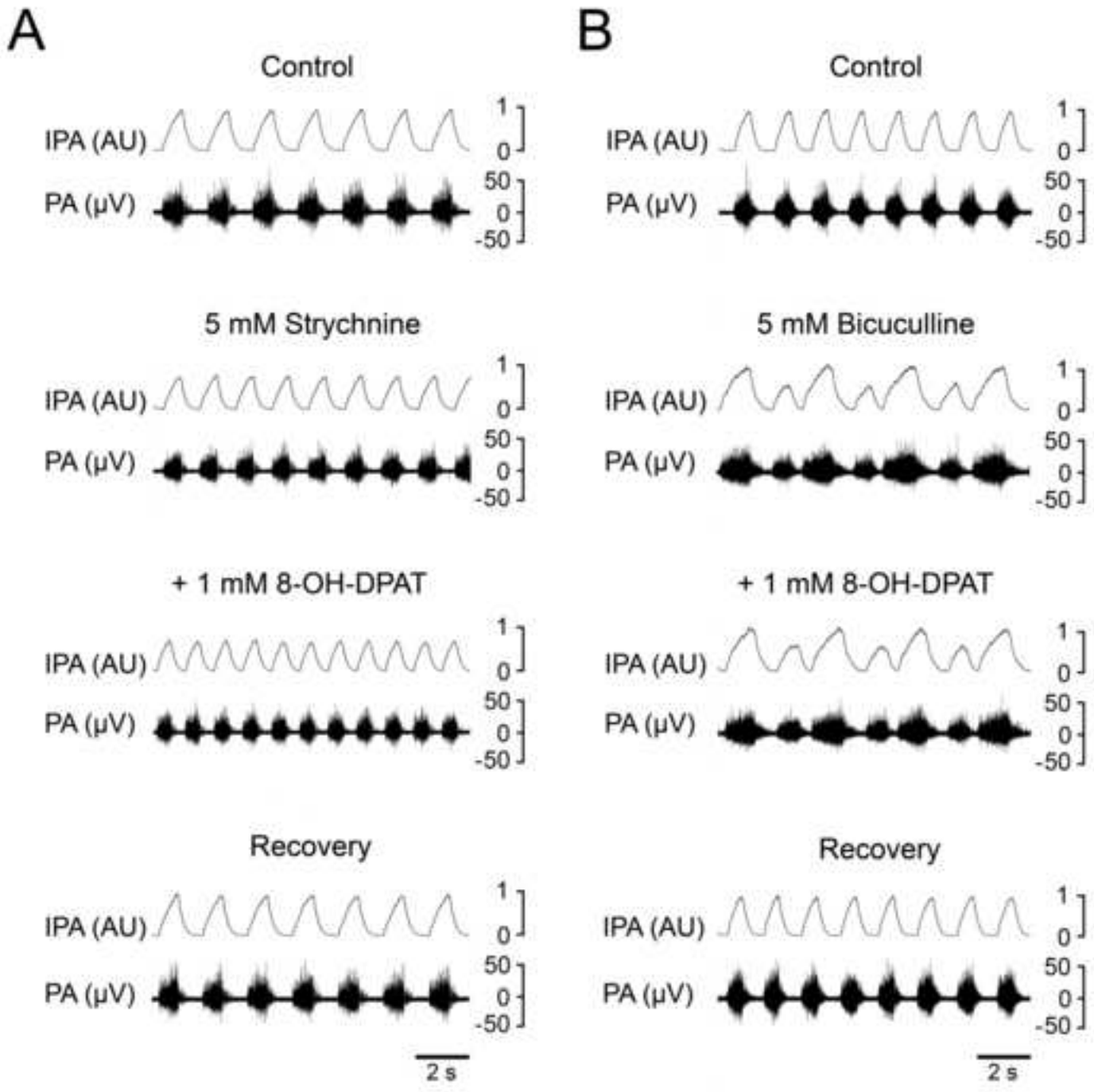

Figure 6

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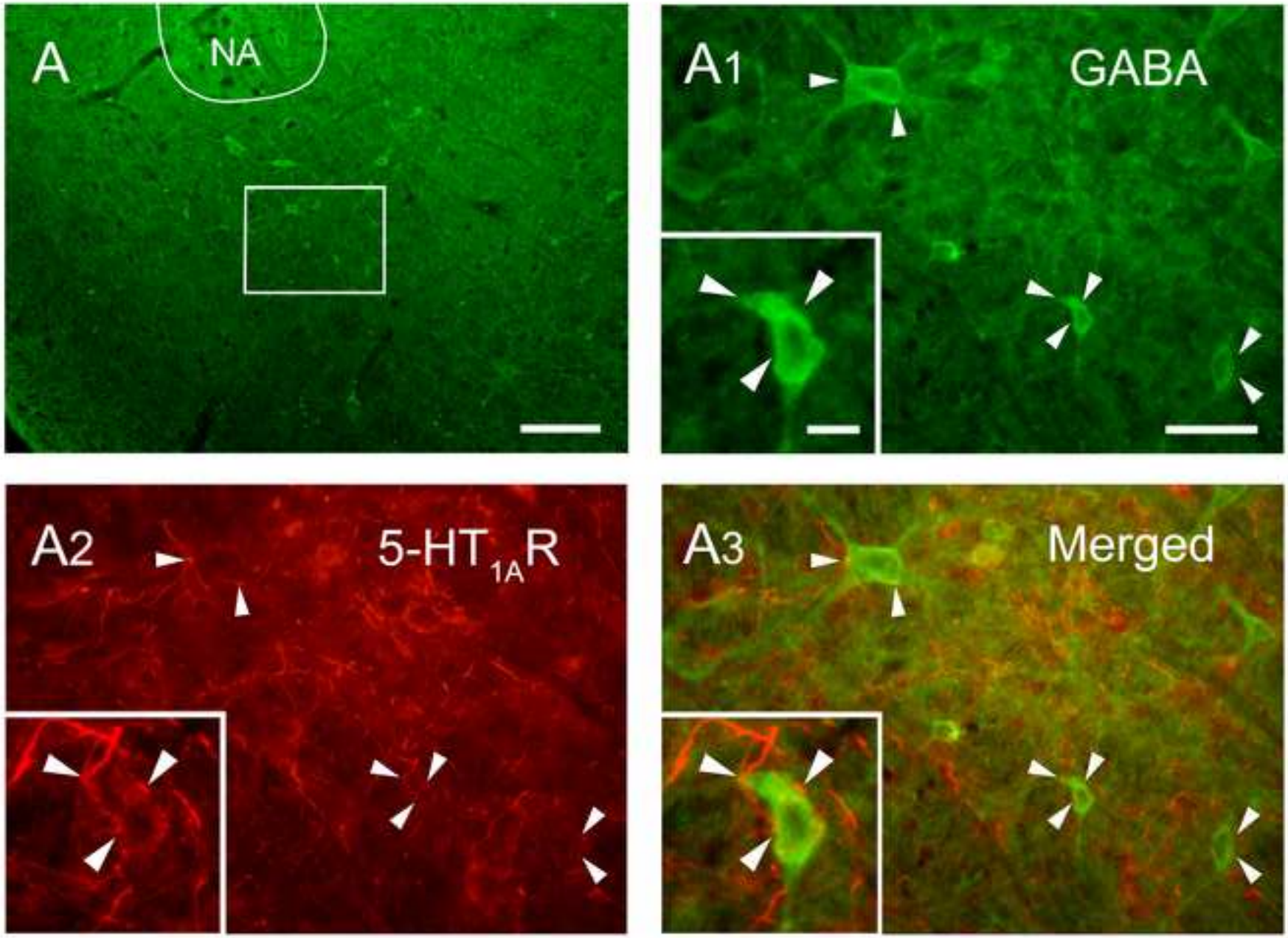

Figure 7

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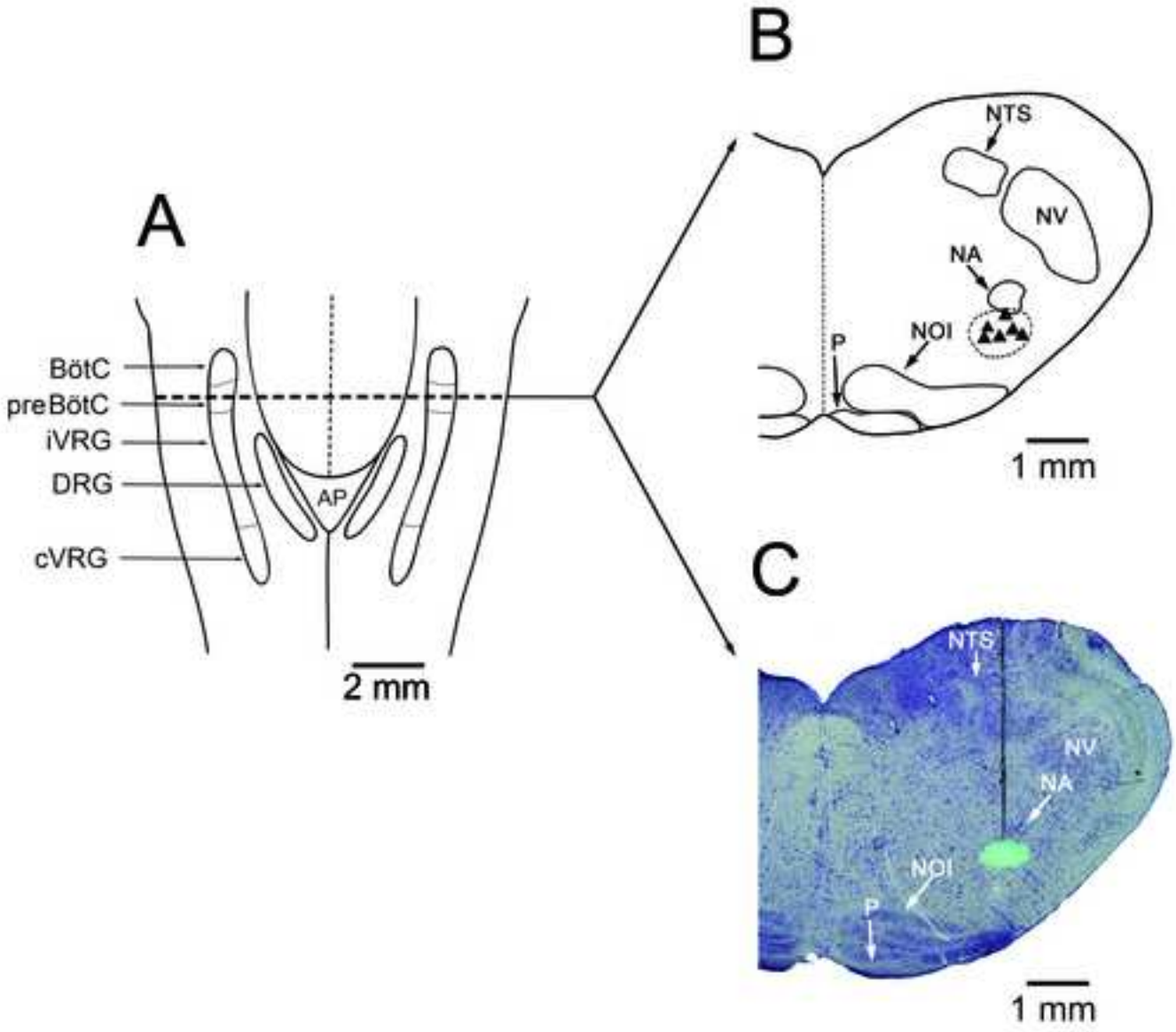

Figure 8

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