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RESEARCH ARTICLE

Orexin-A inhibits fictive air breathing responses to respiratory stimuli in the bullfrog tadpole (*Lithobates catesbeianus*)

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ABSTRACT

In pre-metamorphic tadpoles, the neural network generating lung ventilation is present but actively inhibited; the mechanisms leading to the onset of air breathing are not well understood. Orexin (ORX) is a hypothalamic neuropeptide that regulates several homeostatic functions, including breathing. While ORX has limited effects on breathing at rest, it potentiates reflexive responses to respiratory stimuli mainly via ORX receptor 1 (OX_1R). Here, we tested the hypothesis that OX_1Rs facilitate the expression of the motor command associated with air breathing in pre-metamorphic bullfrog tadpoles (*Lithobates catesbeianus*). To do so, we used an isolated diencephalic brainstem preparation to determine the contributions of OX_1Rs to respiratory motor output during baseline breathing, hypercapnia and hypoxia. A selective OX_1R antagonist (SB-334867; 5–25 µmol l^{-1}) or agonist (ORX-A; 200 nmol l^{-1} to 1 µmol l^{-1}) was added to the superfusion media. Experiments were performed under basal conditions (media equilibrated with 98.2% O_2 and 1.8% CO_2), hypercapnia (5% CO_2) or hypoxia (5–7% O_2). Under resting conditions gill, but not lung, motor output was enhanced by the OX_1R antagonist and ORX-A. Hypercapnia alone did not stimulate respiratory motor output, but its combination with SB-334867 increased lung burst frequency and amplitude, lung burst episodes, and the number of bursts per episode. Hypoxia alone increased lung burst frequency and its combination with SB-334867 enhanced this effect. Inactivation of OX_1Rs during hypoxia also increased gill burst amplitude, but not frequency. In contrast with our initial hypothesis, we conclude that ORX neurons provide inhibitory modulation of the CO_2 and O_2 chemoreflexes in pre-metamorphic tadpoles.

KEY WORDS: Chemoreception, Hypercapnia, Hypoxia, Orexin, Pre-metamorphic bullfrog tadpole, Respiratory control

INTRODUCTION

Orexins A and B (ORXs), also known as hypocretins, are small neuropeptides produced by distinct groups of neurons located in the dorsomedial, lateral and perifornical hypothalamus. As they project throughout the central nervous system, ORX neurons play important roles in diverse homeostatic functions such as sleep–wake states, food intake, metabolism and cardiorespiratory control (Barnett and Li, 2020; Sakurai et al., 1998). The molecular structures of these neuropeptides is highly conserved (Volkoff, 2012) and their actions on the regulation of breathing are consistent in the various groups of adult vertebrates tested to date (Dutschmann et al., 2007; Fonseca et al., 2016; Peyron et al., 1998; Sakurai, 2007; Sun et al., 2016; Yokota et al., 2016; Zhang et al., 2005).

Central administration of ORX-A stimulates breathing in rats, cats and mice. In mammals and adult toads (*Rhinella schneideri*), administration of ORX receptor antagonists have limited effects on resting ventilation, thus indicating that ORXs make a limited contribution to the basal respiratory drive (Carrive and Kuwaki, 2017; Fonseca et al., 2016). Conversely, inactivation of ORX receptors or genetic deletion of ORX attenuates the ventilatory response to hypercarbia in adult rats, mice and toads (Carrive and Kuwaki, 2017; Deng et al., 2007; Dias et al., 2009, 2010; Fonseca et al., 2016; Iigaya et al., 2012b; Vicente et al., 2016). Deletion of ORX neurons and administration of ORX antagonists also reduce the ventilatory response to hypoxia, but the attenuation is more modest (Fonseca et al., 2016; Han et al., 2010; Nakamura et al., 2007). As the activity of ORX neurons is highest during wakefulness (Lee et al., 2005; Mileykovskiy et al., 2005), the potentiation of respiratory reflexes by ORXs is more noticeable during the animal's active phase. These consistent data indicate that in adult vertebrates, ORX has limited effects on respiratory activity at rest but potentiates respiratory reflexes.

This consensus contrasts with the results from studies addressing the role of ORX in the regulation of respiratory function in early life. The variability of the results is surprising considering that research has been limited to rats and mice using reduced preparations both *in vivo* and *ex vivo* (Corcoran et al., 2010, 2015; Loiseau et al., 2019; Sugita et al., 2014). For instance, Loiseau et al. (2019) reported that bath application of ORX-A (10^{-3} to 10^{-2} µmol l^{-1}) onto diencephalon–brainstem–spinal cord preparations augments basal phrenic burst frequency and potentiates the fictive breathing response to CO_2 and H^+. Conversely, application of ORX-A (0.3 µmol l^{-1}) onto medullary slices reduces fictive breathing frequency, increases tonic hypoglossal discharge, but has no effect on the response to CO_2 and H^+ (Corcoran et al., 2015). The ORX system undergoes important anatomical and functional changes during the transition from fetal to extra-uterine life (Ogawa et al., 2017). In rats and mice, ORX neurons are detected in the hypothalamus at embryonic day 12; their number then augments until they peak at postnatal day 7 (Amiot et al., 2005; Ogawa et al., 2017). During that period, the spontaneous firing of ORX cells augments progressively (Ogawa et al., 2017). In *Xenopus*, the first ORX cells are detected in the hypothalamus at early embryonic stages. Throughout larval development, the number of ORX-immunoreactive (OX-ir) cells increases significantly and a widespread fiber network innervating the main areas of the forebrain and brainstem is formed progressively. The final distribution pattern is achieved through metamorphic climax

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(López et al., 2016). Considering that experiments were performed on preparations from newborn animals (postnatal days 0–3), it is unlikely that age is a factor. Thus, the presence/absence of ORX neurons in the different preparations, along with the need to use elevated [K^+] in the solution superfusing the slice to maintain rhythmicity, more likely explains those differences (Ren and Greer, 2006). Regardless, our understanding of the contribution of ORXs to respiratory control in an immature vertebrate remains limited.

To address this gap in knowledge, we measured the changes in fictive breathing in response to bath application of ORX-acting agents onto *ex vivo* preparations from pre-metamorphic bullfrog tadpoles (*Lithobates catesbeianus*). The frequency and pattern of the respiratory motor output produced by these preparations are similar to those observed *in vivo* (Gdovin et al., 1998; Kinkead et al., 1994). The neural correlates of gill and lung ventilation can be recorded at a physiologically relevant temperature and the superfusate does not require high [K^+] to generate robust respiratory rhythms. Thus, this approach reduces the limitations normally associated with *ex vivo* studies with mammalian models, while providing novel insights from non-rodent species.

Emergence of the motor command driving air breathing is a fascinating, yet poorly understood aspect of bullfrog physiology. Based on the knowledge that the neural networks generating lung ventilation are present but actively inhibited in the early tadpole stages (Straus et al., 2000), we hypothesized that ORX facilitates the expression of fictive air breathing in *ex vivo* preparations from pre-metamorphic animals; experiments were performed under basal conditions and during acute chemosensory stimuli. Our experiments focused on the specific involvement of OX_1Rs using a specific antagonist (SB-334867) and high-affinity, endogenous agonist (ORX-A) of the receptor. By manipulating activation of OX_1Rs in these *in vitro* preparations, our data reveal an important role for ORXergic signalling in modulating respiratory motor output in response to both hypercapnia and hypoxia.

MATERIALS AND METHODS

Animals

Experiments were performed on American bullfrog tadpoles [*Lithobates catesbeianus* (Shaw 1802)] of both sexes according to the guidelines of the Canadian Council on Animal Care. Tadpoles were obtained from a commercial supplier (Island Bullfrog, Nanaimo, Canada) and housed in the animal care facility of the Institut Universitaire de Cardiologie et de Pneumologie de Québec. Animals were kept in aquaria filled with flowing de-chlorinated water at 19–22°C under a 12 h:12 h light:dark photoperiod. The animals were fed frozen spinach and goldfish food (Goldy Royal, Sera) daily; naturally occurring algae were available *ad libitum*. We used early- to mid-stage tadpoles ranging between TK stages IV–XII (Taylor and Kollros, 1946) for all experiments. The specific number of animals in each group is reported in the figure legends.

Ex vivo preparations and electrophysiological recordings

The central nervous system was isolated as described previously by Fournier et al. (2007). Briefly, tadpoles were anesthetized by immersion in tricaine methane sulfonate (MS-222, 0.06 g l^{-1}, buffered to pH 7.0 with $NaHCO_3$). The brain and spinal cord (rostral to spinal nerve II) were carefully dissected, keeping the cranial nerves (CNs) intact (Fig. 1). During the dissection, tissue was perfused with cold (0–5°C) artificial cerebrospinal fluid (ACSF, in mmol l^{-1}: 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$ and 10 D-glucose) equilibrated with 1.8% CO_2 and 98.2% O_2 to pH 7.90±0.06.

Following dissection, preparations recovered for 40–60 min in oxygenated ACSF, delivered at 6–7 ml min^{-1}. Once a stable signal was achieved, bursts of respiratory-related motor activity were recorded simultaneously from the rootlets of the trigeminal (V) and vagus (X) CNs using suction electrodes. All solutions were maintained at room temperature (19–21°C).

Experimental protocols

Protocol 1: Participation of the diencephalon in respiratory motor output at 'rest'

In adult bullfrogs, descending inputs from the diencephalon and brainstem regions affect the neural correlates of breathing recorded *ex vivo* (Reid et al., 2000). This encephalic region contains many groups of neurons that produce ORX and we do not know if its presence/absence influences respiratory motor output in tadpoles. Thus, two types of *ex vivo* preparations were tested initially: (1) transected between the optic tectum and the cerebellum – removing the hypothalamic ORX neurons and other structures known to modulate breathing, including the locus coeruleus and the nucleus isthmi (medulla only), and (2) transected rostral to the optic chiasma to keep ORX neurons intact (preparations with diencephalon and brainstem) (Fig. 1A,B). To obviate concerns about the impacts of multiple transections on the same brain, initial experiments were performed on distinct preparations. We then compared responsiveness to stimuli between preparations. To do so, respiratory motor output was first recorded from both preparation types during baseline perfusion with oxygenated ACSF (10 min, 1.8% CO_2, 98.2% O_2, pH 7.9±0.1; 6–7 ml min^{-1}). This was followed by a 20 min perfusion of ACSF equilibrated with hypoxia (1.8% CO_2, 98.2% N_2, pH 7.9±0.1; 10 ml min^{-1}) or hypercapnia (95% O_2, 5% CO_2, pH 7.4±0.05; 6–7 ml min^{-1}). The finding that preparation type influenced respiratory motor output during resting and stimulated (i.e. hypoxia or hypercapnia) conditions informed our decision to consider only preparations with the diencephalon intact for all subsequent protocols outlined below.

Protocol 2: Participation of OX_1R in basal respiratory motor output

To assess the contribution of OX_1Rs to respiratory drive under 'resting' conditions, respiratory motor output was first recorded while the preparation was perfused with oxygenated ACSF for at least 10 min (Fig. 1C; Protocol 2). Next, ACSF containing the selective OX_1R antagonist SB-334867 (Tocris, Bristol, UK) was continuously perfused through the recording chamber for 20 min followed by 30–40 min of wash-out. Based on previous literature (Dergacheva et al., 2016; Klisch et al., 2009; Soffin et al., 2002), three concentrations were tested: 5, 10 and 25 µmol l^{-1}. Aliquots containing SB-334867 were prepared according to Deng et al. (2007) and diluted in 200 ml of ACSF. Solutions containing antagonist were used for a maximum of 10 days without loss of potency.

Protocol 3: Effects of ORX-A on fictive breathing during resting conditions

ORX-A is the natural ligand of the ORX system that preferentially binds to OX_1Rs with high affinity (10- to 100-fold higher versus OX_2R; Sakurai et al., 1998). Respiratory-related motor output was recorded during baseline conditions and during subsequent perfusions with ACSF containing 200 nmol l^{-1} and 1 µmol l^{-1} of ORX-A (Orexin-A, *Xenopus* SS; Sigma-Aldrich, St Louis, MO, USA). Concentrations were chosen based on the literature (Deng et al., 2007; Young et al., 2005) and both were applied in random order to each preparation. Each drug treatment lasted 25 min and was followed by 30–40 min of wash-out (Fig. 1C; Protocol 3).

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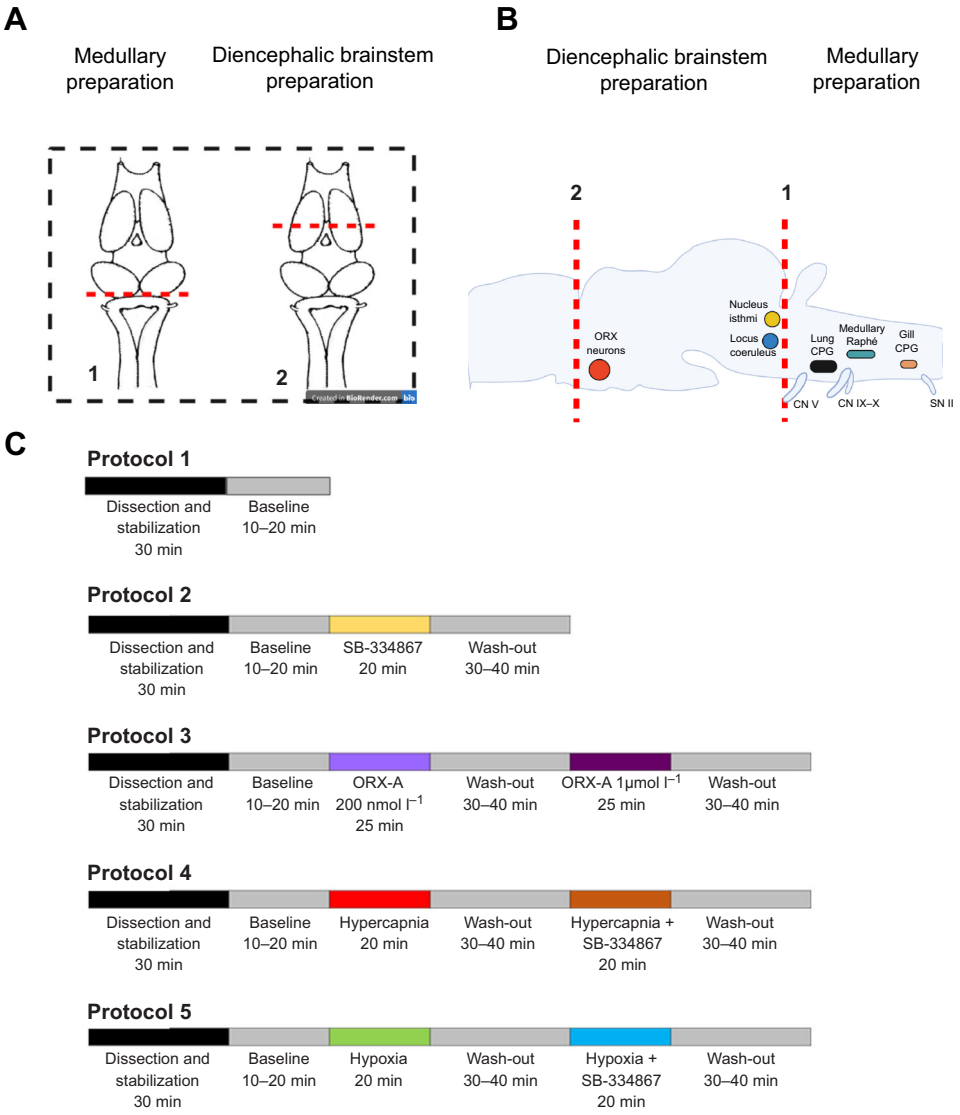

Fig. 1. Types of *ex vivo* preparations and experimental protocols. Schematic diagram showing the two types of isolated (*ex vivo*) central nervous system preparations of *Lithobates catesbeianus* used in the study. (A) Dorsal view; (B) sagittal view; preparation 1, transected between the optic tectum and the cerebellum (medullary preparations); preparation 2, transected rostral to the optic chiasma, keeping the hypothalamus intact (preparations with the diencephalon+brainstem). The schematic representation illustrates the location of cranial nerves (CN V and IX–X) and spinal nerve II (SN II). It also presents the location of key groups of neurons modulating respiratory function, including orexin neurons (ORX), the locus coeruleus, and the medullary raphé. The location of the central pattern generators (CPG) generating lung and gill rhythm are also presented. Panels A and B were created with Biorender. (C) Protocols 1–5: schemes illustrating specific experimental protocols; see Materials and Methods for additional details.

Preliminary analyses showed that preparations fully recovered and that the order of the application did not influence outcomes. The ORX-A was dissolved directly in distilled water and subsequently diluted in 200 ml ACSF. Agonist solutions were used for up to 4 days (maximum of four preparations) with no loss of potency.

Protocol 4: Contribution of OX_1R to the hypercapnic chemoreflex

In this series of experiments, basal respiratory motor output was recorded for 10 min before the perfusate was switched to an ACSF equilibrated with a hypercapnic gas mixture (95% O_2, 5% CO_2; 6–7 ml min^{-1}; pH 7.3–7.4; Fournier et al., 2013) for 20 min (Fig. 1C; Protocol 4). Following wash-out (30–40 min), the preparation was exposed to hypercapnic ACSF containing SB-334867 (5 or 10 µmol l^{-1}) for 20 min. Only two concentrations were used, as data from Protocol 2 showed that application of 25 µmol l^{-1} resulted in tonic bursting that interfered with fictive breathing. A final period of wash-out with baseline ACSF ended the protocol.

Protocol 5: Contribution of OX_1R to the hypoxic chemoreflex

Contributions of OX_1Rs to the hypoxic chemoreflex were assessed in a distinct group of *ex vivo* preparations by replicating Protocol 4, but the stimulated condition utilized ACSF equilibrated with a hypoxic gas mixture (1.8% CO_2, 98.2% N_2; pH 7.8–7.95; 10 ml min^{-1}; Fig. 1C; Protocol 5). Based on previous experiments, this procedure resulted in chamber ACSF containing 5–7% O_2. Only concentrations of 5 and 10 µmol l^{-1} SB-334867 were used for this protocol.

Data analysis

Ex vivo preparations derived from pre-metamorphic tadpoles produce two patterns of respiratory-related neural activity: high-frequency, low-amplitude motor output driving gill ventilation, and low-frequency, high-amplitude output driving lung ventilation in the intact animal (Gdovin et al., 1998, 1999; Liao et al., 1996; Torgerson et al., 1998). Respiratory-related motor output was analysed using LabChart software (version 7, AD Instruments, Colorado Springs, CO, USA). Lung bursts were identified as large-amplitude bursts (at least double the preceding gill burst amplitude) that coincided with bursting on CN X indicative of glottis activation. Lung burst frequency was obtained by analysing the last 5–10 min of each condition, while gill burst frequency was analysed during the last 1 min of each condition (both values are expressed per minute). Because gill-related bursting is more frequent and more rhythmic than lung-related activity, analysis of a longer recording is necessary to consider more lung bursting activity and thus ensure a

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more reliable (and more representative) mean value for each preparation under each condition. Analysis of the respiratory pattern and identification of lung burst episodes was conducted according to criteria proposed by Kinkead et al. (1994). The number of lung bursts within an episode was obtained by counting the number of large-amplitude lung bursts occurring in succession with no pause longer than the length of two bursts between them.

Statistics

Data distribution (normality) was tested using a Shapiro–Wilk test; data did not pass this test on three occasions, and in these cases, data were analysed using a non-parametric approach. The effects of drug treatments (SB-334867, ORX-A) and stimuli (CO_2, hypoxia) on respiratory variables were assessed with analysis of variance (ANOVA); a repeated measures design was used when appropriate. Prior to this analysis, homogeneity of variance was tested with Levene's test. Specifically, differences in respiratory motor output between preparations with and without the intact diencephalon were assessed using an independent samples *t*-test; a non-parametric Mann–Whitney *U*-test was used when data did not meet normality criteria. Potential dose-dependent effects of SB-334867 for respiratory motor output under resting conditions were assessed using a mixed ANOVA with a Bonferroni correction for multiple comparisons. Dose-dependent effects of ORX-A on respiratory motor output were tested using a repeated measures (RM) ANOVA with a Bonferroni correction. For the hypercapnic condition (Protocol 4), differences between baseline, 5% CO_2+ACSF, 5% CO_2+5 µmol l^{-1} SB-334867 and 5% CO_2+10 µmol l^{-1} SB-334867 were compared using a mixed ANOVA with a Bonferroni correction. Similar statistics were run for hypoxia data (Protocol 5). All statistical tests were performed using SPSS 13.0 for Windows (IBM, Markham, Ontario, Canada) and JASP (version 0.14.1; University of Amsterdam, Netherlands).

RESULTS

Participation of the diencephalon in respiratory motor output during resting conditions

Fig. 2 shows representative traces of integrated motor output recorded from the trigeminal nerve (CN V) of *ex vivo* preparations, with diencephalon removed or intact (Fig. 2A,B). In both preparation types, low-amplitude gill bursts are periodically interrupted by high-amplitude lung bursts (black arrowhead in expanded trace in Fig. 2B), which can occur as episodes (open arrowheads). Under resting conditions (i.e. 98% O_2 and 2% CO_2), lung and gill burst frequencies were higher in preparations with the diencephalon intact (lung: P=0.03; gill: P<0.0001; Fig. 2C–F). Fewer lung burst episodes were observed in preparations without the diencephalon, but the number of bursts per episode did not differ between preparation types (Fig. 2C–F). These data confirm that, under resting conditions, brain regions comprising the diencephalon and brainstem provide excitatory drive to modulate respiratory motor output in tadpoles. Given that the diencephalon (1) contains hypothalamic ORX-producing neurons and (2) significantly modulated respiratory motor output under resting conditions, we

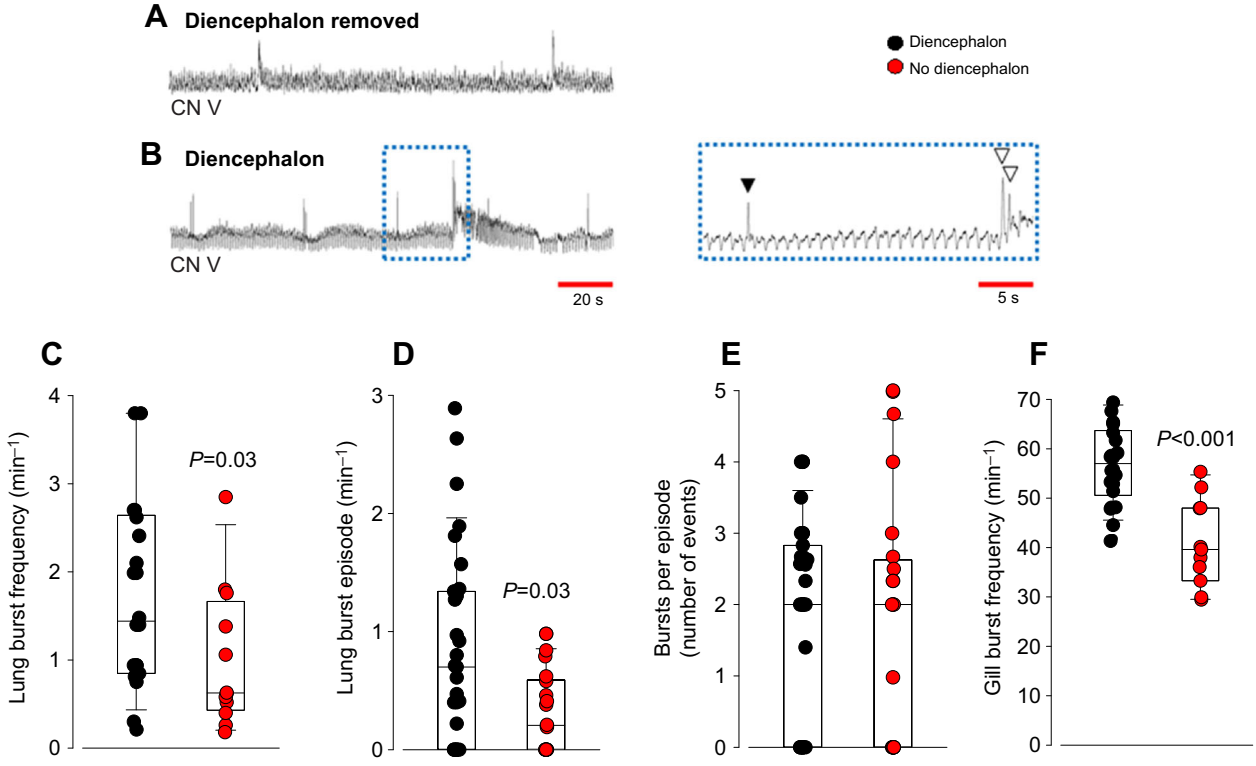

Fig. 2. Respiratory motor output receives excitatory drive from the diencephalon under resting conditions. Representative recordings of respiratory motor activity from the trigeminal nerve (CN V) in two preparation types derived from early-stage tadpoles: without (A: stage V) and with (B, left: stage IX) the diencephalon intact. The expanded trace on the right in B shows low-amplitude gill bursting punctuated by high-amplitude lung busts (▼) that can occur in episodes (▽) of two bursts or more. (C–F) Lung and gill burst-related variables. The frequency of lung bursts (C; diencephalon, *N*=22; medullary, *N*=12) and lung burst episodes (D; diencephalon, *N*=27; medullary, *N*=18; middle) was higher in preparations with an intact diencephalon. (E) The number of lung bursts comprising an episode was similar between preparation types (diencephalon, *N*=27; medullary, *N*=18). (F) Gill burst frequency (diencephalon, *N*=22; medullary, *N*=11). In each plot, the boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles, respectively.

subsequently limited our experiments to preparations possessing an intact diencephalon and brainstem.

Contributions of OX_1Rs to respiratory motor output during resting conditions

The left trace in Fig. 3A is a representative recording of resting lung and gill motor output in a preparation derived from a stage IX tadpole with the diencephalon intact. Inactivation of OX_1Rs with 10 µmol l^{-1} SB-334867 produced no significant change in lung motor output (Fig. 3A, right). Indeed, perfusion with 5, 10 or 25 µmol l^{-1} SB-334867 did not alter lung burst frequency or amplitude (Fig. 3B,C), the incidence of lung burst episodes (Fig. 3D) or the number of lung bursts per episode (Fig. 3E). Gill burst frequency was not altered by SB-334867 (Fig. 3F) but a dose-dependent increase in gill burst amplitude was observed (Drug×Dose: $F_{2,14}$=4.957; P=0.02; Fig. 3G). We noted that 25 µmol l^{-1} SB-334867 frequently resulted in large-amplitude bursts of long duration that could not be conclusively defined as respiratory related (not included in analysis). We subsequently limited our investigation to 5 and 10 µmol l^{-1} SB-334867 concentrations.

Contributions of ORX-A to respiratory motor output during resting conditions

Bath application of the OXR agonist, ORX-A, affected fictive breathing but the effects were not dose dependent (Fig. 4A–C). Despite a suggestive trend, the effect of ORX-A on lung burst frequency was not significant (ORX-A effect: $F_{1,6}$=3.25; P=0.15; Fig. 4B) but compared with baseline, lung burst episodes were less frequent during bath application of 200 nmol l^{-1} ORX-A (ORX-A effect: $F_{1,6}$=9.683; P=0.02; Fig. 4D) and were associated with fewer bursts per episode (ORX-A effect: $F_{1,6}$=13.504; P=0.01; Fig. 4E). ORX-A did not affect lung burst amplitude (Fig. 4C). Gill burst frequency and amplitude both increased in response to 200 nmol l^{-1} and 1 µmol l^{-1} ORX-A (ORX-A effect on: frequency: $F_{1,8}$=32.212; P<0.001, Fig. 4F; amplitude: $F_{1,8}$=44.624, P<0.001, Fig. 4G).

Contribution of OX_1R to the hypercapnic chemoreflex

Fig. 5A shows representative recordings of respiratory motor output during baseline, hypercapnia, and hypercapnia in the presence of 10 µmol l^{-1} SB-334867. Perfusion of 5% CO_2 without drug (ACSF) produced minimal effects on respiratory motor output (Fig. 5B–G). However, the combination of 5% CO_2 with 5 or 10 µmol l^{-1} SB-334867 increased lung burst frequency compared with baseline and 5% CO_2 ACSF (CO_2×Dose: $F_{2,22}$=12.344; P<0.001; Fig. 5B). CO_2 increased lung burst amplitude compared with baseline (CO_2 effect: $F_{1,12}$=8.682; P=0.01; Fig. 5C). Co-perfusion of 5% CO_2 with 10 µmol l^{-1} SB-334867 did not increase the incidence of lung burst episodes or the number of bursts per episode; Fig. 5D,E). Perfusion of 5% CO_2 with SB-334867 did not alter gill burst frequency, but augmented gill burst amplitude; the largest increase was observed at 10 µmol l^{-1} (CO_2 effect: $F_{1,12}$=9.131; P=0.01; Fig. 5F,G).

Contribution of OX_1R to the hypoxic chemoreflex

Representative traces of motor output depicted in Fig. 6A show that hypoxia stimulated fictive lung breathing in tadpole brainstem preparations by increasing lung burst frequency. Co-perfusion of hypoxia with SB-334867 further increased lung burst frequency (Hypoxia×Dose: $F_{2,20}$=3.504; P=0.05; Fig. 6B). Lung burst amplitude augmented also (hypoxia effect: $F_{1,21}$=28.907; P<0.001) but this response was not influenced by SB-334867 (Fig. 6C). Neither hypoxia nor its combination with SB-334867 altered the frequency of lung burst episodes, although the number of bursts per episode was enhanced by hypoxia alone (hypoxia effect: $F_{1,20}$=5.199; P<0.03; Fig. 6D,E). SB-334867 also potentiated the gill burst amplitude response to hypoxia (Hypoxia×Dose: $F_{2,20}$=3.504, P=0.04) but no effect on gill burst frequency was observed (Fig. 6F,G).

DISCUSSION

We tested the hypothesis that OX_1Rs facilitate the expression of the motor command producing air breathing in pre-metamorphic bullfrog tadpoles (*L. catesbeianus*). Experiments performed in the process allowed us to investigate the contributions of ORX-A and the OX_1R to central respiratory motor activity and their participation in the O_2 and CO_2 chemoreflexes in this species. We show that the ORX system has modest effects on fictive air breathing at 'rest' and the effects of SB-334867 are greatest during hypercapnic and hypoxic stimulation. These data are generally in line with those obtained in other groups of vertebrates, but the robust potentiation of the lung burst responses following inactivation of OX_1R clearly oppose results reported in adult vertebrates. The selective stimulatory actions of ORX-A on gill-related (but not lung-related) activity are novel observations that strengthen the notion that the neural networks driving water and air breathing are functionally distinct. Although original, our data therefore do not support our hypothesis but demonstrate that ORX neurons inhibit the fictive air breathing responses to CO_2 and O_2 in the pre-metamorphic tadpole. These data point to an important developmental change in the modulatory influence of ORX neurons on respiratory motor control that probably contributes to the ontogeny of air breathing in this species.

The diencephalon, orexin neurons, and their effects on basal respiratory motor output and pattern

The basal lung and gill frequencies produced by preparations with the diencephalon and brainstem were greater than those without. Thus, as in mammals (Iigaya et al., 2012a; McDowall et al., 2007; Redgate and Gellhorn, 1958), this rostral brain structure exerts a stimulatory tone on the respiratory rhythm at rest. These preparations also presented a higher number of lung burst episodes compared with the medullary preparations in which the diencephalon was absent. The essential elements explaining the occurrence of episodes in anurans is still to be elucidated (Gargaglioni and Milsom, 2007), but the structures present in the diencephalon and brainstem play a role in clustering of lung bursts into episodes in early stage tadpoles of *L. catesbeianus* and adult bullfrogs (Milsom et al., 1997; Reid et al., 2000). The diencephalon is an encephalic region that contains several structures, many of which contain ORX neurons. Thus, brain transection does not provide the anatomical precision necessary to comment on the specific role of ORX neurons *per se* to the differences in motor output observed between the two types of preparations. Under resting conditions, ORX neurons are under strong tonic inhibition (Li and van den Pol, 2006) and their influence on basal breathing of adult animals is limited. This, and our data showing that bath application of SB-334867 do not affect lung bursting significantly, indicate that the stimulatory influence originates from other groups of neurons. While the present study does not allow us to identify them, the paraventricular nucleus of the hypothalamus is a plausible candidate owing to its numerous projections to medullary structures that generate and regulate breathing in mammals (Tenorio-Lopes and Kinkead, 2021).

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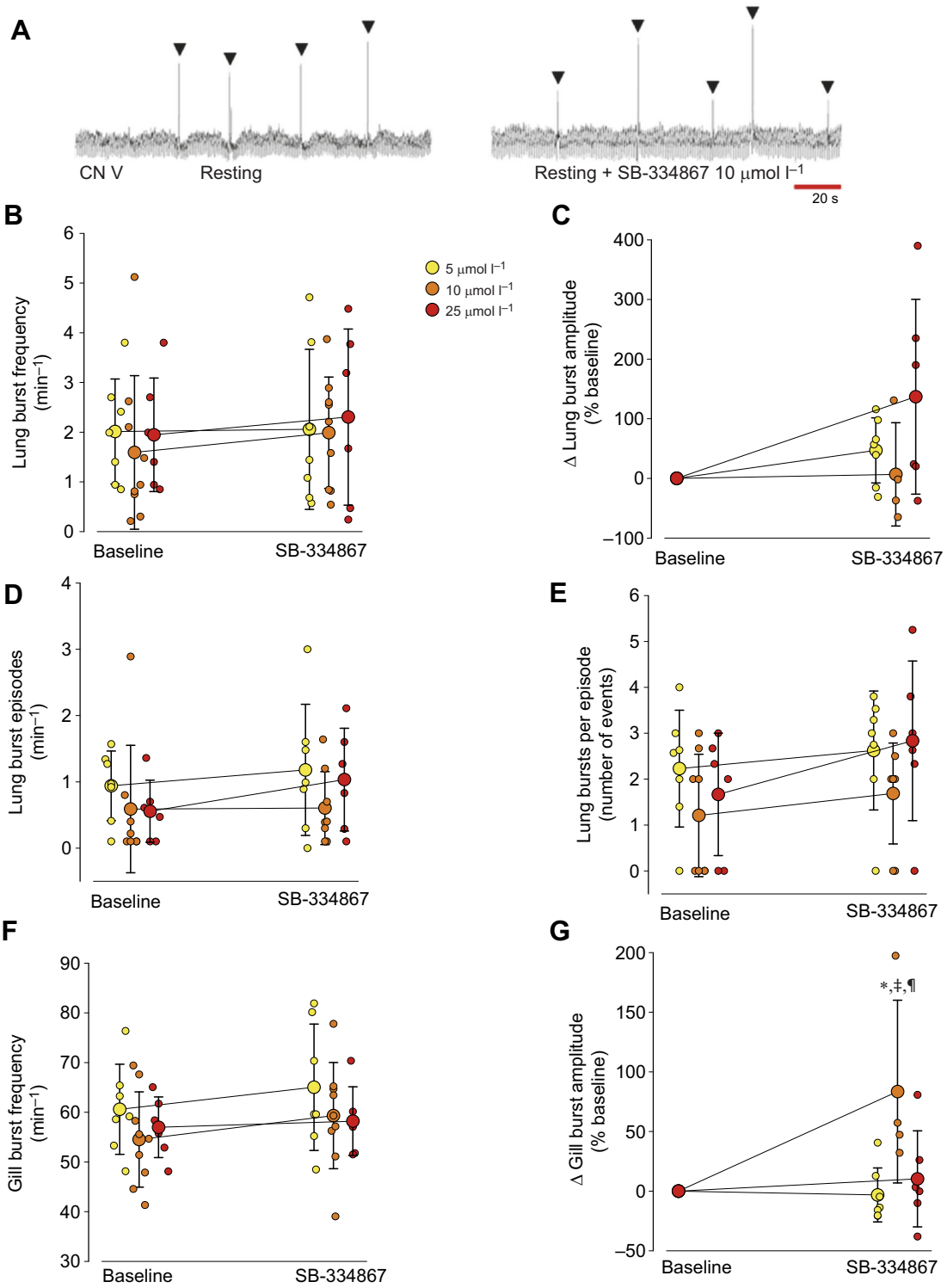

Fig. 3. OX_1Rs do not play a major role in tadpole respiratory motor output under resting conditions. (A) Representative traces of motor activity from a stage IX tadpole *ex vivo* preparation with the diencephalon and brainstem intact under resting conditions (left) and in the presence of 10 µmol l^{-1} SB-334867 (right). Lung bursts are denoted by ▼. Lung burst frequency (B), lung burst amplitude (C), episode frequency (D), bursts per episode (E) and gill burst frequency (F) did not show dose dependence in response to SB-334867. (G) Gill burst amplitude was enhanced by 10 µmol l^{-1} SB-334867; 5 µmol l^{-1}: N=7 (except: bursts per episode, N=6); 10 µmol l^{-1}: burst frequency, N=9; burst amplitude, N=4; episodes, N=8; bursts per episode, N=6; 25 µmol l^{-1}: N=6 (except: bursts per episode, N=5). Each plot reports individual data and mean±s.d. Significant *post hoc* differences for 10 µmol l^{-1} SB-334867: *P=0.027 compared with baseline, ‡P=0.003 compared with 5 µmol l^{-1}, ¶P=0.017 compared with 25 µmol l^{-1}.

Exposing the brains to ORX-A allowed us to evaluate the action of this natural agonist on the respiratory network. Although the lung burst frequency (and amplitude) data were not statistically significant, the concurrent reduction in episodic bursting observed in the presence of ORX-A points to an overall drop in respiratory drive (Fong et al., 2009). These results contrast with the clear

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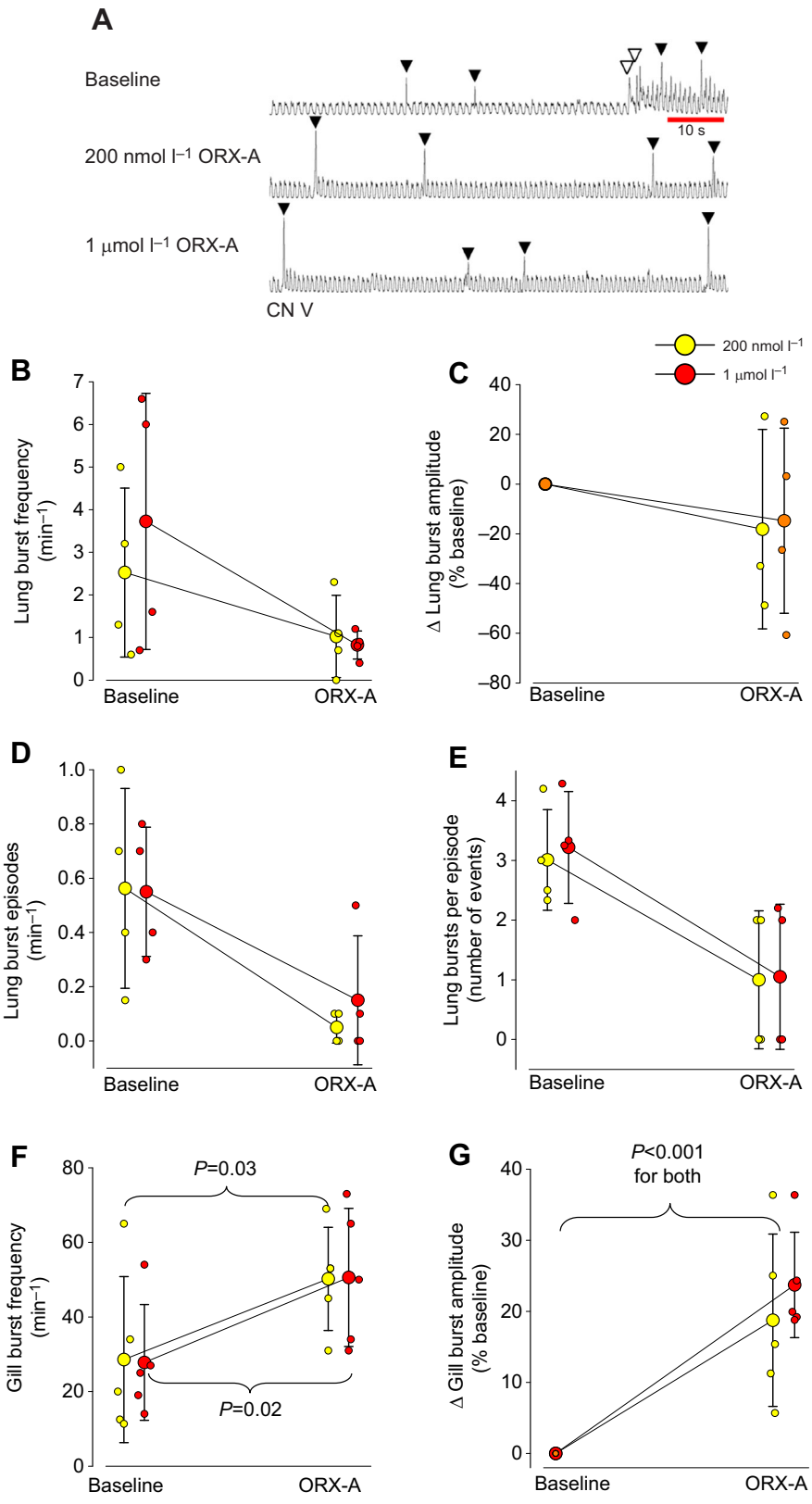

Fig. 4. ORX-A affects gill and lung motor output in tadpole brainstems under resting conditions. (A) Representative recordings of respiratory motor output from CN V in a stage IV tadpole *ex vivo* preparation with the diencephalon intact during baseline, 200 nmol l^{-1} ORX-A and 1 µmol l^{-1} ORX-A. Lung burst frequency (B) and amplitude (C) were not affected by ORX-A. (D,E) The incidence of lung burst episodes and number of bursts per episode was decreased by 200 nmol l^{-1} ORX-A. (E,F) ORX-A at 200 nmol l^{-1} increased gill burst frequency and amplitude, while 1 µmol l^{-1} ORX-A increased gill burst frequency only. Lung data, N=4; gill data, N=5. ▼, lung bursts; ∇, episodes.

increase in gill bursting (both frequency and amplitude) that followed ORX-A application. Without additional data, this result is difficult to explain, but suggests the existence of a distinct (and local) mechanism that allows tonic ORX release near cells regulating fictive gill breathing. This postulate has merit, as the neural oscillators driving gill and lung motor output are anatomically distinct in amphibians (Baghdadwala et al., 2015). ORX-A could also act on lung-related activity indirectly via GABA/glycine neurons, which, at this developmental stage, is excitatory (Fournier et al., 2007).

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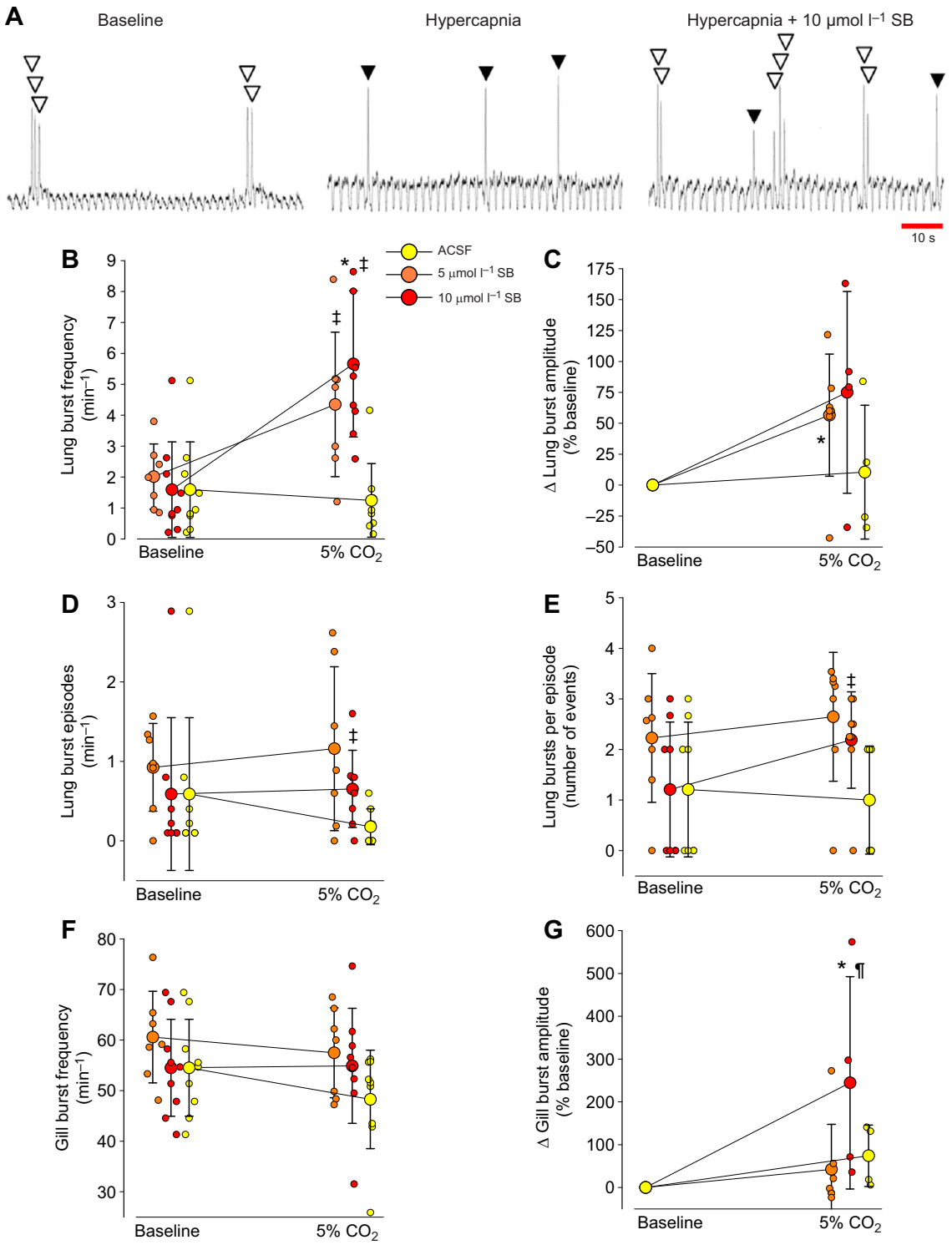

Fig. 5. Antagonizing OX_1Rs during hypercapnia reveals an excitatory effect on lung and gill motor output. (A) Representative recordings of motor output from CN V in a stage V tadpole *ex vivo* preparation with the diencephalon intact. Lung and gill motor output is shown during baseline, hypercapnia (5% CO_2) and hypercapnia+10 µmol l^{-1} SB-334867 (SB). The combination of hypercapnia with 5 or 10 µmol l^{-1} SB-334867 increased lung burst frequency (B) and amplitude (C). (D,E) Hypercapnia+10 µmol l^{-1} SB-334867 increased lung burst episodes and bursts per episode. Perfusion with 5% CO_2 without drug ('ACSF') had no effect on respiratory motor output except to decrease lung burst episodes (D). (F) Gill burst frequency was not affected by SB-334867. (G) Hypercapnia+10 µmol l^{-1} SB 334867 increased gill burst amplitude; 5 µmol l^{-1}: N=7; 10 µmol l^{-1} and ACSF: frequency, N=9; amplitude, N=4; episodes, N=8; bursts per episode, N=7. ▼, lung bursts; ∇, episodes. Significant *post hoc* differences: *compared with baseline; ‡compared with ACSF; ¶compared with 5 µmol l^{-1} SB, all at P<0.05.

ORX attenuates CO_2- and O_2-related chemoreflexes

The ventilatory response to CO_2 of vertebrates undergoes significant changes during early life (Eugenín et al., 2006; Hempleman and Pilarski, 2011; Putnam et al., 2005; Whitaker-Fornek et al., 2019). In rodents, the response is greatest at birth and then declines to nearly undetectable values near postnatal day 5; it

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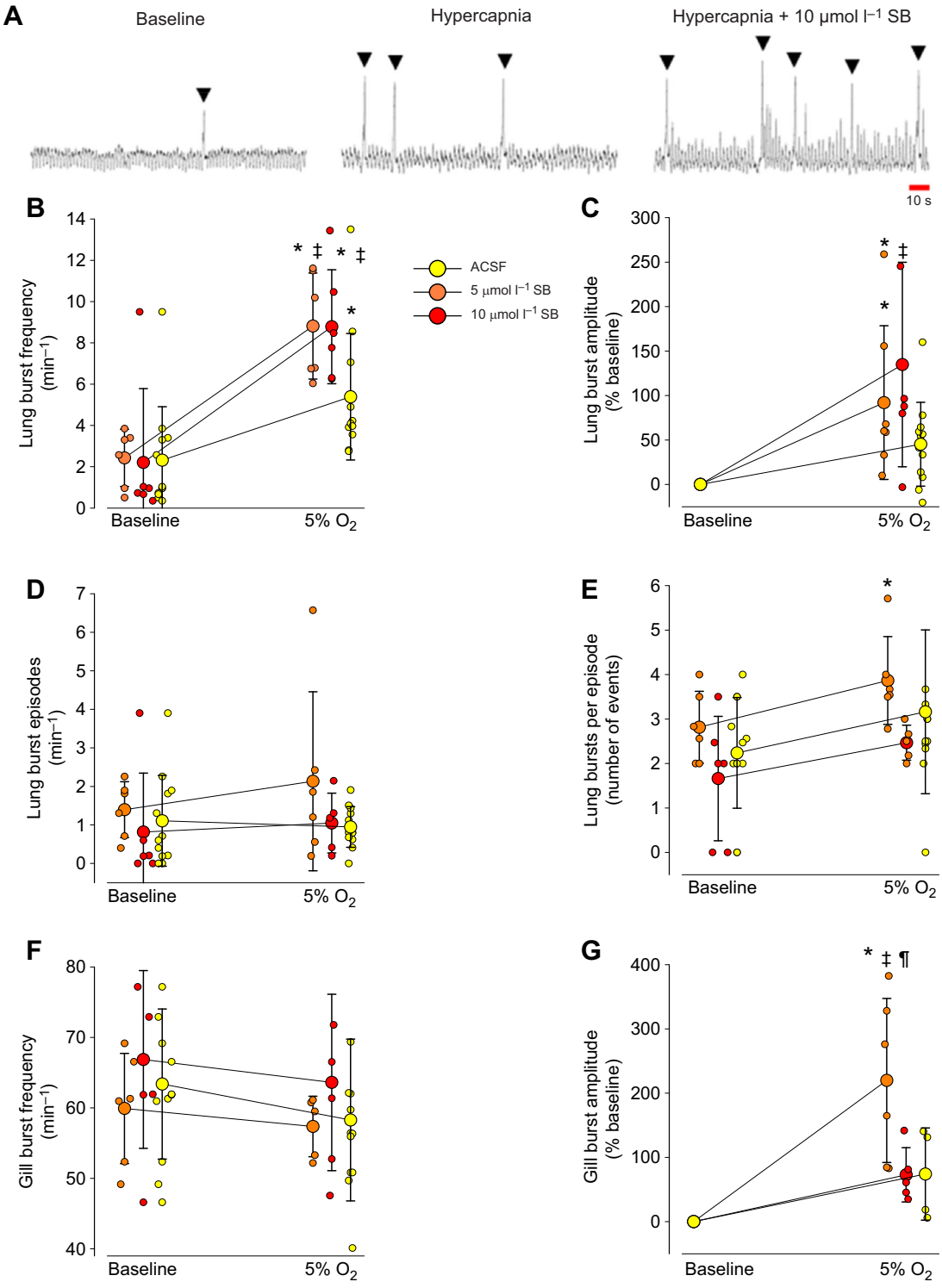

Fig. 6. Antagonizing OX_1Rs during hypoxia enhances respiratory-related motor output. (A) Representative recordings of motor output from CN V in a stage VII tadpole *ex vivo* preparation with the diencephalon intact shown during baseline, hypoxia (5% O_2) and hypoxia+10 µmol l^{-1} SB-334867 (SB). (B) Hypoxia without drug (ACSF) increased lung burst frequency and its combination with 5 or 10 µmol l^{-1} SB-334867 further enhanced frequency. (C) Lung burst amplitude increased during co-perfusion of hypoxia with 5 or 10 µmol l^{-1} SB-334867. (D,E) Hypoxia+SB-334867 did not alter the incidence of lung burst episodes but increased the number of bursts per episode. (F) Gill burst frequency was not altered by SB-334867. (G) Hypoxia+5 µmol l^{-1} SB-334867 increased gill burst amplitude; 5 µmol l^{-1}: *N*=6; 10 µmol l^{-1}: *N*=6 (except gill amplitude, *N*=5). ▼, lung bursts. Significant *post hoc* differences: *compared with baseline; ‡compared with ACSF; ¶compared with 10 µmol l^{-1} SB, all at $P<0.05$.

then rises progressively until postnatal day 15 and then stabilizes until adulthood (Hempleman and Pilarski, 2011; Putnam et al., 2005). In birds, the response is complex; depending on the embryonic stage, acidosis and alkalosis can both stimulate fictive breathing (Whitaker-Fornek et al., 2019). The stimulatory action of CO_2 and H^+ on fictive breathing measured from reduced

preparations is weak in comparison with the response measured in age-matched intact animals (Eugenín et al., 2006; Kinkead et al., 1994; Kinkead and Milsom, 1994; Loiseau et al., 2019). In frogs, the lack of vagal feedback from pulmonary stretch explains this discrepancy (Kinkead et al., 1994). In our experiments, exposing *ex vivo* preparations to hypercapnia (5% CO_2) had limited effects and led to a modest decrease in the number of lung burst episodes. As our preparations were taken from early- to mid-stage tadpoles, this poor response tends to agree with the notion that CO_2 chemosensitivity (and CO_2- and H^+-related respiratory drive) increase during bullfrog development (Torgerson et al., 1997). Although other studies performed on animals of a similar developmental stage report a significant increase in lung burst frequency during CO_2 exposure (Rousseau et al., 2016), we must keep in mind that most (if not all) of those experiments were performed on preparations that did not have the diencephalon or the brainstem. Regardless, exposing the preparations to hypercapnia in the presence of SB-334867 doubled the lung burst frequency response and the propensity for episodic bursting versus ACSF alone, thus indicating that, at this developmental stage, ORX inhibits the CO_2 chemoreflex in this species. This observation contrasts with the excitatory effect of ORX on the hypercarbic chemoreflex of adult mammals and toads (Dias et al., 2010; Fonseca et al., 2016), but strengthens the notion of a developmental shift in the ORX modulation of CO_2 chemoreflex.

Activation of ORX receptors generally leads to an increase in intracellular Ca^{2+} levels through $G\alpha_{q/11}$ activation, which leads to postsynaptic excitation that can last several minutes (Gotter et al., 2012). The responses reported here therefore suggest that other (indirect) mechanisms are responsible for the attenuation of respiratory reflexes and ORX neurons can regulate the presynaptic release of other transmitters (GABA, glutamate, serotonin) which, in turn, can inhibit lung bursting (Chou et al., 2001; Davies et al., 2009; Gotter et al., 2012; Vasilakos et al., 2005).

Unlike the CO_2 and H^+ chemoreflex, the ontogenic changes in the fictive breathing response to hypoxia are distinct and consistent in developing bullfrogs (for a review, see Janes et al., 2019). In pre-metamorphic tadpoles, central hypoxia has clear stimulatory effects on fictive lung ventilation. This result is consistent with previous reports (Fournier et al., 2007; Janes and Kinkead, 2018; Taylor et al., 2013; Winmill et al., 2005), and data from our laboratory show that noradrenergic signalling (probably originating from the locus coeruleus, LC) is necessary for this response (Fournier et al., 2007; Fournier and Kinkead, 2006, 2008). Again, bath application of SB-334867 prior to such challenge further increased the response by a factor of 2, with no net effect on bursting pattern. The interactions between the LC and ORX neurons raises the possibility that this drug inactivated receptors located on rhythmogenic neurons (direct effect) or other structures like the LC (indirect effect). This result also differs from those obtained in adult toads in which i.c.v. administration of this selective antagonist attenuated the hypoxic ventilatory response (Fonseca et al., 2016). Whole-cell recordings performed in juvenile rats show that hypoxia hyperpolarizes ORX neurons (Dergacheva et al., 2016). Considering that ORX-A tends to inhibit lung bursting at rest, our results suggest that hypoxia could have a similar effect on ORX neurons of tadpoles. As a result, the hypoxic response observed in the presence of SB-334867 may reflect the withdrawal of an inhibitory influence.

Conclusion

The highly conserved organization of ORX neurons and receptors distribution appears to contribute to the consistency of its multiple roles among vertebrates (Volkoff, 2012). Here, we revealed the inhibitory actions of ORX on chemoreflexes of pre-metamorphic tadpoles, an effect that differs substantially from the potentiation described in adults, but brings support to the hypothesis that ORXs contribute to metamorphic events in amphibians (Volkoff, 2012). In the context of respiratory control, developmental changes in ORXergic regulation of chemoreflexes may contribute to the emergence of air breathing during amphibian metamorphosis. This hypothesis is promising and warrants further studies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.M.F., T.A.J., R.K.; Methodology: E.M.F., T.A.J., S.F., R.K.; Validation: E.M.F., T.A.J., R.K.; Formal analysis: E.M.F., T.A.J., S.F., R.K.; Investigation: E.M.F.; Data curation: R.K.; Writing - original draft: E.M.F., T.A.J., R.K.; Writing - review & editing: E.M.F., T.A.J., S.F., L.H.G., R.K.; Supervision: T.A.J., S.F., L.H.G., R.K.; Project administration: S.F., R.K.; Funding acquisition: R.K.

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