**PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO
EM CIÊNCIAS FISIOLÓGICAS - UFSCar/UNESP**

ELISA MAIOQUI FONSECA

**PARTICIPAÇÃO DA NEUROTRANSMISSÃO OREXINÉRGICA NAS
RESPOSTAS RESPIRATÓRIAS À HIPERCARBIA E HIPÓXIA EM
IGUANAS VERDES E GIRINOS DE RÃ-TOURO**

**"CONTRIBUTION OF OREXIN NEUROTRANSMISSION TO THE RESPIRATORY RESPONSES TO
HYPERCARBIA AND HYPOXIA IN GREEN IGUANAS AND BULLFROG TADPOLES"**

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Tese de doutorado apresentada ao Programa Interinstitucional de Pós-graduação em Ciências Fisiológicas da Universidade Federal de São Carlos/Universidade Estadual Paulista "Júlio de Mesquita Filho" (UFSCar/UNESP) como parte dos requisitos para obtenção do título de Doutora em Ciências Fisiológicas.

Orientadora: Profa. Dra. Luciane Helena Gargaglioni Batalhão

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JOINT GRADUATE PROGRAM IN PHYSIOLOGICAL SCIENCES

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Thesis submitted to the Joint Graduate Program in Physiological Sciences PIPGCF - UFSCar/UNESP in partial fulfillment of the requirements for the degree of "Doctor in Physiological Sciences".

Supervisor: Dr Luciane Helena Gargaglioni Batalhão

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UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
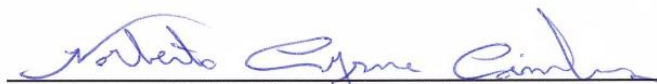Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas
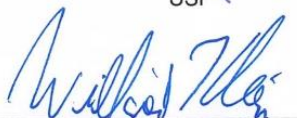

Folha de Aprovação

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USP

I dedicate this work to my grandmother Idalina Nicola Maiochi,

a great biologist who has never studied biology.

And that taught me to love life in its fullness.

Dedico esse trabalho a minha avó Idalina Nicola Maiochi,

uma grande bióloga que nunca estudou biologia.

E que me ensinou o amor pela vida em sua plenitude.

What you got, what you got in your hand? - a father said to son
I got the whole world here, daddy, between my fingers and my thumb
Well you take care of it please – it's the only one
Well it'd take me a lifetime old man to undo what you've done
To undo what you've done

Oh come on now boy think what would Jesus do?
He'd shake his head like an angry mother – spoke the boy and say I did what I could do
But you take care of it please - it's the only one you got
And it'd take ten lifetimes boy to undo what I've done

Boy shrugged walked away
The man stood and watched as he was leaving
Boy just walked away
The man stood alone thinking

One hand is bleeding and the other hand holds a gun
While everything is open everything is shut down, down, down
Begin to ending is really just a go round and round and round
As I stand here - the ground beneath is nothing more than one point of view

...

Raven – Dave Matthews

Gosto de pensar na ciência da mesma forma como penso sobre a vida: como uma estrada. Ampla e vasta. Com inúmeros caminhos que levam a todos os lugares. Com muitos desvios, atalhos e voltas. Obstáculos, desafios. Que caminhos distintos podem levar aos mesmos lugares. Penso eu que nossas estradas podem se encontrar em determinados trechos. E se separar depois. Ou talvez juntos podemos pegar uma rota outra. E que a sua estrada pode me fazer chegar mais rapidamente em um outro destino. Voltar é bom também, é permitido. Mas o que mais me faz achar graça nesse todo é pensar que o caminho, na verdade, se faz ao caminhar. O caminhante, passo a passo, trilha a sua senda. Pegada por pegada. E aqui nesse documento, nesse final de ciclo, quero que você, leitor, pense nas palavras dessa tese como as minhas pegadas. Da ciência, e da vida.

Agradeço a Deus por me dar uma nova oportunidade de compreender a vida durante esses cinco anos de doutorado. Por ter colocado tantas mudanças no meio do caminho que acabaram me mudando. Por cuidar dos meus sonhos, transformando-os em realidade sempre me surpreendendo, fazendo muito mais do que eu posso idear. Por ser amor, sem cessar, a todo instante, abundante e transbordante.

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Bioética, Química e muito mais. Obrigada pela convivência diária sempre alegre. Ao Euclides, Damares e Dona Ângela pelo trabalho excepcional!

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Ao PIPGCF e à UNESP-FCAV por tornarem esse sonho possível, e em especial, ao Alexandre, pelo trabalho competente e dinâmico.

E por último, porém primordiais, fundamentais e imprescindíveis nessa jornada, agradeço à minha família: Tina, Tino e Dedé. Vocês são a minha vida. Esse trabalho é tão de vocês, quanto é meu. E é por vocês e para vocês. A conquista de um de nós é a conquista de todos e a batalha de um também é a batalha de todos. O meu maior obrigada é pra vocês. Eu amo vocês!

E se você chegou até aqui, parabéns, agora chuta aí quantas vezes eu falei a palavra "obrigada"! Certeza que eu esqueci uma galera, mas vocês sabem que eu vivo no mundo da Luba!

"For a large number of problems there will be some animal of choice or a few

such animals on which it can be most conveniently studied."

August Krogh

Figura 8. Atividade dos músculos intercostais de *Uromastyx aegyptius microlepis* demonstrando como acontece o ciclo respiratório de lagartos. Figura de Al-Ghamdi et al., 2001.

List of Figures - Chapter 1

Figure 1. Schematic diagram showing the two types of preparations used in the study. Dorsal view (A) and sagittal view (B): Preparation 1, transected between the optic tectum and the forebrain; and preparation 2, transected rostral to the optic chiasma, keeping the hypothalamus. Adapted from Rugh, 1951 (A) and López et al., 2016 (B).

Figure 2. Photos showing the dissection of the tadpole (A and B) and the limb bud to stage the animal according to the criteria of Taylor-Kollros, 1946 (C).

Figure 3. Photo showing the brainstem preparation 2 (with the hypothalamus). The nerves V and X inside the suction electrodes, ready for being recorded.

Figure 4. Scheme illustrating the experimental protocols.

Figure 5. Diagram showing a representative trace demonstrating the difference between the gill and the lung bursting.

Figure 6. Representative recording of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* before and after SB-334867 10 µM exposure.

Figure 7. "Box Plot" showing the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in 'resting conditions' of "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to SB-334867 (5, 10 and 25 µM). Amplitude data is expressed as % change to baseline. * means different from correspondent group in the

preparations with the hypothalamus, [+] means different from baseline, # means different from 5 µM and @ different from 25 µM. (n = 12)

Figure 8. Relative change of lung burst frequency (A); buccal burst frequency (C) and percentage change from baseline of lung burst amplitude (B) and buccal burst amplitude (D) from *in vitro* preparations of premetamorphic tadpoles exposed to ORX-A 20 nM and 1 µM. Two preparations were used, with the hypothalamus (open circles) and without the hypothalamus (painted circles). (n = 5)

Figure 9. Relative change of lung burst (A) and buccal burst (B) frequency versus the developmental stage of the premetamorphic tadpoles. In blue the preparations with only the brainstem and in orange the preparations with the hypothalamus intact. (n = 5)

Figure 10. Representative recordings of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* exposed to 5% CO_2 only (A) and with 10 µM SB-334867 (B).

Figure 11. "Box Plot" showing the effect of SB-334867 (5, 10 and 25 µM) on the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to 5% CO_2. Amplitude data is expressed as % change to baseline. * means different from baseline and [+] means different from hypercapnia. (n = 10)

Figure 12. Representative recordings of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* exposed to moderate hypoxia only (A) and with 10 µM SB-334867 (B).

Figure 13. "Box Plot" showing the effect of SB-334867 (5, 10 and 25 µM) on the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to moderate hypoxia. Amplitude data

14

is expressed as % change to baseline. * means different from baseline, [+] means different from hypoxia and [#] means different from 5 µM. (n = 8)

List of Figures - Chapter 2

Figure 1. Photo showing detail of the guide cannula implanted on the lateral ventricle through stereotactic surgery. Carefully implanted in a place not to cover the modified transparent scale or the "parietal eye". The eye is a photoreceptive structure and is associated with the pineal gland, regulating circadian rhythmicity.

Figure 2. Reptile brain showing the site of the microinjection in red. Adapted from Parker (1990).

Figure 3. Photo showing the iguana with the guide cannula implanted and the mask equipped with the pneumotachograph in detail.

Figure 4. Scheme representing the experimental protocol.

Figure 5. Recording demonstrating the times from which the data analysis calculations were made.

Figure 6. A photomicrograph showing one of the transections (A) where were found the most prominent group of ORX-labeling and a scheme illustrating the slice from Dominguez et al., 2009 (B). The red circle indicates the localization of the cell bodies where were found the ORX-neurons. The level of the transections in the reptile brain (C).

Figure 7. Photomicrographs of ORX-A labeling.

Figure 8. Photomicrographs showing the ORX-B labeling in detail.

Figure 9. Daily variation of the plasma levels of ORX-A in the green iguana. The sun symbols represent the light phase of the diurnal cycle.

Figure 10. Representative trace from normocarbic normoxia during light phase.

Figure 11. Trace from normocarbic normoxia during dark phase.

Figure 12. Representative trace from hypercarbia (5% CO_2) and recovery.

Figure 13. Representative from hypoxia 5% O_2.

Figure 14. Effect of the i.c.v. injection of SB-334867 and its vehicle on f_R, V_T and $\dot{V}_I$ in normocapnic normoxia in green iguanas during light or dark phases. * means different from light phase.

Figure 15. Effect of the i.c.v. injection of SB-334867 and its vehicle on f_R, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypoxia (5% O_2) during light or dark phases.

Figure 16. Effect of the i.c.v. injection of SB-334867 and its vehicle on f_R, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypercarbia (5% CO_2) during light or dark phases. * means different from vehicle.

Figure 17. Effect of the i.c.v. injection of Almorexant and its vehicle on f_R, V_T and $\dot{V}_I$ in normocapnic normoxia in green iguanas during light and dark phases. * means different from light phase.

Figure 18. Effect of the i.c.v. injection of Almorexant and its vehicle on f_R, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypoxia (5% O_2) during light or dark phases.

Figure 19. Effect of the i.c.v. injection of Almorexant and its vehicle on f_R, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypercarbia (5% CO_2) during light or dark phases. * means different from vehicle.

Figure 20. Effect of the i.c.v. injection of SB-334867 and its vehicle on T_{NVP} in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Figure 21. Effect of the i.c.v. injection of SB-334867 and its vehicle on the number of breaths per episode in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Figure 22. Effect of the i.c.v. injection of SB-334867 and its vehicle on the frequency of episodes in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Figure 23. Effect of the i.c.v. injection of Almorexant and its vehicle on T_{NVP} in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Figure 24. Effect of the i.c.v. injection of Almorexant and its vehicle on the number of breaths per episode in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom). * means different from correspondent stimulus of the vehicle group.

Figure 25. Effect of the i.c.v. injection of Almorexant and its vehicle on the frequency of episodes in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Table 1. Values of f_R, V_T and $\dot{V}_I$ of *I. iguana* microinjected with vehicle, SB-334687 or Almorexant exposed to room air, acute hypoxia or hypercarbia during light or dark phases.

aCSF – artificial cerebrospinal fluid/líquor artificial

AMB – nucleus ambiguus

ATP – adenosine triphosphate/adenosina trifosfato

C – caudal

CNS/SNC – central nervous system/sistema nervoso central

CO_2 – carbon dioxide/dióxido de carbono

cVLM – caudal ventrolateral medulla/bulbo ventrolateral caudal

DMH – dorsomedial hypothalamus/hipotálamo dorsomedial

DMSO - dimethyl sulfoxide/dimetilsulfóxido

DR – dorsal raphe/rafe dorsal

FN – fastigial nucleus/núcleo fastigial

f_R – respiratory frequency/frequência respiratória

ir – immunoreactive/imunorreativo

KO - knockout

LC - locus coeruleus

LHA – lateral hypothalamus/hipotálamo lateral

M – medial

NTS – nucleus tractus solitarius/núcleo do trato solitário

NO – nitric oxide/óxido nítrico

NOS - nitric oxide synthase/óxido nítrico sintase

NREM – non-rapid eyes movement

O_2 – oxygen/oxigênio

ORX – orexin/orexina

ORX-A – orexin-A/orexina-A

ORX-B – orexin-B/orexina-B

OX_1R – orexin receptor-1/receptor de orexina-1

OX_2R - orexin receptor-2/ receptor de orexina-2

$PaCO_2$ – arterial partial pressure of CO_2/pressão parcial arterial de CO_2

PaO_2 – arterial partial pressure of O_2/pressão parcial arterial de O_2

PBC – pre-Bötzinger complex/complexo pré-Bötzinger

PBS - phosphate buffer solution/tampão fosfato-salino

pCO_2 - partial pressure of CO_2/pressão parcial de CO_2

PFA – perifornical area/área perifornical

Pn – pons/ponte

pO_2 - partial pressure of O_2 or oxygen tension/pressão parcial de O_2

PVN – paraventricular nucleus/núcleo paraventricular

R – rostral

REM – rapid eyes movement

RTN/pFRG – retrotrapezoid nucleus/parafacial group/núcleo retrotrapezoide/grupo respiratório parafacial

rVLM – rostral ventrolateral medulla/bulbo ventrolateral rostral

rVRG – rostral respiratory group/grupo respiratório rostral

s.e.m. - standard error of the mean/erro padrão da média

SO – oliva superior

$\dot{V}_I$ - inspired ventilation rate/ventilação inspirada (fR x V_T)

VII – facial nucleus/núcleo facial

V_T – tidal volume/volume corrente

4v – 4th ventricle/4º ventrículo

7N – facial nerve/nervo facial

SÍNTESE

O presente estudo trata das orexinas e seu papel na ventilação basal e na modulação das respostas respiratórias à hipercarbia e hipóxia em girinos pré-metamórficos de rãs-touro (*Lithobates catesbeianus*) e em iguanas verdes (*Iguana iguana*).

Para facilitar o entendimento, o estudo foi dividido em uma síntese, que tem como finalidade reunir todas as informações relativas ao assunto, sumarizar as descobertas e discuti-las; e em mais dois capítulos detalhados, cada um referente a uma parte do estudo. O capítulo 1 teve como objetivo entender a participação das orexinas na atividade motora respiratória em girinos de rãs-touro ("*in vitro*"); enquanto o capítulo 2 se destinou a avaliar a participação das orexinas nas respostas respiratórias à hipercarbia e hipóxia em iguanas verdes em animais não anestesiados ("*in vivo*").

As orexinas (ORXs) são peptídeos produzidos por um grupo de neurônios do hipotálamo que regulam diversas funções fisiológicas - como o ciclo sono vigília, o comportamento de se alimentar, a pressão arterial e até mesmo a respiração. Esses neurônios são responsivos a alterações na pCO_2/pH e à hipóxia; e seus efeitos na respiração envolvem a participação nos quimiorreflexos. A grande maioria dos estudos a respeito das ORXs e da sua contribuição para a modulação respiratória foram realizados em mamíferos, principalmente em roedores. O único trabalho realizado em vertebrados não-mamíferos, foi um trabalho do nosso grupo que demonstrou que o receptor-1 de ORX (OX_1R) contribui para os quimiorreflexos à hipercarbia e à hipóxia em sapos adultos. Essa tese, portanto, se destinou a avaliar o papel das ORXs no controle respiratório em girinos de rã-touro através de registros da atividade respiratória em preparações "*in vitro*" do tronco encefálico; e em iguanas verdes através de registros "*in vivo*" da ventilação.

Nós observamos que as ORXs participam das respostas ventilatórias à hipercarbia nessas duas classes de vertebrados, mas apenas nos girinos, participam também das respostas ventilatórias à hipóxia também. No caso dos girinos, nossos experimentos demonstraram que as ORXs atuam nos quimiorreflexos ao O_2 e CO_2 de maneira inibitória, enquanto nas iguanas, atuam no quimiorreflexos ao CO_2 de maneira excitatória.

Orexines (ORXs) are peptides produced by a group of neurons of the hypothalamus which regulate various physiological functions - such as the sleep-wake cycle, eating behavior, blood pressure, and respiration. These neurons are responsive to changes in pCO_2/pH and hypoxia; and its effects on breathing involve participation in chemoreflexes. The vast majority of studies on ORXs and their contribution to respiratory modulation have been performed in mammals, especially rodents. The only work conducted in a non-mammalian vertebrate is one work from our group that demonstrated that ORX receptor-1 (OX_1R) contributes to hypercarbia and hypoxia chemoreflexes in adult toads. This thesis, therefore, was designed to evaluate the role of ORXs in respiratory control in bullfrog tadpoles by recording respiratory activity in in vitro brainstem preparations; and in green iguanas through "*in vivo*" ventilation recordings.

We observed that ORXs participate in ventilatory responses to hypercarbia in these two classes of vertebrates; but only in tadpoles they participate in ventilatory responses to hypoxia as well. In the case of the tadpoles, our experiments have shown that ORXs act in an inhibitory manner on O_2 and CO_2 chemoreflexes, while in iguanas, they act on CO_2 chemoreflexes in an excitatory way.

Respiração

Poucos processos são tão essenciais para a vida quanto a respiração. A definição fisiológica de respiração difere da definição bioquímica. A definição bioquímica é o processo metabólico pelo qual um organismo obtém energia em forma de ATP – adenosina trifosfato. Essa energia é oriunda, justamente, das ligações que unem os fosfatos na molécula de ATP, ligações de alta energia que, quando necessário para alguma função ou reação do corpo, são quebradas liberando energia suficiente para esses eventos. A respiração celular pode ser de dois tipos: aeróbia – realizada na presença de oxigênio – ou anaeróbia – realizada na ausência de oxigênio. A respiração aeróbia (que é sobre a qual vamos falar no presente estudo), ou fosforilação oxidativa, é a obtenção de energia (ATP) por meio da oxidação de nutrientes.

Fisiologicamente, a respiração diz respeito à difusão e transporte de metabólitos entre o organismo e o ambiente externo. Nossas células necessitam de um suprimento contínuo de oxigênio (O_2) para que, por meio da respiração celular, possam gerar a energia necessária para seu perfeito funcionamento e produção de trabalho. O sistema respiratório tem como função básica fornecer O_2 aos tecidos e retirar o dióxido de carbono (CO_2) produzido pelo metabolismo celular, regulando o processo de troca gasosa para manter as pressões parciais desses gases no sangue arterial ($PaCO_2$ e PaO_2) relativamente constantes. Dessa maneira, as trocas gasosas são equilibradas em função da demanda do metabolismo (Gilmour, 2001).

O ritmo respiratório é gerado no sistema nervoso central (SNC) e é dependente de aferências que trazem ao SNC informações sobre as pressões parciais dos gases sanguíneos, o pH dos líquidos corporais, os gases inspirados e o estado de estiramento pulmonar, informações detectadas por químio e mecanorreceptores. A integração destas aferências no SNC resulta na

modulação das eferências aos músculos respiratórios (Wang et al., 1999) que acontece para que os gases sanguíneos do meio interno sejam mantidos dentro de valores fisiológicos de acordo com a demanda metabólica relacionada a um dado estado de atividade.

Figura 1. Figura representativa do controle respiratório por quimiorreceptores.

Para que o sistema funcione devidamente, os componentes muscular e neuronal do sistema respiratório devem estar funcionais possibilitando a geração de um ritmo que permita as trocas gasosas e integrando os outros comportamentos relacionados à respiração. A circuitaria neural respiratória deve estar bem coordenada com outros movimentos que geram fluxo de ar de acordo com a espécie em questão, como vocalização, deglutição, reflexos das vias aéreas como tossir ou espirrar, ou até mesmo a locomoção (Greer et al., 2006; Klein e Codd, 2010). Esses circuitos devem estar estáveis e responsivos aos estados metabólicos, que afetam as concentrações de O_2 e CO_2, e pH no organismo, como vigília, exercício, sono, altitude, se enterrar/entocar, hibernação etc. Da mesma forma, alterações em longo prazo que

possam ocorrer no organismo (ganho ou perda de peso, fase reprodutiva ou doença) demandam ajustes respiratórios para acomodar mudanças físicas associadas (Feldman et al., 2003).

Respiração em vertebrados

Estima-se que os vertebrados compreendam cerca de 56 mil espécies viventes hoje em dia - distribuídas em seis classes que ocupam praticamente todos os ecossistemas do planeta: desde as profundezas dos oceanos até as maiores altitudes (Pough et al., 2008). Todavia, para que os animais habitem habitats tão diversos, é necessário que apresentem adaptações morfofisiológicas que os possibilitem viver com o que o ambiente oferece. De acordo com Shelton et al. (1986), essa diversidade fica óbvia no caso dos mecanismos respiratórios e seus sistemas de controle associados - que relacionam a respiração tanto ao ambiente quanto às necessidades dos animais.

Ao longo da evolução, os vertebrados enfrentaram desafios similares fundamentais para aquisição de oxigênio para o metabolismo aeróbio. Sob limitações e restrições impostas por fatores tais como filogenia, comportamento, tamanho corpóreo e o ambiente, esses animais responderam diferentemente desenvolvendo estruturas respiratórias ótimas. A refinada ideia de que as adaptações são resultado do processo de seleção natural ao longo de várias gerações seguidas de mudanças é revelada através do desenvolvimento da perfeita relação entre estrutura e desempenho do sistema respiratório no decorrer da história. Em cada táxon, a complexidade e a eficiência respiratórias aumentam de acordo com a capacidade metabólica e a necessidade por oxigênio. Por exemplo, pequenos endotérmicos superativos tem superfícies de trocas gasosas mais refinadas do que ectotérmicos grandes e letárgicos (Maina, 2002). Dessa forma, consequentemente, existem particularidades nos sistemas respiratórios dos vertebrados ectotérmicos de acordo com seus modos de respiração (Taylor et al., 2010).

De acordo com Kinkead (1997), o modelo atual de controle respiratório é composto por um gerador de ritmo e um circuito de formação do padrão respiratório, mas a diferença entre ritmo e padrão respiratórios nem sempre é óbvia: o ritmo respiratório refere-se à frequência respiratória, enquanto o padrão respiratório refere-se aos componentes motores que podem ser alterados de maneira relativamente independente do ritmo respiratório (Kinkead e Milsom, 1997).

Os padrões respiratórios da maioria dos peixes, aves e mamíferos são contínuos, enquanto grande parte dos peixes de respiração aérea, anfíbios e répteis apresenta ventilação intermitente ou episódica (Milsom, 1991). Estes, podem exibir dois tipos de respiração episódica: um em que eventos individuais são espaçados (relativamente uniformes) ou um em que episódios de ventilação contínua são separados por longos períodos não-ventilatórios (Milsom, 1991). Como a respiração intermitente típica dos ectotérmicos apresenta pausas não-ventilatórias, flutuações importantes na gasometria arterial ocorrem nesses organismos, mas não são prejudiciais pelo fato do metabolismo desses indivíduos ser baixo. Desta forma, ectotérmicos toleram muito bem situações de hipóxia e hipercarbia (Kinkead, 1997).

Figura 2. Comparação entre registros de ventilação de vertebrados ilustrando a variação nos padrões respiratórios (contínuos e episódicos). Figura de Milsom, 1991.

Todos os vertebrados possuem áreas geradoras de ritmo localizadas no tronco encefálico (Taylor et al., 2010) e, no geral, essas áreas parecem ser altamente conservadas ao longo da evolução (Hedrick et al., 2005). Em peixes ciclostomados, parece estar localizada no núcleo do nervo trigêmeo. Em peixes de respiração aérea e anfíbios aparentemente existem osciladores distintos para ventilação branquial e pulmonar na formação reticular bulbar ventral. Já em répteis, existem evidências de múltiplas áreas geradoras de ritmo (Taylor et al., 2010; Hedrick et al., 2005). Em aves e em mamíferos a geração do ritmo está localizada na superfície ventral do bulbo, destacando a região parafacial (pFRG) e o complexo Pré-Bötzinger (PBC) (Milsom et al., 2004).

De maneira geral, o "*drive*" respiratório representa a integração das aferências periféricas (receptores de estiramento pulmonar, quimiorreceptores arteriais, quimiorreceptores olfatórios) com essas regiões centrais ritmogênicas, produzindo assim os padrões respiratórios.

Quimiorrecepção em vertebrados

Os quimiorreceptores centrais e periféricos são células especializadas em detectar alterações na pCO_2, pO_2 e pH, e promover ajustes na ventilação via SNC, garantindo a homeostase de acordo com a demanda do metabolismo (Jones e Milsom, 1982; Milsom, 1990; Smatresk, 1990; Ballam, 1984, 1985, Coates e Ballam, 1987). A hipercarbia é caracterizada por um aumento da pCO_2 (pressão parcial de dióxido de carbono) no ar inspirado; e a hipóxia, pela diminuição na pO_2 (pressão parcial de oxigênio) no ar inspirado. A hipercapnia é o aumento da $PaCO_2$ (pressão parcial arterial de dióxido de carbono) nos líquidos corporais e a hipoxemia é a diminuição da PaO_2 (pressão parcial arterial de oxigênio) nos mesmos.

Quimiorrecepção ao O_2

Os quimiorreceptores periféricos são sensíveis a variações na PaO_2, na $PaCO_2$ e no pH; eles estão presentes em todos os vertebrados estudados até o momento (Milsom e Burleson, 2007). Os quimiorreceptores de O_2 estão localizados na periferia, mas existem evidências de que existem também receptores centrais de O_2, entretanto, sua participação na resposta sistêmica ainda não foi bem compreendida (Neubauer e Sunderram, 2004; Winmill et al., 2005).

Em peixes, esses sensores estão presentes nas brânquias e respondem a diferentes concentrações desse gás tanto no ambiente quanto no sangue arterial (Milsom e Burleson, 2007; Perry et al., 2009). Também há evidências de sítios receptores fora do aparato branquial, os receptores venosos de O_2 (Barrett e Taylor, 1984) e os receptores extra branquiais (Butler et al.,

1977). Evolutivamente, os receptores associados ao primeiro e segundo arcos branquiais de peixes tornaram-se os receptores periféricos primários em todos os demais vertebrados, portanto, os quimiorreceptores dos tetrápodes adultos são considerados homólogos aos dos peixes (Milsom, 2002; Milsom e Burleson, 2007). Existe uma possível redução na distribuição dos quimiorreceptores periféricos de O_2 de múltiplos e dispersos sítios, organizados em pequenos "*clusters*", como em peixes, anfíbios e répteis, para poucos sítios, arranjados em "*clusters*" maiores e dominantes como os das aves e mamíferos. Essa evolução na organização desses sítios provavelmente está associada à quantidade de O_2 disponível no ambiente (ar ou água) e à demanda energética (Reyes et al., 2015). Em mamíferos e aves os quimiorreceptores periféricos para O_2, CO_2/pH estão localizados nos corpos carotídeos e arco aórtico.

A resposta frente à hipóxia ambiental ou arterial nos vertebrados adultos é, em geral, uma hiperventilação. A informação dos quimiorreceptores é levada ao tronco encefálico (principalmente grupos respiratórios bulbares), onde ocorre a integração das aferências com neurônios de áreas específicas do bulbo, essas regiões fazem parte do processamento deste sinal e da modulação da resposta (Nattie e Li, 2006; Takakura et al., 2006), resultando em aumento da ventilação (Gonzalez et al., 1995).

Mais detalhes sobre a quimiorecepção ao O_2 em anfíbios e répteis em: *Respiração em anfíbios* e *Respiração em répteis*.

Quimiorrecepção ao CO_2

Em relação às respostas ao CO_2, os quimiorreceptores sensíveis ao CO_2/pH podem ser classificados como periféricos e centrais. Segundo Guyenet et al. (2010), os quimiorreceptores centrais são células sensíveis a variações na pCO_2 e no pH encefálicos e contribuem para a estimulação da ventilação causada por hipercapnia ou acidose metabólica.

Os quimiorreceptores centrais já foram encontrados em todos os grupos de tetrápodes (Milsom, 2002). Estes receptores são banhados pelo líquor e isolados do sistema circulatório pela barreira hematoencefálica, que é pouco permeável ao íon H^+, protegendo o encéfalo de variações no pH sistêmico, porém, altamente permeável ao CO_2 (Hlastala e Berger, 2001).

O surgimento dos quimiorreceptores centrais está associado à redução dos sítios de quimiossensibilidade periférica ao CO_2/pH. Uma vez que surgiram na história evolutiva, sua atividade predomina sobre a dos demais receptores no controle da ventilação em condições basais (Milsom, 2002). Em mamíferos, estudos indicam que os corpos carotídeos são responsáveis por cerca de 30% da resposta ventilatória à hipercapnia sistêmica (Dempsey e Forster, 1982; Pan et al., 1998; Forster et al., 2008), sendo o restante realizado pelos quimiorreceptores centrais (Forster e Smith, 2010).

Em peixes, os quimiorreceptores de CO_2 são encontrados nas brânquias, sendo ativados apenas quando aumenta-se a concentração desse gás no ambiente (Perry e Mckendry, 2001; Perry et al., 2009; Reid et al., 2000), o que está de acordo com o fato da $PaCO_2$ destes animais ser muito baixa (Howell, 1970; Cameron e Randall, 1972). Já nos tetrápodes, os quimiorreceptores de CO_2/pH no sistema vascular encontram-se geralmente em congruência com os de O_2 e sua estimulação gera uma resposta hiperventilatória (Milsom, 2002). A informação sensorial dos quimiorreceptores centrais sensíveis a CO_2/pH é fundamental para o controle da respiração, os quais afetam a geração do ritmo respiratório e a modulação do seu padrão para proteger o encéfalo das alterações no CO_2 e no pH.

Existem receptores periféricos encontrados nas vias aéreas (na pele ao redor das narinas e da boca) no caso de anfíbios e répteis sensíveis às variações no CO_2 ambiental: são os chamados quimiorreceptores olfatórios (Jones e Milsom, 1982, Milsom et al., 2004). Esses

receptores inibem a respiração e prolongam a retenção da respiração quando os níveis ambientais de CO_2 são altos.

Ainda existem os receptores de estiramento pulmonar e receptores intrapulmonares encontrados nos pulmões sensíveis ao CO_2 e que regulam o padrão respiratório de uma maneira que reduz a ventilação do espaço morto e aumenta a eficiência da excreção de CO_2 sob condições de hipercarbia ambiental e/ou reduz a perda de CO_2 pela hiperventilação (Milsom et al., 2004).

Mais detalhes sobre a quimiorrecepção ao CO_2 em anfíbios e répteis em: *Respiração em anfíbios* e *Respiração em répteis*.

Respiração em anfíbios

Os primeiros anfíbios surgiram no período devoniano, na Era Paleozoica, há cerca de 350-400 milhões de anos. A classe Amphibia é representada atualmente pelas ordens Urodela (salamandras), Gyminophiona (cecílias) e Anura (sapos, rãs e pererecas). Mesmo apresentando várias similaridades, esses grupos são muito diferentes no que se refere à estrutura esquelética e aos modos de vida. São animais que ocupam vários ecossistemas, tais como: florestas tropicais equatoriais, ambientes gelados, montanhas, pântanos, cavernas, desertos, entre outros.

Os anfíbios possuem a fase larval totalmente aquática e, na sua maioria, fase adulta terrestre. Portanto, esse grupo é um modelo bastante interessante para o estudo do controle da ventilação em vertebrados. Esta compreensão pode revelar algumas propriedades fundamentais que estão associadas à emergência da respiração aérea. Geralmente, as espécies de anuros passam por uma metamorfose antes de se tornarem adultos, que prepara a larva aquática para a vida terrestre. Logo após o nascimento, em girinos pré-metamórficos, as trocas gasosas são

realizadas através da pele e brânquias, passando a ocorrer em três locais concomitantemente durante a metamorfose (pele, brânquias e pulmões) para, no adulto, ocorrer através da pele e pulmões (Burggren e West, 1982).

Em rãs adultas da espécie *Lithobates catesbeianus*, a 20°C os pulmões são responsáveis por cerca de 80% da captação de O_2, mas por apenas 20% da excreção de CO_2 durante o repouso (Burggren e West, 1982) como exemplifica a figura 3. Em *Anaxyrus americanus*, o pulmão capta 59% do O_2 e excreta 21% do CO_2 (Ultsch, 1996).
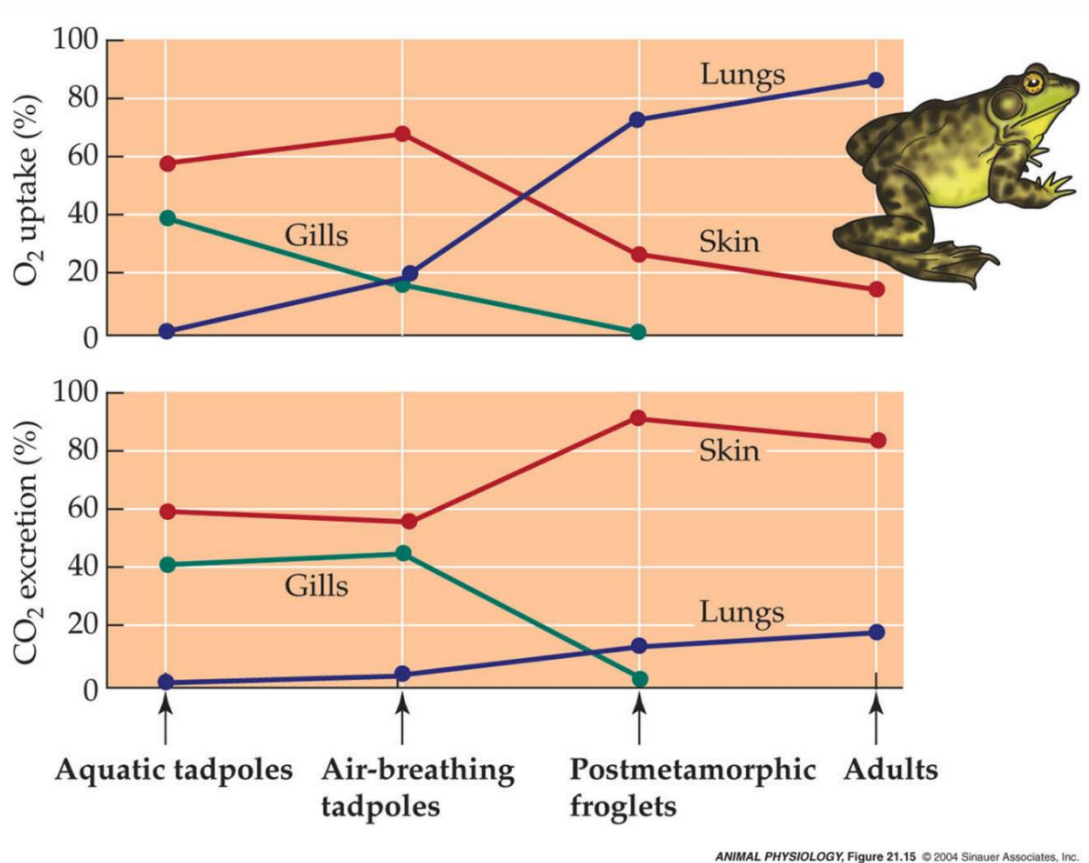

Figura 3. Representação da contribuição de cada órgão para a troca gasosa (captação de O_2 e excreção de CO_2) ao longo do desenvolvimento de *Lithobates catesbeianus*. Figura de Hill et al. (2004).

Nesses animais, a ventilação funciona através de uma "bomba respiratória" dependente da harmônica atividade das aferências neuromusculares para os músculos respiratórios, as narinas e a glote, produzindo fluxo de água pelas brânquias ou de ar pelos pulmões. Os músculos respiratórios em anfíbios são inervados por nervos cranianos.

Os girinos usam bombas de força bucal e faríngea para produzir um fluxo unidirecional de água através das brânquias. A cavidades bucal e a faríngea são separadas por um "*velum*" (seria uma estrutura correspondente à glote dos adultos). Quando o assoalho da cavidade bucal é deprimido, a pressão dentro desta câmara diminui, enchendo a câmara de água através da boca e narinas. A pressão negativa fecha o "*velum*", impedindo a entrada de água na cavidade faríngea. Perto do final da fase de inspiração bucal, a constrição faríngea provoca um aumento da pressão dentro da cavidade faríngea, que mantém o "*velum*" fechado e expele a água através das brânquias. Em seguida, o assoalho bucal é elevado, elevando a pressão dentro desta câmara e fechando o "*velum*". Isso empurra o "*velum*" e força a água para dentro da cavidade faríngea, que se expande e juntamente com a compressão bucal reabastecem a cavidade faríngea. O efeito líquido não é apenas para preencher a câmara faríngea, mas para deslocar a água sobre as brânquias, uma vez que a ejeção bucal excede a taxa de expansão faríngea. Como consequência, como ocorre com os peixes que respiram água, há um fluxo unidirecional de água sobre as brânquias durante as duas fases do ciclo ventilatório (Gradwell, 1972; Wassersug e Hoff, 1979; Gargaglioni e Milsom, 2007). A ventilação pulmonar nos adultos ocorre também através do uso da musculatura do assoalho bucal enquanto a glote encontra-se aberta, impulsionando a entrada de ar para os pulmões (West e Jones, 1975; Vitalis e Shelton, 1990; Gargaglioni e Milsom, 2007).

Figura 4. Esquema ilustrando a "bomba respiratória" em girinos e anuros adultos. Figura da esquerda de Gargaglioni e Milsom (2007) e da direita de Hill et al. (2004).

O padrão ventilatório pulmonar varia dentre os diferentes grupos de anuros. Em rãs, os períodos de apneia são geralmente interrompidos por eventos pulmonares individualizados e com o aumento do "*drive*" respiratório, a ventilação passa a ser episódica, podendo tornar-se contínua (Boutilier e Toews, 1977; Milsom, 1991; Kinkead e Milsom, 1994; Kinkead, 1997). Já em sapos do gênero *Rhinella*, mesmo com pouco estímulo para a ventilação, esta já ocorre em episódios, que se constituem primeiramente de eventos de deflação, seguidos por eventos balanceados e por fim, eventos de inflação pulmonar, seguindo-se um período de apneia maior do que os observados em rãs (Branco et al., 1992; Wang, 1994; Coelho e Smatresk, 2003; Gargaglioni e Milsom, 2007).

Conforme já mencionado, os anfíbios, assim como os outros tetrápodes, apresentam quimiorreceptores periféricos e centrais que detectam as alterações nos gases sanguíneos, levando a ajustes ventilatórios para a manutenção das pressões parciais de CO_2 e O_2 no sangue arterial e do equilíbrio ácido-base.

Nesses animais, os quimiorreceptores de O_2 estão localizados no labirinto carotídeo, na bifurcação das artérias carótidas interna e externa, no arco aórtico e na artéria

pulmocutânea (Gargaglioni e Milsom, 2007). Em anfíbios, a hipóxia induz um aumento da ventilação, que ocorre principalmente pela estimulação dos quimiorreceptores localizados no labirinto carotídeo (Boutilier e Toews, 1977; Van Vliet e West, 1992; Wang et al., 1994). O labirinto carotídeo é um leito vascular em forma de labirinto que surge a partir da artéria carótida (Toews et al., 1982; Kusakabe, 2002, 2009). É considerado homólogo aos quimiorreceptores presentes no primeiro arco branquial de peixes e ao corpo carotídeo de mamíferos, com base na sua origem embrionária e inervação por ramos dos nervos cranianos IX e X (Ishii et al., 1966; Van Vliet e West, 1987; West e Van Vliet, 1992; Kusakabe, 2002, 2009; Milsom e Burleson, 2007). Contudo, a denervação bilateral do labirinto carotídeo não abole completamente a resposta ventilatória à hipóxia (Van Vliet e West, 1987). Duas outras áreas, o arco aórtico e a artéria pulmocutânea também são considerados sítios quimiossensíveis em anfíbios (Lillo, 1980; Hoffmann e de Souza, 1982; Ishii et al., 1985; Wang et al., 2004). Um estudo recente de Reyes et al., (2014) demonstrou a presença de células imunorreativas para tirosina hidroxilase e serotonina no labirinto carotídeo, no arco aórtico e na artéria pulmocutânea de rãs (*Lithobates catesbianus*) e uma subpopulação de células imunorreativas para ambos no labirinto carotídeo.

Em relação aos quimiorreceptores centrais, em mamíferos e anfíbios, em um primeiro momento, foram demonstradas áreas quimiossensíveis na superfície ventrolateral do bulbo, sendo banhadas pelo quarto ventrículo encefálico (Coates et al., 1993; Torgerson et al., 2001). A contribuição relativa de cada uma dessas áreas para o controle respiratório em mamíferos vem sendo amplamente debatida (Nattie e Li, 2006; Guyenet et al., 2008). Contudo, com os estudos em mamíferos, várias evidências começaram a indicar que os quimiorreceptores centrais estão amplamente distribuídos no SNC, e não somente no bulbo, e seriam áreas quimiossensíveis o núcleo do trato solitário (NTS), o núcleo fastigial (FN), o núcleo

retrotrapezóide (RNT) Mulkey et al., 2004; Guyenet et al., 2005), a rafe rostral bulbar, o locus

coeruleus (LC) (Nattie, 2001; Biancardi et al., 2008) e os neurônios orexinérgicos (ORXs) do

hipotálamo (Dias et al., 2009; Dias et al., 2010; Williams e Burdakov, 2008; Kuwaki et al.,

2010; Williams et al., 2007). A figura 5 ilustra a visão antiga a respeito e a visão mais atual.

Figura 5. Localização esquemática dos quimiorreceptores centrais. Em azul do lado esquerdo a visão clássica: quimiorreceptores localizados na superfície bulbar ventral; e em vermelho do lado direito a visão atual: quimiorreceptores amplamente distribuídos no SNC. Figura de Nattie e Li, 2012. Na figura, as abreviações: rostral (R), medial (M), caudal (C), hipotálamo lateral (LHA), rafe dorsal (DR), núcleo fastigial (FN), 4º ventrículo (4v), locus coeruleus (LC), nervo facial (7N), núcleo do trato solitário caudal (cNTS), núcleo ambiguus (AMB), núcleo facial (VII), oliva superior (SO), complexo pre-Bötzinger (PBC), grupo respiratório rostral (rVRG), bulbo ventrolateral caudal (cVLM), núcleo retrotrapezoide/grupo respiratório parafacial (RTH/pFRG) e ponte (Pn).

O mesmo vem acontecendo para os anfíbios. Estudos evidenciaram algumas áreas quimiossensíveis no SNC desses animais que não pertencem à superfície ventrolateral do bulbo: o locus coeruleus/núcleo do istmo (Kinkead et al., 1997; Noronha-De-Souza et al., 2006; Santin e Hartzler, 2013) no mesencéfalo, duas regiões bulbares - uma "área quimiossensível rostral" na altura dos nervos trigêmeos, e uma "área quimiossensível caudal" na altura do nervo vago (Taylor et al., 2003; Torgerson et al., 2001) - e os neurônios ORXs no hipotálamo (Fonseca et al., 2016). Contudo, outras regiões sensíveis a variações na pCO_2/pH provavelmente existem (Reed et al., 2018).

Transição da respiração aquática para a aérea

As principais modificações nos sistemas respiratórios durante a evolução dos vertebrados são inquestionavelmente aquelas associadas à mudança da respiração na água para a respiração do ar (Shelton et al., 1986). A conquista do ambiente terrestre veio acompanhada da transição do modo de ventilação bimodal (água e ar) para o modo unimodal (ar), que foi de suma importância na história evolutiva dos vertebrados. Segundo Gans (1970), a respiração aérea provavelmente surgiu em peixes que nadavam rente à superfície durante épocas com baixa disponibilidade de O_2 durante o período Devoniano. Essa transição afetou diretamente diversos sistemas e seus mecanismos de controle devido às diferenças das propriedades físicas entre o ar e a água.

Devido à baixa capacitância da água ao oxigênio, os vertebrados de respiração aquática produzem uma taxa elevada de fluxo de água pelas brânquias, a fim de se obter O_2 suficiente para suprir as demandas metabólicas (Milsom, 2002). Como consequência, o CO_2 produzido metabolicamente é rapidamente excretado pelas brânquias, resultando em uma baixa pressão parcial arterial de CO_2 (Milsom, 2002).

A concentração de O_2 no ar atmosférico nos dias de hoje é cerca de 30 vezes maior do que na água à 15°C, e a água é 800 vezes mais densa e 50 vezes mais viscosa que o ar atmosférico (Schmidt-Nielsen, 2002), possibilitando aos animais de respiração aérea uma menor taxa ventilatória da superfície respiratória (Ultsch, 1996). Essa menor ventilação nos animais de respiração aérea está associada à uma menor eliminação de CO_2 comparada aos animais que respiram água, sendo que os animais de respiração aérea são considerados retentores de CO_2. Desta forma, a transição do ambiente aquático para o terrestre resultou em maiores valores de pCO_2 (Nattie e Li, 2012). Este fato, associado à perda das brânquias como local primário de eliminação do CO_2, gerou um novo desafio aos vertebrados terrestres: a eliminação do CO_2 e regulação do pH (Ultsch, 1996).

Juntamente com essa mudança, uma grande transformação ocorreu nos mecanismos de controle da ventilação nos vertebrados: em animais de respiração aquática o O_2 exerce o papel dominante no controle químico da ventilação (Milson, 2002). Contudo, com o aumento da $PaCO_2$ em animais de respiração aérea, aumentou-se a sensibilidade a esse gás, que passa agora a ser o principal estímulo ventilatório durante o repouso (Milsom, 2002). A regulação do CO_2 é essencial, já que ele está associado ao pH e os organismos costumam ser pouco tolerantes a alterações no pH dos líquidos corporais.

Os anfíbios são animais interessantes para estudar a transição da respiração aquática para a aérea pois passam por mudanças extraordinárias durante os estágios da vida. A prevalência da ventilação branquial sobre a pulmonar durante os primeiros estágios larvais se inverte completamente durante a metamorfose (Burggren e West, 1982; Burggren e Doyle, 1986). Para serem completamente funcionais, essas transformações no sistema respiratório exigem modificações também na rede neural que gera e modula a atividade respiratória. Estudar

essas mudanças tão intrigantes pode revelar propriedades fundamentais associadas ao surgimento da respiração aérea.

Respiração em répteis

Os répteis evoluíram a partir dos anfíbios labirintodontes aproximadamente 60 milhões de anos depois que os primeiros anfíbios surgiram. Foram os primeiros vertebrados que apresentaram os requisitos para dominar totalmente o ambiente terrestre, incluindo o aparecimento do ovo terrestre, das membranas embrionárias e de um tegumento resistente à dessecação (Hildebrand, 1995). Os répteis atuais são representados por quatro ordens: Testudinata, Crocodilia, Squamata e Rhynchocephalia. Essa classe é altamente polifilética, apresentando assim grande diversidade quanto aos mecanismos respiratórios. Nesse contexto, esse é um grupo muito interessante para estudos relacionados à respiração aérea e fornecer "*insights*" a respeito da evolução dos mecanismos respiratórios que possibilitaram a conquista do ambiente terrestre.

Como os lagartos correm formando um S, usando músculos segmentais da lateral do corpo, acreditava-se que que eles eram incapazes de respirar enquanto correm, entretanto, foi demonstrado que alguns lagartos usam um modo alternativo de ventilação envolvendo a "bomba gular" alternado à "bomba torácica" (Taylor et al., 1999; Taylor et al., 2010). Essa bomba costuma ser usada em situações de hipóxia ou exercício. A figura 6 mostra simplificadamente como aconteceu a aquisição desses caracteres respiratórios durante a evolução.

Figura 6. Cladograma simplificado da evolução da "bomba respiratória" nos vertebrados. Figura de Brainerd (1999).

Em relação ao padrão ventilatório de répteis, ele pode ser contínuo ou intermitente, sempre iniciado por uma expiração como mostra a Figura 7 (Glass e Wood, 1983). O padrão se torna complexo em se tratando desses animais pois incluem períodos não-ventilatórios que podem ser combinados com submersão. Pouco se sabe a respeito dos mecanismos que geram os padrões respiratórios "arrítmicos" em répteis, mas sabe-se que aferências periféricas são importantes na determinação do padrão respiratório nesses respiradores episódicos (Shelton et al., 1986).

Figura 7. Registros demonstrando a variedade de padrões respiratórios em répteis. Figura de Glass e Wood (1983).

Existem evidências de que existem múltiplas áreas geradoras de ritmo em répteis (Johnson et al., 2007; Taylor et al., 2010). Johnson et al. (2015) demonstrou atividade respiratória espontânea (células marca-passo) em preparações isoladas do tronco encefálico de tartarugas. Eles identificaram e classificaram os neurônios respiratórios baseados no tipo de disparo: a maioria dos neurônios eram neurônios expiratórios, enquanto neurônios inspiratórios, pós-inspiratórios e pré-expiratórios eram menos comuns. A maior parte dos neurônios marca-passo foram localizados na altura da raiz do nervo vago (X). Ainda não havia sido demonstrada a existência de neurônios marca-passo relacionados a atividade respiratória em animais ectotérmicos, e os autores hipotetizaram que esses neurônios existem, são conservados evolutivamente e devem ser potenciais células ritmogênicas que contribuem para a respiração, pelo menos em tartarugas.

A Figura 8 ilustra o ciclo respiratório do lagarto *Uromastyx aegyptius microlepis*. O ciclo é trifásico, com expiração ativa, inspiração ativa e depois a uma expiração parcial, seguida por uma pausa ventilatória com os pulmões inflados, terminados pelo ciclo seguinte.

Figura 8. Atividade dos músculos intercostais de *Uromastyx aegyptius microlepis* demonstrando como acontece o ciclo respiratório de lagartos. Figura de Al-Ghamdi et al., 2001.

Cieri et al. (2014) demonstrou que iguanas verdes apresentam um fluxo unidirecional de ar nos pulmões. Até então acreditava-se que essa era uma característica exclusiva de aves e que era uma adaptação relacionada ao voo. Presumia-se que era uma adaptação para aumentar a eficiência das trocas gasosas e que era característica de animais com capacidades aeróbicas expandidas, para voar e manter um metabolismo endotérmico. As iguanas verdes são lagartos com fisiologia e hábitos notavelmente diferentes aos das aves, um animal ectotérmico com baixa capacidade aeróbica. Jacarés e lagartos da família Varanideae

43

são outros exemplos de vertebrados não-aves que também apresentam fluxo unidirecional de ar nos pulmões (Cieri et al., 2014; Farmer, 2015; Farmer e Sanders, 2010; Schachner et al., 2014).

Na maioria dos répteis, estudos relatam evidências indiretas que sugerem que os quimiorreceptores periféricos estejam localizados na artéria carótida interna, oriunda do arco carotídeo, sendo ela inervada pelo ramo laríngeo superior do nervo vago e, talvez, também do nervo glossofaríngeo (Jones e Milsom, 1982; Milsom, 1990; Smatresk, 1990). Mais recentemente, em sua tese, Reyes localizou células quimiossensíveis ao O_2 em *Trachemys scripta elegans* e em *Crotalus durissus* e viram que o arranjo e localização dessas células eram parecidos. Elas ficam na bifurcação da artéria carótida comum, aorta e artéria pulmonar. O "*truncus arteriosus*" de tartarugas não aparentou ser uma área quimiossensível (Reyes et al., 2015; Reyes, 2014).

Répteis apresentam receptores olfatórios que são sensíveis ao CO_2, como já mencionado, que estão localizados na pele ao redor das narinas e da boca, nas vias aéreas superiores, sendo sua resposta a inibição da ventilação (Jones e Milsom, 1982; Ballam, 1984, 1985; Coates e Ballam, 1987; Ballam e Coates, 1989; Coates et al., 1991, 1998; Coates, 2001). Acredita-se que a função desses quimiorreceptores seja inibir a respiração enquanto os animais procuram um ambiente melhor.

Os quimiorreceptores intrapulmonares tem sido descritos nos pulmões de várias espécies de répteis. Eles estão localizados dentro dos pulmões, inervados pelo nervo vago e têm uma descarga que é inversamente proporcional à pCO_2. Seu papel se dá principalmente na modulação do padrão, para melhorar a eficiência da excreção de CO_2 sob condições de hipercarbia ambiental e contribuir na finalização da inspiração (Banzett e Burger, 1977;. Milsom et al., 1981).

Os quimiorreceptores centrais sensíveis ao CO_2/H^+ em répteis não têm recebido muita atenção. Baseado em estudos de respostas reflexas ventilatória que emprega desnervação dos quimiorreceptores periféricos e técnicas de perfusão encefálica, a presença dos quimiorreceptores centrais foi demonstrada em tartarugas (Hitzig e Jackson, 1978; Hitzig, 1982; Hitzig e Nattie, 1982; Davies e Sexton, 1987) e jacarés (Branco et al., 1993). Novos estudos apontam ainda o locus coeruleus como uma região quimiossensível também em lagartos monitores (*Varanus exanthematicus*) (Zena et al., 2016).

Orexinas (ORXs)

Como já mencionado, os neurônios ORXs são considerados quimiossensíveis ao CO_2/pH em mamíferos e um estudo do nosso grupo verificou que, em anfíbios, esses neurônios também têm participação na modulação quimiorreflexa da ventilação (Fonseca et al., 2016). As ORXs, também conhecidas como hipocretinas, são pequenos neuropeptídios produzidos por neurônios no hipotálamo. Existem dois subtipos desses neuropeptídios: a ORX-A e a ORX-B (hipocretina-1 e hipocretina-2, respectivamente) ambas clivadas a partir de um precursor comum, a prepro-orexina (De Lecea et al., 1998; Sakurai et al., 1998), e ligam-se a dois tipos de receptores acoplados à proteína G: receptor-1 (OX_1R) e receptor-2 (OX_2R) (Smart et al. 1999, 2001).

Ainda não estão claras as diferentes funções de cada ORX. O receptor do tipo OX_1R é altamente seletivo à ORX-A, enquanto o OX_2R comporta-se como um receptor não-seletivo, ligando-se aos dois subtipos de ORXs (de Lecea et al. 1998; Sakurai et al. 1998).

Em mamíferos, os corpos celulares dos neurônios ORXs encontra-se somente no hipotálamo (hipotálamo lateral, posterior e núcleo perifornical), contudo projetam seus axônios por todo o encéfalo, exceto para o cerebelo (Peyron et al., 1998; Nambu et al., 1999). Esta

propriedade anatômica explica a grande multiplicidade de funções que são moduladas pelas ORXs como, por exemplo: a sensação de fome, o estado de sono-vigília, resposta ao estresse, nocicepção, bem como o controle cardiovascular e respiratório (Dube et al., 1999; Sakurai et al., 1998; Haynes et al., 2000; Bingham et al., 2001; Duxon et al., 2001).

Esse neurotransmissor está envolvido com uma ampla gama de processos fisiológicos como o ciclo sono-vigília, alimentação, estresse, homeostase energética, dor, metabolismo, sistema de busca-e-recompensa, situações de luta-e-fuga e até mesmo com a secreção hormonal (de Lecea et al., 1998; Sakurai et al., 1998).

A regulação do sono/vigília é a função fisiológica que mais vem sendo estudada. Anatomicamente, neurônios ORXs estão situados entre neurônios promotores do sono no núcleo pré-óptico ventrolateral e neurônios estimuladores da vigília localizados no tronco encefálico. Neurônios do núcleo pré-óptico ventrolateral têm um papel crucial na iniciação do sono NREM (do inglês, "*non rapid eye movement*") e na manutenção dos sonos NREM e REM (Sherin et al., 1998). Esses neurônios apresentam uma alta taxa de disparo durante o sono, e têm sua taxa de disparo diminuída durante a fase de vigília. Por outro lado, neurônios monoaminérgicos do núcleo motor do nervo trigêmeo, do locus coeruleus e da rafe dorsal desempenham importante papel na manutenção da vigília. Esses neurônios disparam tonicamente durante a vigília e se silenciam durante o sono REM (Sherin et al., 1998; Vanni-Mercier et al., 1984). Os neurônios ORXs, por sua vez, agem realizando uma ligação entre esses neurônios e estabilizando estados comportamentais, ativando regiões relacionadas ao despertar durante a vigília e prevenindo indesejáveis transições entre o sono e a vigília.

As concentrações de ORX no líquor variam durante o ciclo diário. Em roedores, as maiores concentrações ocorrem durante as últimas horas da fase escura (que é a fase ativa nesses animais) e as mais baixas durante a fase clara (inativa) (Desarnaud et al., 2004).

Adicionalmente, a taxa de disparo dos neurônios ORXs está relacionada com o estado de vigília, sendo maior nos estados de maior alerta (Lee et al., 2005; Mileykovskiy et al., 2005). Além disso, tem sido demonstrado que as ORXs exercem um importante papel na modulação cardiorrespiratória em mamíferos (Nattie e Li, 2012).

Orexinas e quimiorrecepção

Recentemente, foi demonstrado que os neurônios ORXs do hipotálamo de ratos são profundamente afetados por alterações no CO_2 e pH, estimulam a ventilação e são altamente sensíveis a variações mínimas no pH (Williams et al., 2007). O papel das ORXs na modulação da respiração é conhecido por ter evidências anatômicas e funcionais. Os axônios dos neurônios ORXs se projetam para áreas envolvidas no controle respiratório, como neurônios simpato-excitatórios do bulbo ventrolateral rostral, complexo Pré-Botzinger, núcleo do trato solitário, núcleo retrotrapezóide, rafe, locus coeruleus, núcleo frênico, núcleo hipoglossal, e seus receptores são expressos em todas essas áreas (Fung et al., 2001; Krout et al., 2003; Machado et al., 2002; Rosin et al., 2006; Volgin et al., 2002; Young et al., 2005; Dias et al., 2009).

Evidências fisiológicas indicam que as ORXs modulam a ventilação. Injeções intracerebroventriculares (i.c.v.) de ORXs aumentam a fR e o V_T de ratos (Zhang et al., 2005). Adicionalmente, a microinjeção de ORX-A no Complexo Pré-Bötzinger e a microperfusão nos motoneurônios frênicos resultam em um aumento na atividade do diafragma (Young et al., 2005).

As ORXs têm papel importante no controle da ventilação nos estados de vigília (Nakamura et al. 2007; Williams e Burdakov, 2008). Recentemente, estudos com ratos transgênicos indicam que possuem participação crucial na quimiossensibilidade ao CO_2/pH. Ratos "*knockout*" para a prepro-orexina apresentam uma atenuação no quimiorreflexo à

hipercapnia durante a vigília, mas não durante sono, e esse efeito é parcialmente recuperado com a administração das ORX-A e -B (Deng et al. 2007; Nakamura et al. 2007).

Além disso, administrações i.c.v. de um antagonista do OX_1R (SB-334867), diminuíram o quimiorreflexo respiratório em ratos (Deng et al. 2007). O disparo dos neurônios ORXs são extremamente sensíveis a mudanças fisiológicas nas concentrações de CO_2 e pH extracelulares "*in vitro*" (Williams et al., 2007). Dias et al. (2008) demonstrou em ratos que a diálise com o antagonista de OX_1R, SB-334867 especificamente na região do núcleo retrotrapezoide causou uma atenuação da resposta ventilatória à hipercapnia, que foi substancialmente maior durante a vigília do que durante o sono NREM.

Localização dos neurônios orexinérgicos em vertebrados

Curiosamente, as sequências de aminoácidos das ORX-A e -B foram altamente conservadas nos diferentes grupos de vertebrados (Alvarez, 2002, Peyron et al., 2000). As ORXs têm sido descritas em todas as classes dos vertebrados e os corpos celulares dos neurônios ORXs estão localizados, em sua grande maioria, no hipotálamo, embora em vários núcleos. As fibras, por sua vez, estão espalhadas por todo o SNC inervando áreas similares.

Em anfíbios, foram encontrados neurônios ORXs no núcleo supraquiasmático nas espécies *Hyla cinérea*, *Pelophylax ridibundus*, e *Rhinella diptycha* enquanto em *Xenopus laevis* esses neurônios estão restritos ao hipotálamo ventral. Isso mostra que distribuição dos neurônios imunorreativos para ORX no SNC é bem semelhante à de mamíferos (López et al., 2009; Singletary et al., 2005; Galas et al., 2001; Shibahara, 1999; Fonseca et al., 2016). As fibras dos neurônios ORXs estão amplamente distribuídas no SNC, sugerindo que este peptídeo pode estar envolvido em várias funções (López et al., 2009), incluindo a metamorfose (López et al., 2016).

López et al. (2016) verificaram a localização dos neurônios ORXs ao longo do desenvolvimento de *Xenopus laevis*. Já nos primeiros estágios foi detectada uma população no hipotálamo, e logo depois, projeções descendentes para a medula espinhal. Conforme o desenvolvimento avança, foram observadas células imunorreativas para ORX no núcleo supraquiasmático e o número dessas células aumentou consideravelmente, bem como a quantidade de fibras - tanto direcionadas ao telencéfalo, quanto ao tronco encefálico - incluindo o locus coeruleus e o núcleo do trato solitário. Durante o clímax metamórfico a distribuição já é muito semelhante à de adultos.

Já em répteis, o mesmo grupo encontrou corpos celulares desses neurônios no núcleo periventricular do hipotálamo e no hipotálamo infundibular em *Gekko gecko* e em e *Pseudemys scripta elegans*. Mas somente no gecko havia marcações também no núcleo hipotalâmico dorsolateral e no núcleo periventricular pré-óptico (Domínguez et al., 2010).

Tendo todas essas informações como base, e considerando a escassez de conhecimento relacionado ao controle da ventilação em vertebrados não-mamíferos, nós hipotetizamos que:

As ORXs, agindo nos receptores -1 e -2 de ORX, potencializam os quimiorreflexos ao O_2 e ao CO_2 tanto em girinos de rã-touro, quanto em iguanas verdes, estimulando a atividade respiratória durante a hipóxia e hipercapnia agudas.

Os modelos animais mencionados (girinos de rãs-touro e iguanas verdes) foram escolhidos, primeiramente, devido à falta de estudos sobre o sistema orexinérgico em outras classes de vertebrados (não-mamíferos). Além disso, explorar o papel das orexinas no controle

respiratório nessas classes, pode trazer "*insights*" a respeito da evolução do controle respiratório e da quimiorrecepção nos vertebrados. Mais que isso, os girinos são um modelo interessante para se estudar a modulação respiratória ao longo do desenvolvimento, especialmente tratando-se de um animal que possui respiração bi- ou até trimodal em alguns estágios (pele, brânquias e pulmões) e com modo de vida ainda dependente do ambiente aquático. As iguanas, por serem répteis lacertílios, são animais terrestres. Portanto, estudar essas classes pode ser interessante para avaliar a evolução do sistema orexinérgico e sua contribuição para a modulação respiratória ao longo da evolução.

Tendo todos esses elementos em mente e buscando entender melhor o controle respiratório em vertebrados ectotérmicos, conforme já mencionado, dividimos o trabalho em dois capítulos cujos objetivos gerais foram:

Capítulo 1

Investigar a contribuição da OXR-A na atividade motora respiratória e sua participação nos quimiorreflexos hipóxico e hipercápnico em girinos pré-metamórficos de rã-touro (*Lithobates catesbeianus*).

Capítulo 2

Avaliar a distribuição dos neurônios ORXs, a variação diária de ORX-A e a participação das ORXs na modulação respiratória basal e nas respostas respiratórias à hipercarbia e hipóxia em iguanas verdes (*Iguana iguana*).

Capítulo 1

A abordagem utilizada no capítulo 1 foi a preparação "*in vitro*" do tronco encefálico dos girinos de rã-touro. Os experimentos foram realizados utilizando dois tipos de preparação: 1- com o hipotálamo, e 2- sem o hipotálamo, onde se encontram os corpos celulares dos neurônios ORXs. As preparações foram então expostas a misturas hipercápnicas ou hipóxicas sozinhas, ou na presença de um antagonista de OX_1R para avaliar os efeitos na atividade motora respiratória.

Capítulo 2

A abordagem do capítulo 2 foi realizar medidas de ventilação "*in vivo*" por meio de pneumotacografia e avaliar a participação dos receptores de ORX através da microinjeção i.c.v. dos antagonistas SB-334867 e Almorexant em iguanas verdes. Também foi realizada imunohistoquímica para determinar a localização dos neurônios ORXs na espécie e dosagem de ORX-A no plasma por meio de teste ELISA para entender a variação desse neuropeptídeo nesses animais ao longo do dia.

Veja os capítulos 1 e 2 para a metodologia detalhada.

De maneira geral, ambos os capítulos mostram que as ORXs parecem não desempenhar um papel na modulação da ventilação basal em anfíbios e répteis, mas aparentam ser importantes em situações específicas, tendo participação nos reflexos hipóxico e hipercápnico. Entretanto algumas diferenças foram observadas.

Capítulo 1

Para girinos pré-metamórficos de rãs-touro, foi observado através de preparações "*in vitro*" do tronco encefálico que: (1) o diencéfalo desempenha um papel no "*drive*" respiratório basal (controle tônico, normoxia normocárbica); (2) a ORX-A não participa do controle respiratório basal; (3) a ORX-A inibe o quimiorreflexo hipóxico e hipercápnico atuando principalmente na frequência pulmonar.

Capítulo 2

Em iguanas verdes, nossas descobertas foram: (1) os neurônios ORXs estão localizados no núcleo periventricular do hipotálamo; (2) esses animais são diurnos e as maiores concentrações de ORX-A plasmática são no início da fase clara; (3) a hipóxia aguda causou alterações na ventilação apenas durante a fase clara desses animais, enquanto a hipercarbia aguda causou um aumento na ventilação após o fim do estímulo, sendo essa resposta mais intensa durante a fase clara; (4) as ORXs participam do quimiorreflexo ao CO_2 em iguanas verdes, mas não do quimiorreflexo hipóxico, de maneira excitatória.

O presente estudo demonstra a contribuição das ORXs nos quimiorreflexos ao O_2 e ao CO_2 em duas classes de vertebrados ectotérmicos: anfíbios e répteis. Nessa sessão, discutiremos de maneira geral e breve os principais resultados encontrados e a maneira com que eles se relacionam no que tange a participação das ORXs no controle respiratório de anfíbios e répteis. Os tópicos serão discutidos de maneira mais específica e detalhada nos capítulos 1 e 2.

Essas duas classes são de extrema relevância para o estudo dos mecanismos relacionados ao controle respiratório em vertebrados, e, até mesmo, para o estudo da evolução desses mecanismos. O desenvolvimento da respiração dos anfíbios fornece informações valiosas referentes à transição da respiração aquática para a respiração aérea; enquanto os répteis revelam as adaptações essenciais para a conquista definitiva do ambiente terrestre, e, com isso, da respiração aérea.

ORXs e parâmetros ventilatórios basais

Nossos dados sugerem que as ORXs não estão envolvidas no *"drive"* respiratório basal - em situações de normóxia normocárbica, nem em girinos de rãs-touro, nem em iguanas verdes.

Os dados coletados nos girinos pré-metamórficos mostraram que a exposição dos encéfalos desses animais a um antagonista de OX_1R não alterou a atividade respiratória pulmonar desses animais, mas a exposição ao agonista ORX-A promoveu uma diminuição nesse parâmetro. Entretanto, no trabalho realizado com as iguanas verdes, utilizamos dois tipos de antagonistas, o SB-334867 (antagonista de OX_1R) e o Almorexant (antagonista de OX_1R e

OX$_2$R). Nenhum dos antagonistas promoveu alterações ventilatórias em situações de normóxia normocárbica nesses animais.

No nosso entendimento, acreditamos que em situações basais, não existe muita quantidade de ORX sendo liberada pelos neurônios, portanto, quando o antagonista se liga a esses receptores, não existe um efeito evidente. Entretanto, quando os encéfalos são expostos ao agonista, aí sim é possível observar um efeito. No caso, o efeito do agonista foi uma diminuição da ventilação pulmonar fictícia, mostrando que em girinos pré-metamórficos de rãs-touro, a ORX-A é inibitória para a ventilação pulmonar. Ainda, a ORX-A pode se ligar aos 2 receptores de ORX, enquanto o antagonista utilizado em nosso trabalho, o SB-334867 antagoniza apenas um dos receptores, ou seja, pode ser que a ausência de efeito que vimos com o antagonista se dê pelo fato de que o receptor-1 não esteja relacionado ao tônus basal, e sim o receptor-2, pelo menos em girinos pré-metamórficos de rãs-touro.

Redgate e Gellhorn (1958) observaram que a lesão do hipotálamo lateral (onde estão localizados os neurônios ORXs) em gatos anestesiados promoveu uma diminuição imediata na frequência e profundidade da respiração, tendo um efeito inibitório na ventilação. Ao contrário do estudo anterior, a administração central de ORX-A em camundongos aumenta o volume corrente (Young et al., 2005; Terada et al., 2008), enquanto em ratos promove um aumento na ventilação (Zhang et al., 2005). Corcoran et al., (2013) realizaram preparações "*in situ*" com ratos neonatos e observaram que a aplicação de ORX-A nos banhos não alterou a frequência e a amplitude do sinal, o único efeito observado foi um aumento na duração dos "*bursts*". Camundongos "*knockout*" para ORXs apresentam respiração atípica, mas a ventilação basal permanece semelhante à do tipo selvagem, independentemente de estarem dormindo ou acordados (Kuwaki, 2008). Estudos em ratos demonstraram que a administração i.c.v. de SB-334867 não altera a ventilação em normocapnia durante a vigília ou o sono (Deng et al., 2007).

Além disso, a diálise de SB-334867 no núcleo retrotrapezoide (Dias et al., 2009) ou na rafe bulbar rostral (Dias et al., 2010) não altera a ventilação basal em ratos. Adicionalmente, a microinjeção de SB-334867 no ventrículo lateral do sapo *Rhinella diptycha* não promoveu alterações na ventilação sob normocarbia (Fonseca et al., 2016).

Portanto, a literatura é um pouco heterogênea no que diz respeito à contribuição das ORXs para o "*drive*" respiratório basal, mostrando um papel um pouco diversificado do agonista e antagonista nas condições de repouso. Contudo, de maneira geral, parece que as ORXs não desempenham um papel nessas condições de repouso, mas podem ser importantes em situações específicas, como situações de estresse – como hipóxia e hipercapnia.

ORXs e o quimiorreflexo ao CO_2

Nos nossos estudos, avaliamos a participação dos receptores de ORX no quimiorreflexo hipercápnico através da utilização de antagonistas. Para o estudo com os girinos, foi usado o antagonista de OX_1R (SB-334867), enquanto no estudo das iguanas, foi utilizado o SB-334867 e o Almorexant, um antagonista dos dois receptores (OX_1R e OX_2R). Enquanto em girinos pré-metamórficos de rãs-touro o antagonista promoveu um aumento no ritmo respiratório pulmonar (fictício) quando os encéfalos foram expostos à hipercapnia, em iguanas verdes, a resposta foi uma diminuição na ventilação pós-hipercarbia, ou seja, efeitos antagônicos.

A hipercapnia sozinha, no caso dos girinos, inibiu a ventilação pulmonar fictícia. Já nas iguanas, o aumento do CO_2 ambiental não alterou a ventilação desses animais durante a exposição, mas causou um aumento significativo na ventilação após a suspensão do estímulo.

Em outros trabalhos com preparações do tronco encefálico "*in vitro*" de girinos de rãs-touro, as respostas observadas frente à hipercapnia aguda são variadas: Togerson et al.,

(1997) observou uma ausência de resposta pulmonar nos girinos dos mesmos estágios de desenvolvimento usados em nosso estudo, enquanto Taylor et al. (2003) observaram que a hipercapnia desencadeia uma resposta ventilatória pulmonar em todos os estágios de desenvolvimento. Entretanto, as concentrações de CO_2 utilizadas foram diferentes, o que pode ter promovido diferentes respostas.

Já para os répteis, o aumento na concentração de CO_2 ambiental evoca respostas variadas. Aparentemente, a hipercarbia aguda é bem tolerada por esses animais e resultam em pequenos aumentos na ventilação. No entanto, alterações na frequência respiratória e no volume corrente podem variar. Squamatas, entretanto, apresentaram um aumento no volume corrente, mas no final, o produto foi uma diminuição da ventilação (Nielsen, 1961; Templeton e Dawson, 1963; Glass e Johansen, 1976; Gratz, 1978). As tartarugas, por outro lado, aumentam a frequência e o volume corrente em resposta a altas concentrações de CO_2, produzindo uma hiperventilação pronunciada (Trevizan-Baú et al., 2018).

Em relação às ORXs, conforme mencionado, vimos respostas opostas relacionadas ao quimioreflexo hipercápnico em iguanas e em girinos pré-metamórficos de rãs-touro na presença de antagonistas. Na literatura é sabido que os neurônios ORXs são altamente sensíveis a alterações na concentração de CO_2 e no pH extracelulares (Williams et al., 2007). Deng et al., (2007) mostrou que a administração de SB-334867 i.c.v. diminui o quimiorreflexo central em ratos. Dias et al. (2008) mostrou também que a microdiálise do mesmo antagonista no RTN atenua a resposta ventilatória à hipercapnia durante a vigília e, com efeito menor, mas ainda assim significativo, durante o sono NREM em ratos. Em sapos *Rhinella diptycha* (o antigo *Rhinella schneideri*), o OX_1R participa da modulação do quimiorreflexo central apenas durante a fase escura (Fonseca et al., 2016).

Nas iguanas, observamos uma resposta menor ao aumento do CO_2 ambiental durante a fase escura, enquanto durante a fase clara a resposta foi bem pronunciada. Enquanto o Almorexant atenuou a resposta ventilatória pós-hipercarbia nas duas fases do ciclo, o SB-334867 atenuou apenas durante a fase clara.

ORXs e o quimiorreflexo ao O_2

Em nossos estudos também observamos diferentes papeis das ORXs em relação à modulação do quimiorreflexo ao O_2 em iguanas e em girinos de rãs-touro.

Em girinos de rã-touro observamos que a hipóxia moderada causa um aumento vigroso na ventilação poulmonar fictícia, enquanto nas iguanas, observamos um pequeno, mas significativo aumento da ventilação em resposta à hipóxia aguda, mas somente durante a fase clara. As iguanas não responderam à hipóxia durante a fase escura.

O que encontramos na literatura é que em anuros adultos a resposta à hipóxia ambiental geralmente é uma hiperventilação, que contece através de uma ativação dos quimiorreceptores periféricos (Van Vliet e West, 1992; Gargalgioni e Milsom, 2007). Já em girinos pré-metamórficos intactos, a hipóxia crônica promove um aumento da ventilação pulmonar (Burggren e Doyle, 1986). Preparações "*in vitro*" de troncos encefálicos de girinos pré-metamórficos de rãs-touro aumentaram a frequência pulmonar em resposta à hipóxia aguda moderada. Entretanto, essa resposta foi ausente no mesmo estágio quando exposto a hipóxia leve (Janes e Kinkead, 2018). Os répteis não apresentam circulação pulmonar e sistêmica completamente separadas. Isso significa que parte do sangue venoso sistêmico pode contornar os pulmões para reentrar na circulação sistêmica, enquanto sangue arterial pode reentrar na circulação pulmonar. Consequentemente, a composição do gás no sangue arterial é afetada pelo grau de mistura de sangue arterial e venoso, em vez da composição de gás pulmonar isolada,

como ocorre nos mamíferos. Isto demonstra uma curiosa possibilidade de que a modulação desses "*shunts*" vasculares possa desempenhar um papel importante no controle da composição do gás no sangue arterial em répteis, independente do controle ventilatório (Taylor et al., 1999, 2010).

Em relação à participação das ORXs na modulação do reflexo hipóxico, os girinos apresentaram um aumento significativo na atividade motora pulmonar quando o SB-334867 foi adicionado à câmara. Já nas iguanas, nem o SB-334867 e nem o Almorexant promoveram alterações no quimiorreflexo ao O_2 nesses animais.

Na literatura, o que encontramos é que em estudos realizados em mamíferos, a injeção i.c.v. de um antagonista de OX_1R não teve nenhum efeito na resposta ventilatória à hipóxia (Deng et al., 2007). Ainda, Nakamura et al. (2007) demonstrou que camundongos "*knockout*" para os genes precursores das ORXs não apresentam resposta à hipóxia diferente dos tipos selvagem. Os dados sugerem que as ORXs não estão envolvidas no quimiorreflexo ao O_2 em roedores, mas em contraste, humanos com narcolepsia/cataplexia (deficiência na produção de ORXs) apresentaram responsividade à hipóxia deprimida (Han et al., 2010). Em sapos *Rhinella diptycha*, o OX_1R participa da modulação do quimiorreflexo periférico apenas durante a fase clara (Fonseca et al., 2016).

Talvez em animais aquáticos, ou que ainda tenham certa dependência do ambiente aquático como acontece com os anfíbios (que apresentam fase larval aquática e ainda dependem da água para a reprodução), as ORXs sejam importantes para a modulação quimiorreflexa do O_2 (principal estímulo para esses animais). Ao passo que para animais terrestres, em que o estímulo principal para o "*drive*" respiratório é o CO_2, talvez as ORXs apresentem uma contribuição maior para a modulação desse quimiorreflexo.

Resumindo, nossos resultados sugerem que as ORXs não participam na modulação do controle respiratório em situações basais, mas podem ser importantes em situações específicas, como situações de hipóxia ou hipercarbia. Ainda, as observações presentes, juntamente com os dados relacionados à localização dos neurônios ORXs nas iguanas e nos outros vertebrados, indicam um considerável grau de conservação filogenética tanto das moléculas de ORXs, quanto da sua localização, das suas funções, e dos seus receptores.

Esses dados corroboram a nossa hipótese de que as ORXs, agindo nos receptores -1 e -2, potencializam o quimiorreflexo ao CO_2 em iguanas verdes, estimulando a atividade respiratória durante a hipercapnia aguda, entretanto, não participam do quimiorreflexo hipóxico.

Já para os girinos pré-metamórficos de rã-touro, acontece o contrário do que propusemos: a ORX-A, agindo no receptor OX_1R, inibe os quimiorreflexos hipóxico e hipercápnico, atenuando a atividade respiratória pulmonar nesses animais.

CHAPTER 1

Participation of orexin receptor-1 in the modulation of respiratory motor

activity in the bullfrog tadpole (_Lithobates catesbeianus_)

ABSTRACT

Orexin (ORX) is a peptide produced by hypothalamic neurons that regulates sleep-wake states, feeding, and breathing. Because ORX-neurons are highly responsive to changes in CO_2/pH and hypoxia, its effects on respiration likely involve participation in chemoreflexes. Recently, we have demonstrated that orexin receptor-1 (OX_1R) contributes to hypercarbic and hypoxic chemoreflexes in adult toads. However, there is no study regarding the role of OX_1R in pre-metamorphic anurans. The main objective of this study was to investigate the contributions of OX_1R to central respiratory motor activity and its participation in the O_2 and CO_2 chemoreflexes in early stage tadpoles of *Lithobates catesbeianus* (Taylor-Kollros: IV-XII). We hypothesized that ORX, acting through the OX_1R, potentiates central O_2 and CO_2 chemoreflexes in tadpoles to stimulate fictive ventilation during acute hypoxia and hypercapnia. Experiments were performed on two types of isolated brainstem preparations: 1. transected between the optic tectum and the forebrain, and 2. transected rostral to the optic chiasma to keep hypothalamic orexinergic neurons intact. Isolated brainstem preparations were exposed to aCSF equilibrated with hypercapnic or hypoxic gas alone, or in the presence of an OX_1R antagonist to evaluate the effects of the antagonist on the respiratory motor activity. Our data showed that baseline respiratory motor activity was not affected by the OX_1R antagonist, but ORX-A inhibited the lung burst frequency. Hypoxia elicits an increase in the lung bursting, while hypercapnia inhibits fictive ventilation in this stage group. However, blocking OX_1Rs during hypoxia or hypercapnia revealed a significant ventilatory response observable as an increase in lung burst frequency mainly. ORX cells inhibit the O_2 and the CO_2 chemoreflex in early stage bullfrog tadpoles.

Amphibians generally have the larval phase totally aquatic, and, in its majority, adult phase terrestrial. These animals undergo remarkable changes during the early life stages, with concurrent and profound changes such as the relative prevalence of gill and lung ventilation that switches during metamorphosis (Burggren and West, 1982; Burggren and Doyle, 1986). Therefore, this group is a very interesting model for studying ventilatory control in vertebrates, this understanding may reveal some fundamental properties that are associated with the emergence of air breathing.

In anurans, the contribution of lung ventilation to gas exchange increases during development. Gill ventilation is a very important mechanism for oxygen acquisition in early stages, and as development proceeds, the lungs become more important, until the animal becomes an obligate air-breathing post-metamorphic tadpole (Burggren e West, 1982). Thus, to be efficient, this implies a growing responsiveness of air breathing to respiratory stimuli, especially CO_2.

Circuits driving lung ventilation are present and functional during early life, however, they are inhibited. Straus et al. (2000) observed a lung central pattern generator early in development, which remains quiescent until metamorphosis, suggesting that the central pattern generator for air breathing exists and it is formed early in development, but remains relatively suppressed until metamorphosis. The authors suggest an age-dependent diminution in the potency of this inhibition that along development allows the major changes resulting in the expression of more frequent lung breaths. Therefore, the questions that arise are: what would turn these circuits on? Or still, what would keep them silent?

Therefore, we decided to study orexins (ORXs). ORXs are small neuropeptides released by hypothalamic neurons. They have broad biological roles, including the modulation of breathing (Peyron et al., 1998; Zhang et al., 2005; Sakurai, 2007; Dutschmann et al., 2007; Yokota et al., 2016). There are two ORX isoforms, orexin-A (ORX-A) and orexin-B (ORX-B), both derived from the prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). These isoforms can bind to two receptors: ORX receptor-1 (OX_1R) and ORX receptor-2 (OX_2R), ORX-A can act on both OX_1R and OX_2R, while ORX-B acts primarily on OX_2R.

In mammals, this neurotransmitter is produced in a small population of neurons in the lateral hypothalamus (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; McGranaghan and Piggins, 2001; Mintz et al., 2001; Novak and Albers, 2002; Sakurai, 2005; Nixon and Smale, 2007). Studies on immunohistochemical localization of orexin immunoreactive (ORX-ir) cells in the brains of vertebrates (López et al., 2009a; Shibahara et al., 1999; Galas et al., 2001; López et al., 2009b; Domínguez et al., 2010; Ohkubo et al., 2002; Singletary et al., 2006; Miranda et al., 2013) has shown the highly phylogenetic conserved molecular structure of these neuropeptides (Shibahara et al., 1999; Wong et al., 2011). In *Xenopus laevis*, a study displayed the spatiotemporal analysis of the localization of ORX-ir cells and fibers through the brain development (López et al., 2016). There is a progressive development of the orexinergic fiber system during larval ontogeny, starting with an early expression of ORX-ir cells in the embryonic period, before hatching, suggesting important developmental roles for these neuropeptides. These cells are present in the subparaventricular region of the alar hypothalamus (newly named by the group), and only late in development, the cells emerge in the preoptic area. The pattern of orexinergic innervation increases notoriously in the premetamorphic period (including projections to respiratory areas such as the locus coeruleus, solitary tract nucleus, and raphe) (López et al., 2016). This neuropeptide recently has

been indicated as a mediator of metamorphic events and maturation in amphibians (Tata, 2006; López et al., 2016) given its interactions between the ORX system and the hypothalamic–pituitary–thyroid.

In mammals, ORX contributes to multiple physiological functions, including respiration. This neuropeptide does not appear to contribute to basal breathing when an animal is at rest and under normal air conditions (Carrive and Kuwaki, 2017); however, in rodents, the hypothalamic neurons respond to CO_2 as well as to hypoxic stimuli (Dillon and Waldrop, 1992). The ORX-neurons are excited by small changes in CO_2/H^+, both *in vitro* and *in vivo* (Williams et al., 2007, Sunanaga et al., 2009), showing that they are chemosensitive. Oral treatment with Almorexant (OX_1R and OX_2R antagonist) decreased ventilatory response to 7% CO_2 (Li and Nattie, 2010) and the microdialysis of SB-334867 into the retrotrapezoid nucleus (RTN) or medullary raphe, reduced the hypercapnic ventilatory response during wakefulness in rats (Dias et al., 2009a; Dias et al., 2010b). Human narcoleptic patients presented attenuated responses to hypoxia, but not to hypercapnia (Han et al., 2010). Further, intermittent but not sustained hypoxia (mimicking sleep apnea) efficiently activated ORX-neurons in mice (Yamaguchi et al., 2015), and long-term facilitation was attenuated or even disappeared in ORX-deficient mice (Terada et al., 2008; Toyama et al., 2009). More specifically, in *Rhinella diptycha* toads, the microinjection of SB-334867 did not promote any changes in ventilation under normocarbic normoxia, but an attenuation of the ventilatory response to CO_2 during the dark phase, and an attenuation of the ventilatory response to hypoxia in the light phase (Fonseca et al., 2016).

Considering this background, we wondered if ORX would play a role in activating the circuits that drive air breathing in amphibians. As mentioned, these circuits are present during early life and are relatively dormant until metamorphosis (Straus et al., 2000). As ORX-neurons potentiates O_2 and CO_2 ventilatory responses stimulating ventilation at least in rodents

and have been pointed as one of the main mediators of metamorphosis in anurans, we hypothesized that ORX in early staged tadpoles stimulates fictive ventilation during acute hypoxia and hypercapnia.

Animals

Seventy-three tadpole brains were used to perform experiments in accordance to the guidelines of the Canadian Council on Animal Care. The *Lithobates catesbeianus* tadpoles were obtained from a commercial supplier (Island Bullfrog, Nanaimo, Canada) and were housed in the animal care facility of the "Institut Universitaire de Cardiologie et de Pneumologie de Québec" as described previously (Janes et al., 2019).

Isolated brainstem preparations

Two types of preparations were used for the experiments: 1. transected between the optic tectum and the forebrain - removing the hypothalamic ORX-neurons, and 2. transected rostral to the optic chiasma - to keep the ORX-neurons intact (Figure 1).
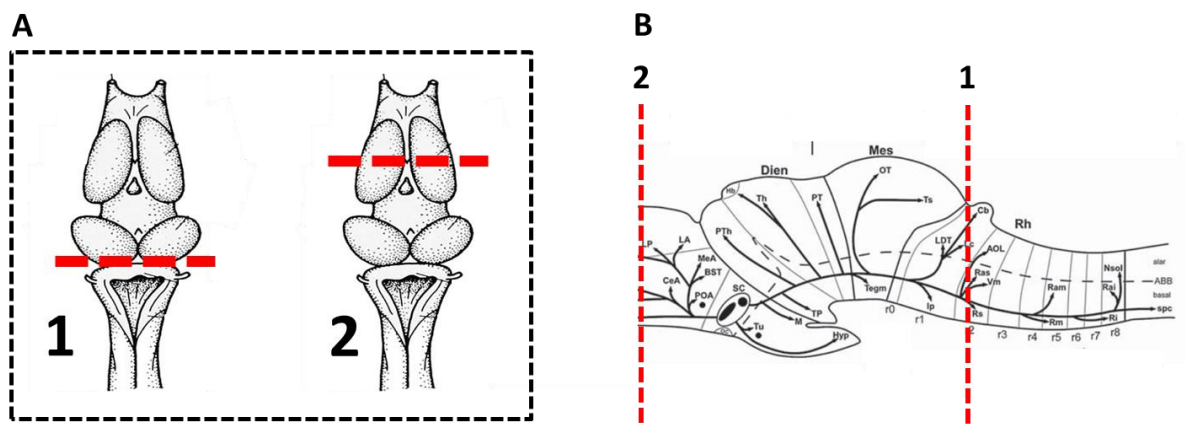

Figure 1. Schematic diagram showing the two types of preparations used in the study. Dorsal view (A) and sagittal view (B): Preparation 1, transected between the optic tectum and the forebrain; and preparation 2, transected rostral to the optic chiasma, keeping the hypothalamus. Adapted from Rugh, 1951 (A) and López et al., 2016 (B).

The isolated brainstem preparations were performed as described previously by Gdovin et al. (1999). *L. catesbeianus* tadpoles from stages IV to XII (premetamorphic, Taylor and Kollros, 1946) were anesthetized by immersion in tricaine methane sulfonate (MS-222, 0.06 g l^{-1}, buffered to 7.0 with $NaHCO_3$) and the brains carefully dissected, keeping the cranial nerves (CNs) intact. During the dissection, the brains were perfused with cold (0-5 °C) artificial cerebrospinal fluid (aCSF) (104 NaCl, 4 KCl, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, 10 D-glucose; in mM) and equilibrated with 1.8% CO_2 + 98.2% O_2 to pH 7.90 ± 0.06. After the dissection, the brains recovered during 40 to 60 minutes in oxygenated room temperature (19-21 °C) aCFS (speed: 6-7 ml min^{-1}). Bursts of respiratory-related motor activity were then recorded simultaneously from the rootlets of the trigeminal (V) and vagus (X) nerve using suction electrodes.
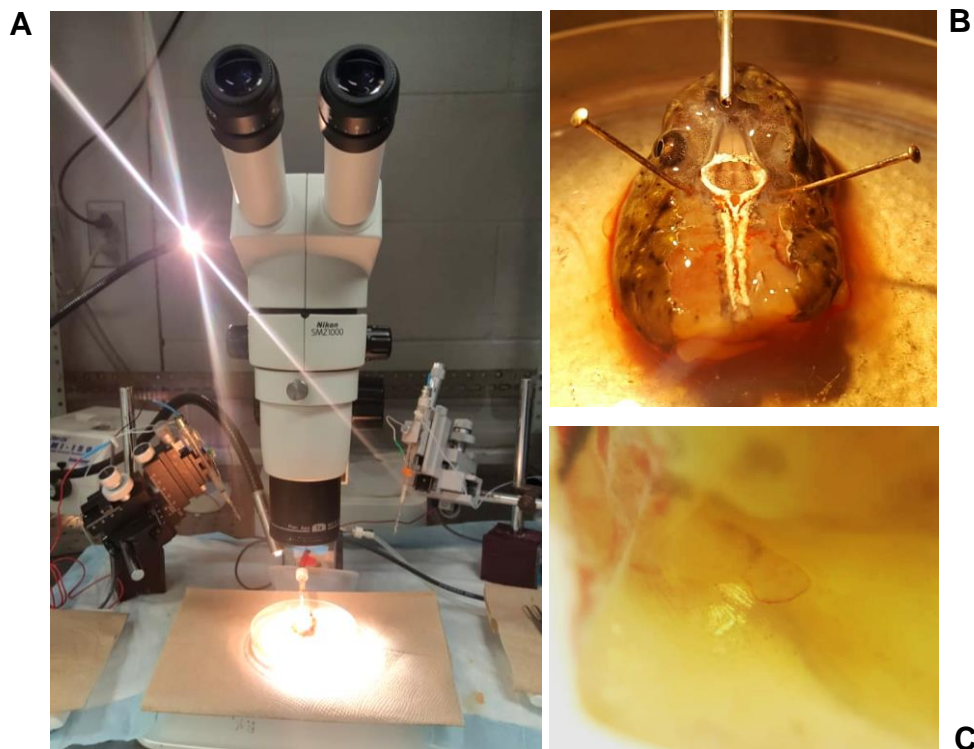

Figure 2. Photos showing the dissection of the tadpole (A and B) and the limb bud to stage the animal according to the criteria of Taylor-Kollros, 1946 (C).

Experimental protocols

To investigate the contributions of OX_1R to central respiratory motor activity and its participation in the O_2 and CO_2 chemoreflexes, the experiments were performed exposing the brains to an OX_1R antagonist: SB-334867 (5 to 25 µM) in the presence of the respiratory stimuli (hypercapnia or hypoxia) or not (Protocols 1, 3 and 4). Moreover, experiments using the agonist (ORX-A) were also performed to verify the reliability of the experiments (Protocol 2). Figure 4 shows a diagram illustrating the experimental protocols.

Thus, after the dissection and the recovery, the fictive motor activity of gill and lung ventilation was recorded. The brains were perfused with aCSF, and once achieving a stable signal, the experiment began. Experiments were performed with the two types of preparations – with hypothalamus and without hypothalamus - according to the following protocols (Figure 3):

Figure 3. Photo showing the brainstem preparation 2 (with the hypothalamus). The nerves V and X inside the suction electrodes, ready for being recorded.

Protocol 1. Participation of OX_1R at 'resting conditions' (basal respiratory drive):

'Resting conditions' (baseline) were recorded for at least 10 minutes, and then a solution containing the antagonist SB-334867 (5, 10 or 25 µM, see item "Chemicals and gas mixtures" above for the drug preparation) was applied to the brainstem chamber for 20 minutes to investigate the contribution of OX_1R to the basal respiratory drive. Both solutions were delivered at the same speed and continuously.

Protocol 2. Participation of ORX-A (OX_1R and OX_2R) at 'resting conditions':

Baseline conditions were recorder for 20 minutes, then the brain was exposed to 200 nM of the agonist ORX-A (see item "Chemicals and gas mixtures" above for the drug preparation) for 25 minutes, followed by 30-40 minutes of washout. After the washout, the brain was exposed over again to more 25 minutes of 1 µM ORX-A and then to another washout period. It was used the speed of 8 ml min^{-1} for this protocol.

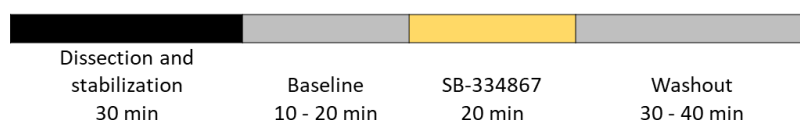*Protocol 3. Contribution of OX_1R to hypercapnic chemoreflex:*

Newly, the baseline was recorded for at least 10 minutes, and then the brain was exposed to hypercapnia (see item 5) for 20 minutes. After a washout time (30-40 minutes), the brain was then exposed to hypercapnia with the solution containing SB-334867 (5, 10 or 25 µM) diluted in aCSF (see item "Chemicals and gas mixtures") for more 20 minutes followed by another washout period. All solutions were delivered on the same speed and continuously.

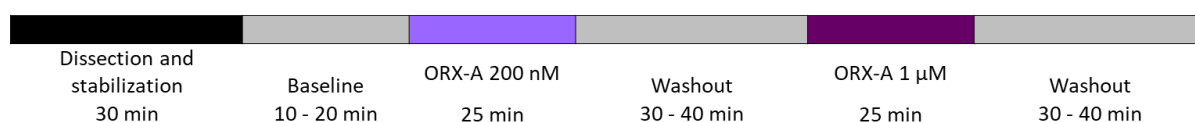*Protocol 4. Contribution of OX_1R to hypoxic chemoreflex:*

For the hypoxia, the same protocol for hypercapnia was used, but replacing the hypercapnic gas mixture for the hypoxic. For the hypoxia, the speed delivery of the aCSF was

10 ml min^{-1} (see "Chemicals and gas mixtures" above). The other solutions were delivered on the same speed and all of them were delivered continuously. Concentrations of 5 and 10 µM of the SB-334867 were used for this protocol.
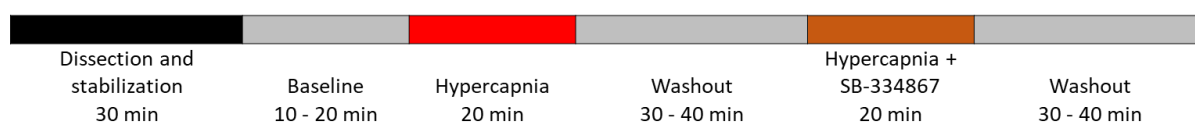
Protocol 1

Dissection and stabilization 30 min	Baseline 10 - 20 min	SB-334867 20 min	Washout 30 - 40 min

Protocol 2

Dissection and stabilization 30 min	Baseline 10 - 20 min	ORX-A 200 nM 25 min	Washout 30 - 40 min	ORX-A 1 µM 25 min	Washout 30 - 40 min

Protocol 3

Dissection and stabilization 30 min	Baseline 10 - 20 min	Hypercapnia 20 min	Washout 30 - 40 min	Hypercapnia + SB-334867 20 min	Washout 30 - 40 min

Protocol 4

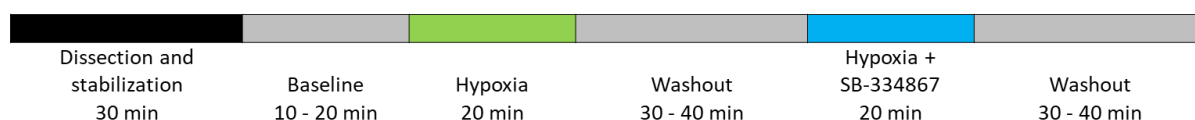|					
Dissection and stabilization 30 min	Baseline 10 - 20 min	Hypoxia 20 min	Washout 30 - 40 min	Hypoxia + SB-334867 20 min	Washout 30 - 40 min

Figure 4. Scheme illustrating the experimental protocols.

Chemicals and gas mixtures

Hypercapnia: The aCSF was equilibrated with 95% O_2, 5% CO_2 and delivered at a speed of 6-7 ml min^{-1} (Fournier et al., 2013).

Hypoxia: aCSF equilibrated with 1.8% CO_2 + 98.2% N_2 at a speed of 10 ml min^{-1} (moderate hypoxia; Janes at al., 2018).

OX₁R antagonist (SB-334867): Aliquots were prepared according to Deng et al. (2007), then diluted in aCSF. Concentrations: 5, 10 and 25 µM. The antagonist solutions were recycled (recirculated) for a maximum of 10 days. Tocris, Bristol, UK.

OXR agonist (Orexin A): Drug dissolved directly in distilled water to make a stock, then diluted in aCSF. The aCSF was equilibrated with 2% CO_2, 98% O_2 and delivered at a speed of 8 ml min^{-1}. Concentrations: 200 nM and 1 µM. The agonist solutions were also recycled (solutions were used for 4 days). Sigma Aldrich, St. Louis, USA.

Data analysis

The tadpole *in vitro* brainstem preparation produces two patterns of respiratory-related neural activity: high frequency, low amplitude, and low frequency, high amplitude, reflecting respectively fictive gill and lung ventilation (Liao et al., 1996; Torgerson et al., 1998; Gdovin et al., 1998) as shown in Figure 5.

Data was analyzed by using software LabChart (version 7, ADInstruments). Lung burst frequency was obtained by analyzing the last 5 to 10 minutes of recording for each condition (values averaged for a 1 min period), while gill burst frequency by analyzing 1 minute. The lung and gill frequencies from protocols 1, 3 and 4 are expressed as absolute values, while the lung and gill amplitudes are expressed as percentage change from the baseline. The analysis of the respiratory pattern, the bursting episodes were determined according to the criteria proposed by Kinkead and Milsom (1994). The frequency of respiratory episodes and the number of events per episode were analyzed. For the analysis of the number of events per episode, the single events were ignored, and only the episode events were considered. The data from the protocols 1, 3 ad 4 are presented as "Box Plot" (Spitzer et al., 2014) and analyzed by using analysis of variance (2-way ANOVA) with Holm-Sidak multiple comparison post test -

P<0.05 considered statistically significant. Data from protocol 2 was expressed as relative change for the frequency and as percentage change for the amplitude.
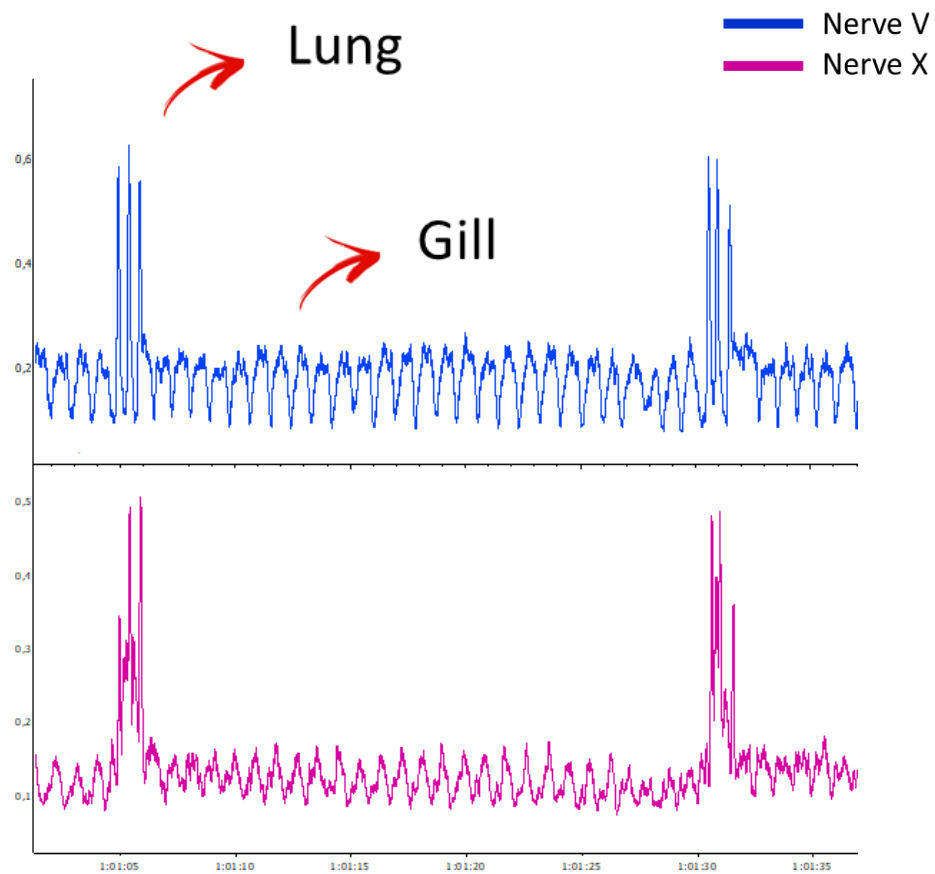

Figure 5. Diagram showing a representative trace demonstrating the difference between the gill and the lung bursting.

These are the description of the results according to the following protocols:

Participation of OX_1R at 'resting conditions' (baseline data)

Figure 6 shows a representative recording. The lung and the gill frequency of the preparations without the hypothalamus was significantly smaller than the preparations with the hypothalamus (Figure 7 A and C; P=0.0259 and P<0.0001, respectively). The SB-334867 did not evoke changes on the lung and gill frequency or in the lung amplitude (Figure 7 A, B and C). There was an increase on the gill amplitude only when the preparations were exposed to 10 µM SB-334867 (with hypothalamus, Figure 7 D; P<0.001). Having the hypothalamus intact augments the frequency of episodes (Figure 7 E; P=0.058). The SB-334867 almost blunted the episode frequency, but the few episodes left had a high number of breaths than the baseline. (Figure 7 E and F; P=0.0025).
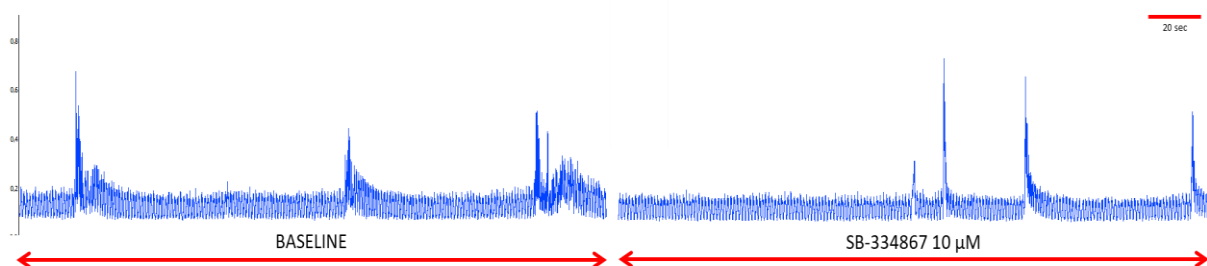

Figure 6. Representative recording of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* before and after SB-334867 10 µM exposure.
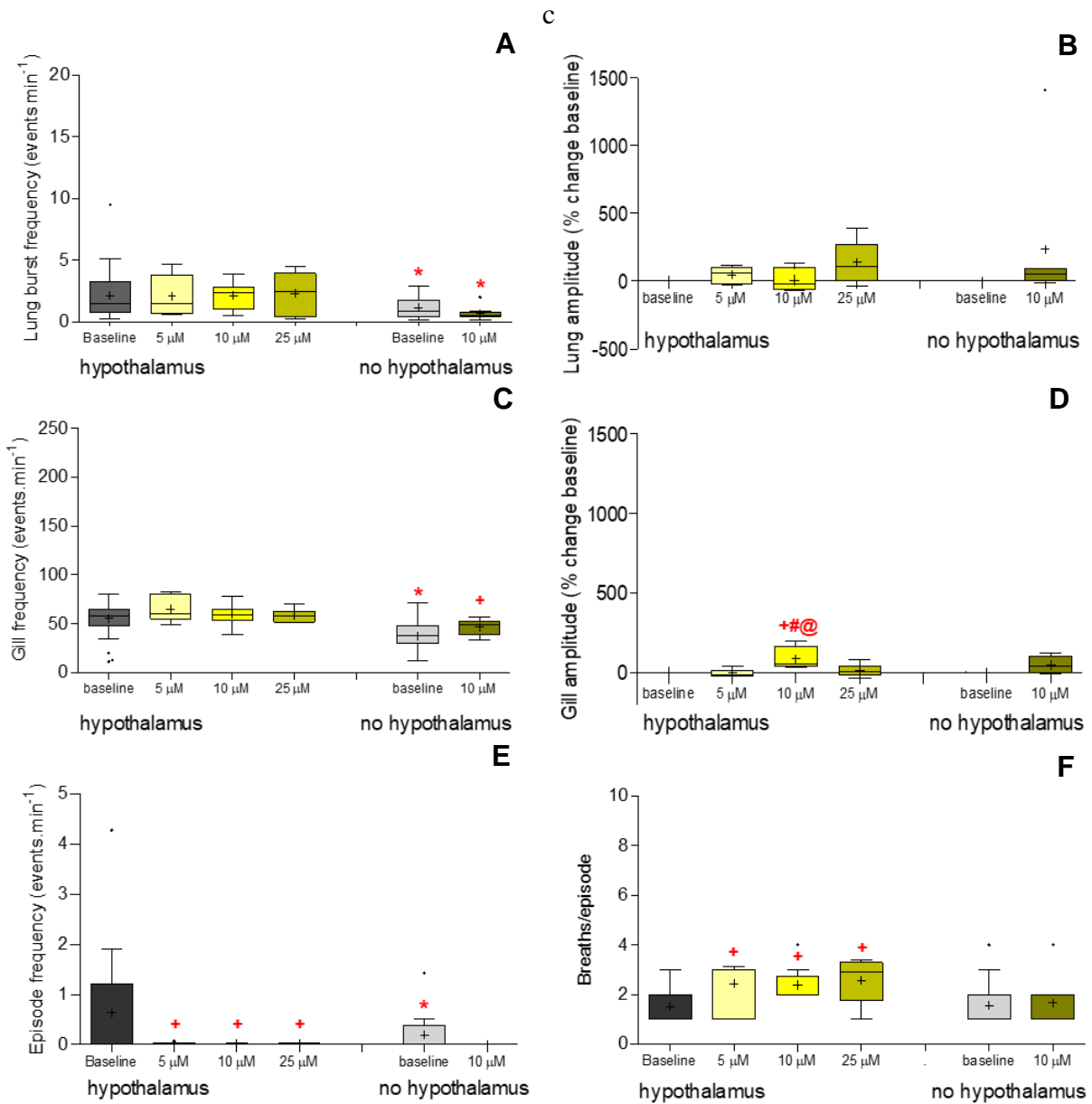
Figure 7. "Box Plot" showing the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in 'resting conditions' of "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to SB-334867 (5, 10 and 25 µM). Amplitude data is expressed as % change to baseline. * means different from correspondent group in the preparations with the hypothalamus, + means different from baseline, # means different from 5 µM and @ different from 25 µM. (n = 12)

Agonist

Contribution of ORX-A to the basal respiratory drive

       When the hypothalamus is present, ORX-A is inhibitory for lung bursting, but excitatory for gill bursting (Figure 8 A and C). It has no effect on the lung amplitude (Figure 8 B) and is excitatory for the gill amplitude. In contrast, when the hypothalamus is absent, the inhibitory effect of the agonist on the lung bursting is much smaller (Figure 8 A), and there is an excitatory effect on the lung amplitude (Figure 8 B) and a potentiation in the excitatory effect on the gill amplitude (Figure 8 D).
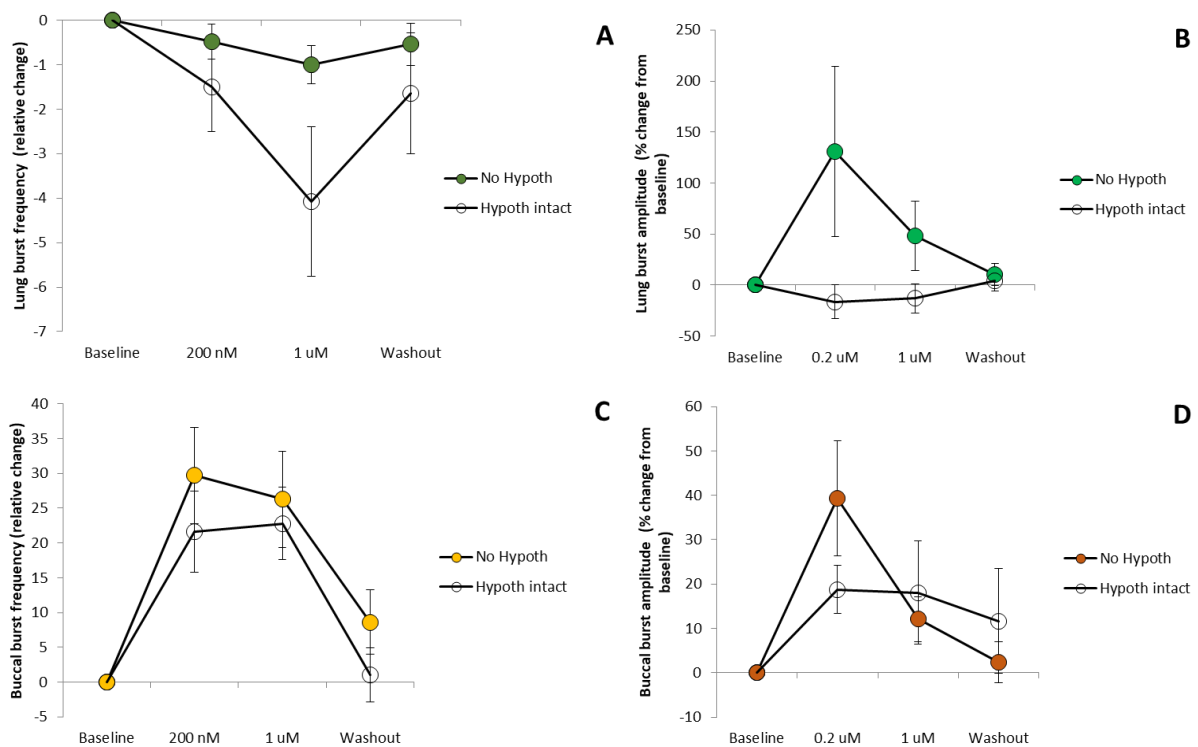

Figure 8. Relative change of lung burst frequency (A); buccal burst frequency (C) and percentage change from baseline of lung burst amplitude (B) and buccal burst amplitude (D) from *in vitro* preparations of premetamorphic tadpoles exposed to ORX-A 20 nM and 1 µM.

Two preparations were used, with the hypothalamus (open circles) and without the hypothalamus (painted circles). (n = 5)

Peak response vs. stage

Further, we plotted the relationship between the frequency response of the lung and gill rhythms to ORX-A (200 nM and 1 uM) compared to the tadpole stage (all premetamorphic, Taylor-Kollros, 1946) in both preparations (with and without the hypothalamus) (relative change, see Figure 9 A and B). The ability of ORX-A to inhibit the lung bursts increases with stage but only when the hypothalamus is intact (Figure 9 A). In contrast, the ability of the agonist to enhance the buccal motor activity increases with age when the hypothalamus is absent (Figure 9 B).
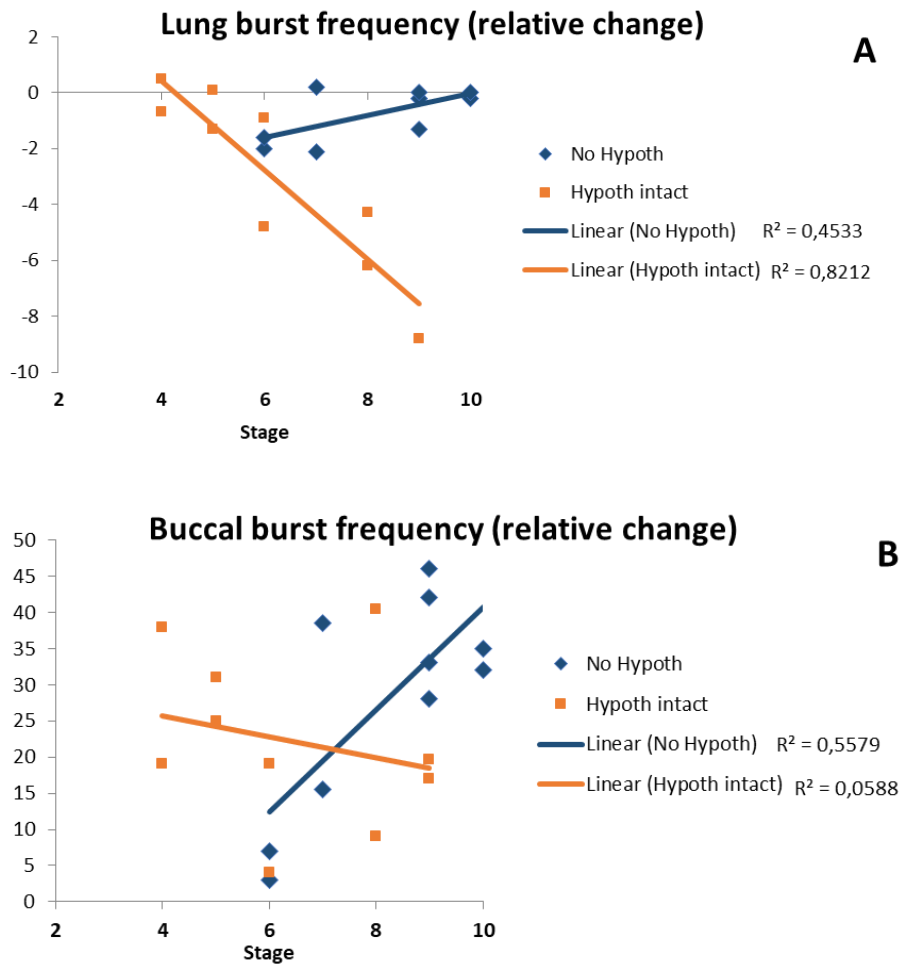
Figure 9. Relative change of lung burst (A) and buccal burst (B) frequency versus the developmental stage of the premetamorphic tadpoles. In blue the preparations with only the brainstem and in orange the preparations with the hypothalamus intact. (n = 5)

Contribution of OX_1R to hypercapnic chemoreflex

Figure 10 shows a representative recording. The hypercapnia alone inhibited the lung and gill frequencies in the preparations when the hypothalamus was present but only the lung frequency when it was absent (Figure 11 A and C; P=0.0481 and P=0.0461 with hypothalamus, respectively; P=0.048 no hypothalamus). High CO_2 also abolished the episodic breathing (Figure 11 E; P=0.0457). But when combined, the hypercapnia with the SB-334867

revealed an excitatory effect in the lung frequency (Figure 11 A; P=0.0022) of the preparations with and without the hypothalamus, while the amplitudes (lung and gill) were not affected (Figure 11 B and D). For the preparations without the hypothalamus, only the 25 µM dose of the antagonist had an excitatory effect on the gill frequency (Figure 11 C; P=0.0008). The drug also partially restored the frequency of episodes in both preparations (Figure 11 E) and augmented the number of breaths per episode when the hypothalamus was present (Figure 11 F; P=0.0007).
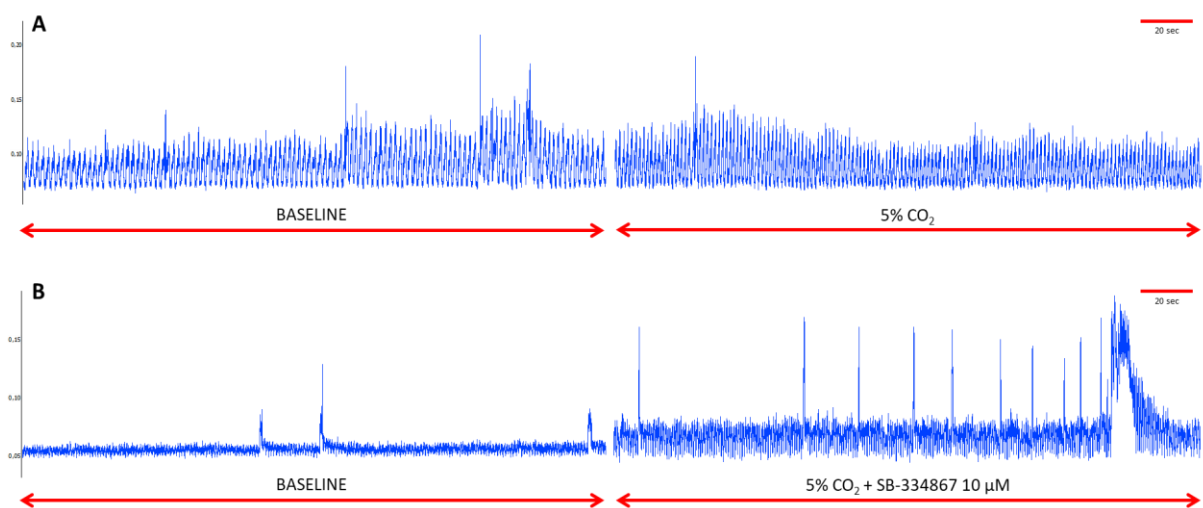

Figure 10. Representative recordings of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* exposed to 5% CO_2 only (A) and with 10 µM SB-334867 (B).

Figure 11. "Box Plot" showing the effect of SB-334867 (5, 10 and 25 μM) on the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to 5% CO_2. Amplitude data is expressed as % change to baseline. * means different from baseline and $^+$ means different from hypercapnia. (n = 10)

Contribution of OX$_1$R to hypoxic chemoreflex:

As shown by the representative traces in Figure 12, hypoxia was excitatory for the lung frequency (Figure 13 A; P=0.0034) and the gill amplitude (Figure 13 D; P=0.0233) but had no effects on the gill frequency independent on the presence of the hypothalamus (Figure 13 C). For the gill amplitude, hypoxia showed also an excitatory effect but only when the hypothalamus was present (Figure 13 D; P=0.0064). The hypoxia also augmented the number of breaths per episode (Figure 13 E; P=0.0002) but has not affected the number of episodes in both types of preparation (Figure 13 F). The SB-334867 is excitatory for the lung burst frequency during the hypoxic chemoreflex with or without the hypothalamus (Figure 13 A; P=0.0049). Only the dose of 10 µM excited the lung amplitude (Figure 13 B; P=0.0166) and the 5 µM excited the gill amplitude (Figure 13 D; P=0.0011) when the hypothalamus was present. The 10 µM SB-334867 increased the number of breaths per episode (Figure 13 F; P=0.0400) in the brains with the hypothalamus.

Figure 12. Representative recordings of respiratory motor activity from nerve V in premetamorphic tadpole of *L. cathesbeianus* exposed to moderate hypoxia only (A) and with 10 µM SB-334867 (B).
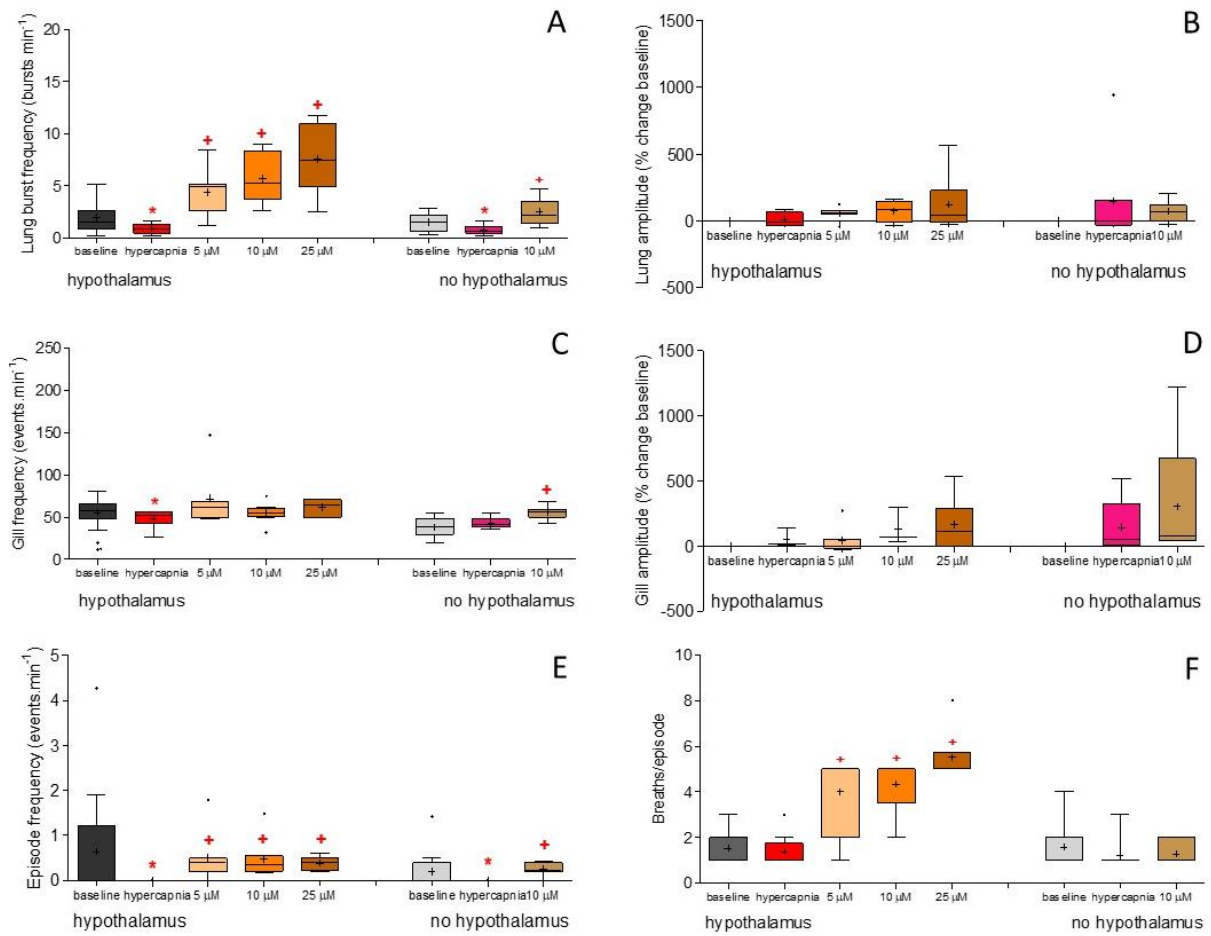
Figure 13. "Box Plot" showing the effect of SB-334867 (5, 10 and 25 μM) on the lung burst frequency (A), lung amplitude (B), gill frequency (C), gill amplitude (D), episode frequency (E) and number of events per episode (F) in "*in vitro*" brainstem preparations with and without the hypothalamus of premetamorphic tadpoles exposed to moderate hypoxia. Amplitude data is expressed as % change to baseline. * means different from baseline, + means different from hypoxia and # means different from 5 μM. (n = 8)

In this study we investigated the contributions of OX_1R to central respiratory motor activity and its participation in the O_2 and CO_2 chemoreflexes in early stage tadpoles of *L. catesbeianus*.

The hypothalamus

In our experiments, the preparations with the hypothalamus had larger lung and gill frequencies than the preparations in which it was absent, suggesting that the diencephalon has a facilitatory influence on the respiratory rhythm generator for early stage tadpoles of *L. catesbeianus*.

Our data corroborate with the studies of Okada et al. (1998) and Voituron et al. (2005) that showed that the ablation of the diencephalon in *in vitro* brainstem preparations of neonatal rats also showed a decreased respiratory frequency. In awake cats, removing the diencephalon including the hypothalamus caused a significant decrease in ventilation (Fink et al., 1962). In mammals, the respiratory nuclei of the hypothalamus that are involved in the respiratory control are the paraventricular nucleus (PVN), the perifornical area (PFA), the dorsomedial hypothalamus (DMH) and lateral and posterior hypothalamus (LHA). There is strong evidence that the hypothalamus plays a role in driving baseline respiration. Duan et al. (1997) stimulated electrically the PVN in anesthetized rabbits and observed an increase in respiratory frequency, and the electrical stimulation of the PFA elicited respiratory augmentation in anesthetized cats (Abrahams et al., 1960). Iigaya et al. (2012) observed that the disinhibition of the PFA neurons augmented respiratory activity in anesthetized rats, and similarly, disinhibition of neurons in the DMH increases the respiratory drive (McDowall et al.,

2007). Lesion of the LHA in cats reduced respiration, and inhibition of the LHA reduced respiration (Redgate and Gellhorn, 1958). These evidences, taken together, suggest that the hypothalamus drives the entire respiratory network at least in mammals, and our finding indicates that the diencephalon plays a similar role in tadpoles.

The episodic breathing pattern is an attribute of breathing in most anuran amphibians, however, the prime elements explaining the variable and inconstant occurrence of episodes in *in vitro* brainstem preparations of tadpoles is still to be elucidated (Gargaglioni and Milsom, 2007). Our data showed that the episode frequency was significantly smaller in the preparations without the hypothalamus. In tadpoles the breathing pattern is usually composed by single breaths and discrete episodes, tending to become more clustered and frequent when there is a respiratory stimulus, such as hypercapnia or hypoxia (Reid et al., 2000). It has been suggested that the episodic breathing emerges as a result of influences descending from higher brain structures acting on the medullary centers (Milsom et al., 1997; Reid et al., 2000).

Reid et al. (2000) showed that transecting the brain rostral do the optic chiasma, the fictive ventilation was more "episodic" (discrete episodes as mentioned before), with more clusters of ventilatory events, and when transecting the brain caudally to the optic chiasma, the number of single breaths increased, essentially eliminated the occurrence of episodes. Gargaglioni et al. (2007) identified sites within the caudal half of the midbrain providing the inputs essential to produce episodic breathing in reduced brainstem preparations of adult bullfrogs. Straus et al. (2000) suggested that a $GABA_B$ dependent pathway may regulate the clustering of breaths into episodes in tadpoles by using baclofen ($GABA_B$ agonist) in *in vitro* brainstem preparations. Another molecule that has been proposed as an important mediator for the production of episodic breathing in the amphibian brainstem is the NO (nitric oxide). The application of L-nitroarginine (a non-specific nitric oxide synthase (NOS) inhibitor) to post-

metamorphic brainstems blocked the production of lung burst episodes (Hedrick et al., 2005). However, the specific sites at which these neurotransmitters/neuromodulators act are still unknown. In rats, the nNOS isoform (the primary enzyme involved in NO production and release in the brain) is found in several brain structures including the PVN, hippocampus, DMH and amygdala (Alderton et al., 2001; Bredt et al., 1991; Forstermann and Sessa, 2012), all structures in the diencephalon. If the same happens in amphibians, this could explain our finding, but further experiments are necessary.

In summary, the data suggest that the diencephalon could also contain structures involved in the production of the episodic breathing pattern. Further, this study also indicates the hypothalamus as one of these sites and ORX as one of the substances that modulate episodic breathing (see more in item 2).

ORX and basal respiratory drive (baseline)

Our data show that the OX_1R-antagonist had no effect on the basal fictive ventilation in the lung and gill rhythms having the hypothalamus or not. Only the 10 µM dose of the SB-334867 had an excitatory effect on the gill amplitude when the hypothalamus was present (Figure 4 A-F). In contrary, the agonist ORX-A promoted a diminution on the lung frequency in the preparations with the hypothalamus, had no effect on the lung amplitude, and an excitatory effect on the gill burst frequency and on the gill amplitude. Without the hypothalamus, there was a much smaller inhibitory effect on the lung burst frequency and an excitatory effect on the lung amplitude, while there was a much stronger excitation on the gill amplitude (Figure 9 A-D).

Redgate and Gellhorn (1958) observed that destroying the LHA in anesthetized cats, promoted an immediate decrease in frequency and/or depth of respiration, having an

inhibitory effect on ventilation, in contrary, the central administration of ORX-A in mice increases the tidal volume (Young et al., 2005; Terada et al., 2008). Still, the central injection of ORX in rats promotes an increase in ventilation (Zhang et al., 2005). Corcoran et al. (2013) performed *in situ* preparations with newborn rats, they observed that applying ORX-A in the baths have not altered the baseline burst frequency and amplitude, the only effect observed was an increase in the burst duration. The antagonist SB-408124 (selective for OX_1R) slightly increased frequency. This study suggest that ORX-A has no impact on ventilation, but only a small suppression of the burst frequency. ORX-knockout mice present respiration atypical, but basal ventilation remained similar in ORX-knockout and wild-type mice, regardless of whether they were asleep or awake, attributing that ORXs do not participate in the basal breathing but could stimulate breathing during stressful situations or some compensatory mechanism could be playing a role in the knockouts (Kuwaki, 2008). Studies in mice demonstrated that the i.c.v. administration of SB-334867 does not change ventilation in normocapnic normoxia during either wakefulness or sleep (Deng et al., 2007). Additionally, the dialysis of SB-334867 in the RTN (Dias et al., 2009) or the rostral medullary raphe (Dias et al., 2010) does not change basal ventilation in rats. More specifically, the microinjection of an ORX antagonist (SB-334867) into the lateral ventricle of toads *Rhinella diptycha* did not promote any changes in ventilation under normocarbic normoxia, suggesting that central ORX does not play a tonic respiratory role in these animals (Fonseca et al., 2016).

The literature is a little contradictory in what concerns the contribution of ORX to the tonic respiratory drive, showing a little diverse role of the agonist and antagonists in resting conditions. But in general, it seems that ORX does not play a role on the baseline breathing, but that it may be important in specific situations, such as stressful situations. In our experiments, the treatment with the ORX antagonist SB-334867 has not promoted important changes in the

fictive ventilation under basal conditions in the early stage tadpoles but exposing the brains to ORX-A agonist showed a decrease in the fictive lung frequency. We assign this diversity to the assumption that during the basal, there may be not much ORX being released by the ORX-neurons, so when the SB-334867 is applied and binds to the OX_1Rs, there is not a strong effect; but when the agonist is administered, then the effect appears because there is an important quantity of the substance acting on the OX_1Rs and also in the OX_2Rs, revealing the inhibitory effect. Therefore, in our understanding, central ORX-A plays an inhibitory role in the basal respiratory drive in early stage tadpoles.

Regarding the respiratory pattern, the antagonist blunted the frequency of episodes in both types of preparation, but the few episodes left on the preparations with the hypothalamus intact had an increased number of bursts per episode (Figure 4 A-F). Thus, we speculate if ORXs might be involved in the episode pattern formation. Still, if ORX is also important in stressful conditions in amphibians, just like happens in mammals, would breathing in bursts, that is, to breath in clustered breaths within a single episode, could save energy for more important efforts during an adversity?

Farther, the data showing the relation between the lung and the gill burst frequency and the stages show that the ability of ORX-A to inhibit the lung bursting increases with the stage, but only when the hypothalamus is intact. This is consistent to what Straus et al. (2000) proposed, that the circuits that drive lung ventilation are working during early life, but they are inhibited. As the animal develops, there is an age-dependent diminution in the potency of the inhibition resulting in the expression of more frequent lung breaths. Our data cohere to this, the inhibitory effect of ORX-A on the lung rhythm augments with age. In contrast, the ability of ORX-A to enhance the gill motor activity increases with age, but in preps without the hypothalamus. This shows the capacity of ORX-A to evoke different effects on the gill and lung

rhythms (excitatory and inhibitory, respectively). Almost like the two rhythms are "uncoupled". Generally, the gill rhythm is very robust, and it is uncommon a drug that can affect the gill breathing in such wise. Accordingly, the data support the hypothesis (Galante et al., 1996) that gill and lung ventilation, may be produced by different central circuits.

ORX and hypercapnia

The amphibian development is evidenced by a progressively shift in the main ventilatory exchange surface from the gills to the lungs and is accompanied by the emergence of CO_2 as the source of the respiratory drive (Torgerson et al., 1997). The system that is completely driven by oxygen changes absolutely to one that is oxygen and acid–base/CO_2-driven (Macintyre and Toews, 1976; Smatresk and Smiths, 1991; Burggren and Pinder, 1991; Branco et al., 1992, 1993; Kinkead and Milsom, 1997; Torgerson et al., 2001; Wang et al., 1999b, 2004). The central chemoreceptors respond to changes in CO_2/pH, they are an important source of respiratory drive and their existence in adult amphibians is well established in the literature (Smatresk and Smits, 1991; Branco et al., 1992; Noronha-de-Souza et al., 2006; Santin and Hartzler, 2013; Fonseca et al., 2016).

In our experiments with the hypothalamus intact, hypercapnia (5% CO_2) was inhibitory for the frequency (lung and gill) but did not affect the amplitudes. When the hypothalamus was absent, 5% CO_2 also inhibited lung burst frequency and has not affected the amplitudes and neither the gill frequency. Still, the hypercapnia blunted the episode frequency in the early stage tadpoles independent on the hypothalamus. These data suggest that hypercapnia inhibits the lung motor activity in early stage tadpoles.

The literature is very inconsistent regarding the ventilatory response to CO_2 in tadpoles: In 1992, Infantino has not observed changes in gill bursting in early stage tadpoles

when exposed to hypercapnia (stages IX-XIV). On the other hand, Torgerson et al. (1997) in contrary, showed that central hypercapnic stimulation in premetamorphic tadpoles (stages X-XIX) promoted a notorious excitation of the gill breathing, but not in metamorphic animals, and Taylor et al. (2003a,b) observed that hypercapnia elicits a lung ventilatory response in all stages of development. The cause of these disparities may be the different levels of CO_2 used and the type of preparation. But even the literature being incongruent, it is evident that CO_2 only stimulates lung ventilation after metamorphosis when the lungs became the predominant site for gas exchange (Infantino 1992; Torgerson et al; 1997). In our study, hypercapnia had an inhibitory effect in the lung rhythm, contradicting what has been reported. What it represents for the physiology of an intact animal is still unclear for us.

In addition to this, when we exposed the preparations to hypercapnia combined with the SB-334867 to verify the role of the OX_1R in the CO_2 chemoreflex, what we observed was an opposite response. The antagonist excited the fictive breathing in the early stage tadpoles, by acting on the lung burst frequency, independent on the presence of the hypothalamus. It also promoted a restauration of the frequency of episodes (that is blunted by hypercapnia itself).

What has been reported in the literature regarding the contribution of ORX receptors to the hypercapnic chemoreflex is an excitatory effect of ORX in ventilation in mammals. ORX-knockout mice exhibit attenuated ventilatory response to hypercapnia compared to wild type animals, and the supplementation with ORX-A or -B partially restored the hypercapnic chemoreflex in the ORX-KO mice (Deng et al., 2007). CO_2 excites ORX-neurons in mice (Sunanaga et al., 2009) and in hypothalamic slice preparations (Williams et al., 2007). Oral administration of almorexant (OX_1R and OX_2R antagonist) caused an attenuation of the CO_2 ventilatory response but only during wakefulness (Li and Nattie, 2010). Microdialysis of SB-334867 in the medullary raphe region (Dias et al., 2009) or RTN (Dias et

al., 2008) causes a reduction in the CO_2 response during wakefulness/active period. In toads, ORX, acting on OX_1Rs, contributes to the hypercarbic chemoreflex during the dark phase of the diurnal cycle (Fonseca et al., 2016).

Our observation that OX_1R antagonism affected the CO_2 response of early stage tadpoles differs with the observations in mammals and toads. We observed an inhibitory role of ORX for the lung motor activity for the CO_2 chemoreflex, thus blocking OX_1R with the antagonist has an excitatory effect during hypercapnia, in contrary to the literature. The understanding of the mechanisms regarding ORX modulation of the hypercapnic chemoreflex in amphibians (and, more specifically, tadpoles) are still in the beginning, but the evidences suggest that one of the underlying mechanisms may involve the effects of ORX at other CO_2 chemoreceptor sites, acting indirectly on ventilation during hypercapnia. Still, the contribution of the OX_2R to the fictive ventilation was not investigated in this study but it might also participate in the ventilatory response to hypercarbic/hypoxic chemoreflex in tadpoles.

ORX and hypoxia

Tadpoles can uptake oxygen by different surfaces: skin, gills and lungs (Burggren and West, 1982; Burggren and Doyle, 1986; Burggren and Infantino, 1994) and along development the importance of each of them changes (Burggren and West, 1982; West and Burggren, 1984). In these animals, mainly during the early life, the primary role of the respiratory system is to guarantee adequate tissue oxygenation, since the CO_2 is easily diffused. Amphibians exhibit peripheral arterial chemoreceptors (Van Vliet and West, 1992; Gargaglioni and Milsom, 2007) and hypoxia in premetamorphic tadpoles evoke an increase in ventilation (Milsom and Burleson, 2007), specially by an increase in the lung burst frequency (Fournier and Kinkead, 2006; Fournier et al., 2007).

Our findings about the effect of hypoxia on the fictive breathing consent with the literature. In our experiments with the hypothalamus, hypoxia induced an increase in the fictive ventilation, but acting on the lung bursting (frequency and amplitude) and on the gill amplitude. Without the hypothalamus, hypoxia promoted an increase in the lung frequency and in the gill amplitude. In both preparations, the hypoxia has not changed the frequency of episodes, but augmented the number of events per episode. In addition, we observed that the SB-334867 amplified the ventilatory response to hypoxia. The effect elicited on the lung burst frequency in both preparations (hypothalamus present or not) was bigger than the effects of the hypoxia itself, and in the preparations with the hypothalamus we saw an increased lung amplitude in response to the dose of 10 µM and an increased gill amplitude only when the 5 µM was applied. Summing up, these data suggest that OXR is inhibitory for the lung rhythm, consequently blockage of OX_1R by SB-334867 has an excitatory effect for the O_2 chemoreflex.

The reports surrounding this subject show that in toads, the i.c.v. injection of SB-334867 attenuated the ventilatory response to hypoxia, but only during the light phase, while in mice, it had no effect on the hypoxic chemoreflex during the light phase (Deng et al., 2007). Besides that, the response of ORX-KO and wild-type mice to hypoxia is very similar (Nakamura et al., 2007). Narcoleptic-cataplectic humans (ORX deficient) show attenuated hypoxic but not hypercapnic ventilatory response (Han et al., 2010). Yamaguchi et al. (2015) related that intermittent but not sustained hypoxia activates ORX-containing neurons in mice, and Dergacheva et al. (2016) found that hypoxia and hypercapnia combined, or hypoxia alone inhibit hypothalamic ORX-neurons in rats.

Summarizing, we found that (1) the diencephalon plays a role in driving tonic breathing; (2) central ORX-A plays an inhibitory role in the basal respiratory drive; (2) hypercapnia inhibits the lung motor activity; (3) hypoxia excites fictive ventilation; (4) ORX

cells inhibit the O_2 and the CO_2 chemoreflex by acting mainly in the lung burst frequency in early stage tadpoles.

CHAPTER 2

Orexin stimulates hypercapnic ventilatory response in the green iguana (*Iguana iguana*) during the dark phase

ABSTRACT

Studies have demonstrated that the hypocretins or ORXs play an important modulation in respiratory control in mammals and in amphibians. It was observed that this modulation is cycle (sleep/awake)-dependent for mammals and at least phase (light/dark)-dependent for amphibians, but little is known about respiratory control in ectothermic vertebrates. To date, no data is available about the role of this neuropeptide in reptiles. Therefore, the aim of this study was: 1. to verify the localization of the ORX-neurons of this species by performing immunohistochemistry; 2. to understand how ORX-A cycles throughout the day in this animal (by ELISA assay), and then, 3. to investigate the participation of the orexinergic neurotransmission in the respiratory modulation in green iguanas (*Iguana iguana*) exposed to normocarbic normoxia, to hypoxia (5% O_2, N_2 balance) or hypercarbia (5% CO_2, 21% O_2, N_2 balance) during light and dark phases. For that, microinjections of the receptor-1 antagonist (SB-334867) and receptor-1 and -2 antagonist (Almorexant) were performed.

Central and peripheral chemoreceptors in reptiles have not received much attention. Based on studies of ventilatory responses using brain perfusion techniques, the presence of central chemoreceptors has been demonstrated in turtles (Hitzig and Jackson, 1978; Hitzig and Nattie, 1982; Davies and Sexton, 1987), alligators (Branco et al., 1992) and lizards (Zena et al., 2016). Recent evidences indicate that central chemoreceptors are widely distributed in the CNS in mammals, and it has been demonstrated that this wide distribution of central chemoreceptors occurs not only in mammals, but seems to occur also in amphibians and reptiles (Noronha-de-Souza et al., 2006; Santin and Hartzler, 2013; Zena et al., 2016). In most reptiles, earlier studies report indirect evidence suggesting that peripheral chemoreceptors are located in the internal carotid artery, derived from the carotid arch, which is innervated by the superior laryngeal branch of the vagus nerve and perhaps also the glossopharyngeal nerve (Jones and Milsom, 1982; Milsom 1990; Smatresk 1990). More recently, Reyes, in her thesis, found O_2-chemosensitive cells in *Pseudemys scripta elegans* and *Crotalus durissus* and observed that their arrangement and location were similar. They lie at the bifurcation of the common carotid artery, aorta, and pulmonary artery. Turtle "truncus arteriosus" did not seem to be a chemosensitive area (Reyes et al., 2015; Reyes, 2014).

It has been shown that in rats, orexinergic neurons from the hypothalamus are profoundly affected by changes in CO_2 and pH (Williams et al., 2007). Orexins (ORXs), also known as hypocretins, include two neuropeptide subtypes, orexin-A (ORX-A) and orexin-B (ORX-B) (hypocretin-1 and hypocretin-2, respectively) both cleaved from a common precursor, prepro-orexin (De Lecea et al., 1998, Sakurai et al., 1998) which binds to two G protein-coupled receptors: orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R) (Smart et

al., 2001). OX_1R is highly selective to ORX-A, whereas OX_2R behaves as a nonselective receptor, binding to the two ORX subtypes with the same affinity (De Lecea et al., 1998; Sakurai et al., 1998).

Interestingly, the amino acid sequences of ORXs -A and B are highly conserved across different groups of vertebrates (Alvarez, 2002). ORX distribution has been described in all classes of vertebrates, and in most groups, ORX-neurons are located in the hypothalamus, but they are not found exclusively within a single nucleus, they are located in various hypothalamic nuclei. Additionally, ORX fibers are widespread, innervating largely similar areas. In rodents, ORX-neurons are found only in the lateral and posterior hypothalamus, however, they project their axons widespread throughout the whole brain (Peyron et al., 1998; Nambu et al., 1999). In amphibians, Singletary et al. (2005) and Galas et al. (2001) observed a single population of ORX-neurons in the suprachiasmatic nucleus in *Hyla cinerea* and *Pelophylax ridibundus*, respectively, while Shibahara (1999) demonstrated that ORX-containing neurons are localized to the ventral hypothalamus in *Xenopus*. Domínguez et al. (2010) demonstrated that in the lizard *Gekko gecko* and in the turtle *Pseudemys scripta elegans*, most ORX immunoreactive neurons were found in the periventricular hypothalamic nucleus and in the infundibular hypothalamus. Only in the gecko, ORX cell bodies were present in the dorsolateral hypothalamic nucleus and the periventricular preoptic nucleus as well. This anatomical property explains the great multiplicity of functions that are modulated by the ORX, for example: the sensation of hunger, the sleep-awake state, feeding behavior, energy homeostasis, nociception, metabolism, the reward system, hormonal secretion, the response to stress, as well as the cardiovascular and respiratory control (Dube et al., 1998, Sakurai et al., 1998, Haynes et al., 2000; Bingham et al., 2001; Duxon et al., 2001; Jones et al., 2001).

ORXs play an important function in the control of ventilation in waking states (Nakamura et al., 2007, Williams and Burdakov, 2008). Studies with transgenic cells indicate that ORXs plays a crucial role in CO_2/pH chemosensitivity in rats. Prepo-orexin "*knockout*" mice showed an attenuation response to hypercapnia during wakefulness, but not during sleep periods, and this effect is partially recovered with the administration of ORX-A and B (Deng et al., 2007; Nakamura et al., 2007). Besides that, intracerebroventricular (i.c.v.) administrations of an OX_1R antagonist (SB-334867), decreased respiratory chemoreflex in rats (Deng et al., 2007). Dias et al. (2009) demonstrated that dialysis with the OX_1R antagonist, SB-334867, specifically in the region of the retrotrapezoid nucleus (RTN) of rats, caused an attenuation of the ventilatory response to hypercapnia, which was substantially higher during wakefulness than during NREM sleep. This result supports the hypothesis that one of the mechanisms by which ORX-neurons modulate respiration is due to its effects on the OX_1Rs of RTN. Recently, our laboratory has demonstrated that the orexinergic projections to the *locus coeruleus* region contribute through the OX_1R in the hypercapnic chemoreflex during wakefulness in the dark phase, and this can be an important connection between the sleep-wake states with the ventilatory response to CO_2 (Vicente et al., 2016).

The vigilance state dependency of the role of ORX on the hypercapnic chemoreflex is in accordance with the fact that ORX levels, which have been measured in the cerebrospinal fluid of rodents, humans and monkeys, vary during the diurnal cycle, with the highest levels occurring during the dark/active phase and the lowest levels during the light/inactive phase (Yoshida et al., 2001; Desarnaud et al., 2004). Therefore, the discharge of ORX-neurons is synchronized according to arousal states, with the highest activity taking place during active arousal (Lee et al., 2005; Mileykovskiy et al., 2005).

Our group has been dedicated to study the central modulation of ventilation in vertebrates (mammals and non-mammals) and one of our studies demonstrated that in toads *Rhinella diptycha*, ORX-A, acting on OX_1R, participates in the respiratory modulation in an excitatory manner in the hypercapnic chemoreflex during the dark phase, and also in an excitatory way in the hypoxic chemoreflex, however, during the light phase (Fonseca et al., 2016). There are no studies regarding the involvement of the ORX system on ventilation in reptiles, which is one of the gaps for which we intend to contribute with this study.

With this background and due to the scarcity of studies on the neural ventilatory control in ectothermic vertebrates, the objectives of this study were:

1. to determine the localization of the ORX-neurons in *Iguana iguana*;

2. to understand how ORX-A cycles during the day in this animal;

3. to investigate the contributions of OX_1R and OX_2R to the basal respiratory drive;

4. to investigate the contributions of OX_1R and OX_2R to the O_2 and CO_2 chemoreflexes in these animals.

Animals

Green iguanas *(Iguana iguana)* of either sex weighing 44.8 ± 2.1 g were obtained from the Jacarezario of the Sao Paulo State University, campus of Rio Claro. The animals were transported and maintained in agreement with SISBIO-ICMBio (animal license 50041) in the Department of Animal Morphology and Physiology of the Sao Paulo State University in the campus of Jaboticabal, where all the experiments were performed. This study was conducted in compliance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and in compliance with the approval of the local Animal Care and Use Committee. The iguanas were maintained at 25°C in large tanks with shavings and twigs mimicking perches and with UV illumination.

There were two groups of iguanas kept in different light/dark cycles: one group maintained under natural light/dark cycle; and another group kept under an inverted artificial light/dark cycle (lights turned on at 12 p.m. and turned off at 0 a.m.). The iguanas kept under the natural cycle were used for the ELISA assays, while the iguanas under the inverted cycle were used for the ventilation experiments. Thus, for the ventilation, the dark phase experiments were performed during the morning and light phase during the evening.

Immunohistochemistry

Four intact animals were used for histological analysis to identify the localization of the ORX-neurons in this species. The iguanas were anesthetized by pentobarbital injection (Thiopental, Cristalia, Brazil) and perfused through the heart with phosphate saline (0.01 M PBS, pH 7.4); perfusion was continued with phosphate buffer (0.01 M PB, pH 7.4) containing

4% paraformaldehyde (PFA). The brains were quickly dissected, post-fixed with the same fixative solution for 4 h and stored in 30% sucrose in PBS at 4ºC overnight. Then, the tissues were placed in an embedding medium (O.C.T. Tissue Tek, Germany) and immediately frozen in isopentane and sliced into 30 µm sections using a cryostat (Leica CM 1850).

Before starting the labeling, the slices were incubated in a retrieval solution (Target Retrieval Solution Ready-to-use, Dako, Denmark) at 70ºC (in water bath in microtubes) for 30 min. To verify the localization of ORX-neurons the sections were incubated for 48 h with a rabbit polyclonal anti-ORX A (1:500; Santa Cruz C-19) or anti-ORX B (1:500; Santa Cruz C-19) followed by a 2 h incubation with a biotinylated goat polyclonal anti-rabbit IgG (1:1000; Vector Laboratories, Burlingame, CA). The biotinylated antibody was complexed with avidin DH-biotinylated horseradish peroxidase (Vector, code PK-4001), and the complex was developed by addition of the peroxidase substrate 3,3-diaminobenzidine tetrahydrochloride according to manufacturer instructions (Sigma-Aldrich). The reaction was terminated by washing out excessive amounts of PBS. Finally, the sections were mounted on gelatin-coated slides, dried, dehydrated through graded concentrations of alcohol, cleared in xylene, and sealed with a coverslip. Photomicrographs of the brain were captured by the optic microscope (LEICA) using the image acquisition software LAS.

ELISA assay

Animals with normal diurnal cycle (natural light, no cycle inversion) were kept at the same conditions in the animal facility at the Sao Paulo State University in Jaboticabal. After one year of adaptation in our research facilities, 24 iguanas were used for measurements of ORX-A plasma levels along the diurnal cycle. For that, the iguanas were anesthetized with isoflurane (Cristalia, Brazil) and the blood was collected from the heart at 4-hour interval (0

a.m., 4 a.m., 8 a.m., 12 p.m., 4 p.m., 8 p.m.). After each collection, the iguanas were euthanized, thus, each iguana was used for only one sample. For each time point, 4 iguanas were used. During the dark phase, the iguanas were anesthetized very quickly, and covered in a heavy black cloth for the blood collection and the collections for this phase were carried out with very little light. The blood was collected with a heparinized syringe, homogenized with aprotinin (Sigma-Aldrich, U.S.A.), centrifuged and the plasma was kept at -80ºC until analysis.

For the assay it was used the ELISA kit Orexin A (Extraction Free EIA Kit, Phoenix Pharmaceuticals, Inc, California, U.S.A.) and the test were performed according to the company protocol. The tests were performed in Dr. Kinkead's laboratory in Quebec, QC, Canada, at the "Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec" (CRIUCPQ), affiliated to "Université Laval".

Surgical procedure (stereotaxic surgery)

Animals were anesthetized with isoflurane. The heads of the animals were then fixed in a David Kopf stereotaxic apparatus (Model 900 Small Animal Stereotaxic, Tujunga, USA), the skin covering the skull was removed using a bone scraper, and an opening was made in the skull above the telencephalon using a small drill (LB100, Beltec, Araraquara, Brazil). For microinjection, a guide cannula prepared from a hypodermic needle segment of 12 mm in length and 0.55 mm in outer diameter was attached to the tower of the stereotaxic apparatus and placed into the lateral cerebral ventricle. These coordinates were adapted according to the brainstem atlas for the lizard *Varanus exanthematicus* (Donkelaar et al., 1987). The displacement of the meniscus in a water manometer confirmed the correct positioning of the cannula within the lateral ventricle. The orifice around the cannula was filled out with a wax made of equal parts of paraffin and glycerin. The cannula was attached to the bone with acrylic

cement mixed to superglue. A tight-fitting stylet was kept inside the cannula to prevent occlusion and infection. The experiments were initiated at 7 days after brain surgery. After stereotaxic surgery, the iguanas were treated with a prophylactic antibiotic (Enrofloxacin, Flotril; Schering-Plough, 5.0 mg/kg, intramuscular) and a nonsteroidal anti-inflammatory drug (Meloxicam, 0.2 mg/kg, s.c.) according to recommended doses for reptiles (Mosley, 2011; Martinez-Jimenez et al., 2007).
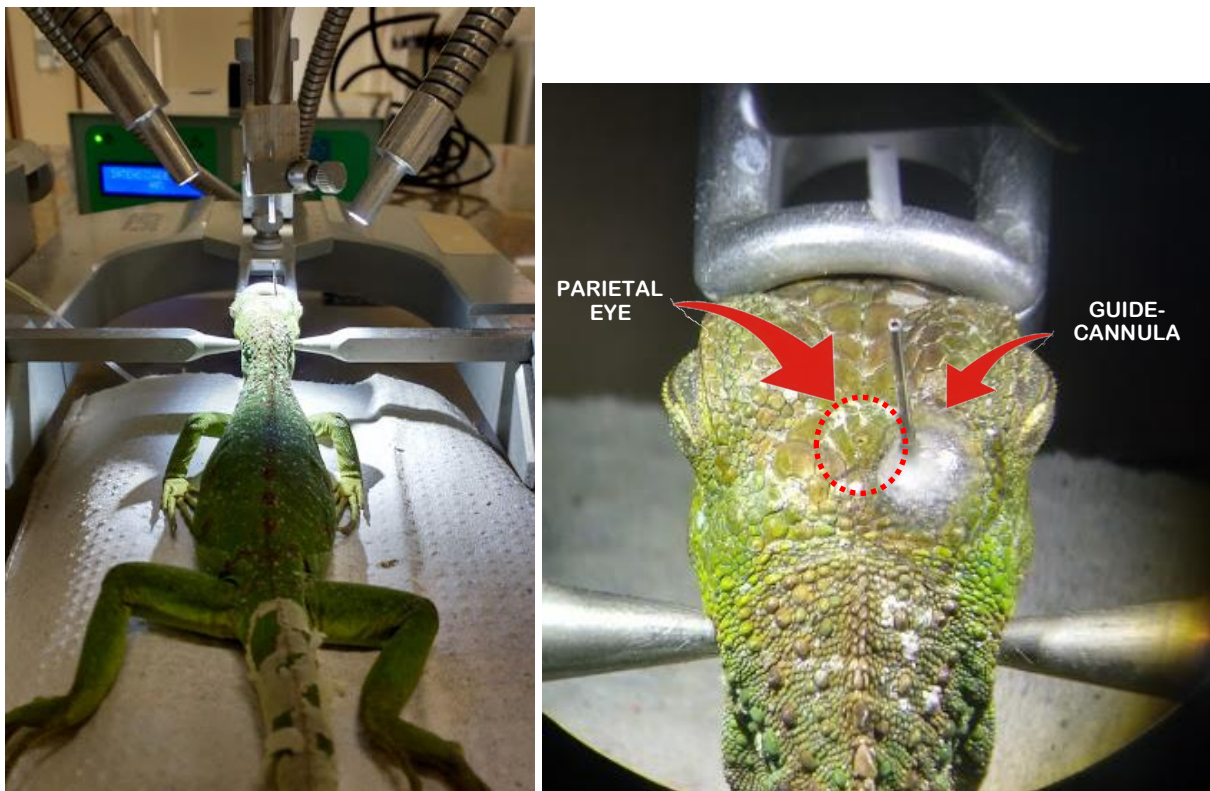

Figure 1. Photo showing detail of the guide cannula implanted on the lateral ventricle through stereotactic surgery. Carefully implanted in a place not to cover the modified transparent scale or the "parietal eye". The eye is a photoreceptive structure and is associated with the pineal gland, regulating circadian rhythmicity.

Intracerebroventricular microinjections

A dental needle (Mizzy, 30-gauge) was inserted until its tip was 0.4 mm below the guide cannula. A volume of 1 μL was injected over a period of 30 s with a 5-μL Hamilton syringe using a microinjection pump (model 310, Stoelting Co., IL, USA). At the end of each experiment, 1 μL of 2% Evans blue solution was microinjected into the lateral ventricle. The animals were euthanized by pentobarbital overdose (AVMA, 2007). Upon dissection, we observed that the dye had diffused into the periventricular tissue and spread throughout the ventricular system.
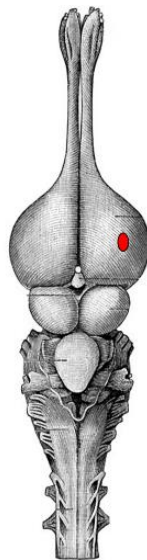

Figure 2. Reptile brain showing the site of the microinjection in red. Adapted from Parker, T (1990).

The OX_1R antagonist SB-334867 (Tocris, Bristol, UK) was dissolved in 4% DMSO, and then, the solution was diluted using 35% (2-hydroxypropyl)-β-cyclodextrin in artificial cerebral spinal fluid (aCSF, pH 7.4 at 25°C). For the vehicle, we used a solution containing 4% DMSO and 35% (2-hydroxypropyl)-β-cyclodextrin in aCSF. For the Almorexant (OX_1R and OX_2R antagonist, Cayman Chemical Company, U.S.A.), same procedure was made, however, saline was used instead of aCSF.

The dose and method of dissolving the drug were chosen based on pilot experiments and previous studies (SB-334867: Deng et al., 2007; Dias et al., 2009; Almorexant: Vazquez-DeRose et al., 2016).

Ventilation measurements

Pulmonary inspired ventilation ($\dot{V}_I$), tidal volume (V_T) and breathing frequency (f_R) were measured using the pneumotachographic method (Glass et al., 1978), which is based on the Poiseuille principle that a laminar flow of a gas is proportional to the pressure gradient across a tube. A lightweight transparent facemask attached to a pneumotachograph was fixed to the animal snout, allowing for inspirations and expirations to be measured continuously. Inspiratory and expiratory gas flows were monitored with a differential pressure transducer connected to a data acquisition system that included specific application software (MLT141 Spirometer, PowerLab System, ADInstruments/LabChart Software, version 7.3, Australia). Calibration for volume was performed during each experiment by injecting known amounts of air into the facemask (0.5 to 5 mL) using a graduated syringe. All measurements of pulmonary ventilation were performed at a constant temperature of 25°C and an atmospheric pressure of 716 mmHg. Ventilatory parameters in the graphs are displayed as percentage changes, and the normocarbic normoxia values (basal values) represent the reference values.
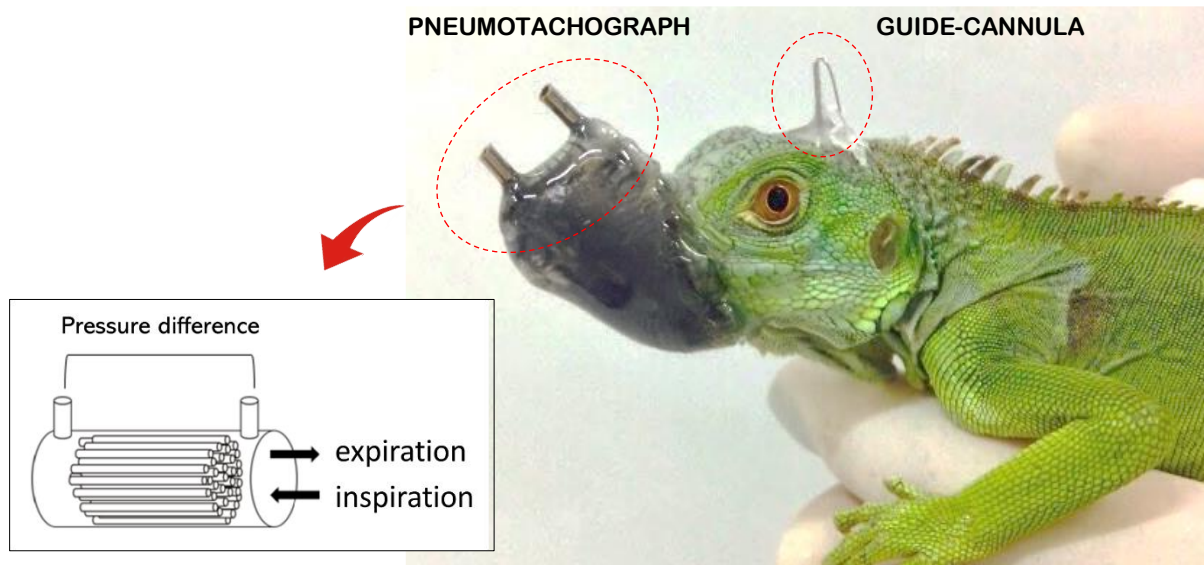
Figure 3. Photo showing the iguana with the guide-cannula implanted and the mask equipped with the pneumotachograph in detail.

Experimental protocols

Protocol 1. Contribution of OX$_1$R to the ventilatory responses in green iguanas

Protocol 1a. Effect of intracerebroventricular microinjection of SB-334867 on $\dot{V}_I$, under normocarbic normoxia during light and dark phases

Seven days after brain surgery, the iguanas were placed in a plastic chamber at 25°C. The chamber was continuously flushed with humidified air (1.5 L/min) for at least 6 h before the experiment. After an acclimatization period, control $\dot{V}_I$ was measured before microinjection. Subsequently, i.c.v. microinjections of vehicle or SB-334867 (5 mM or 10 mM) were performed. $\dot{V}_I$ measurements were continuously recorded during the experiment.

Protocol 1b. Effect of intracerebroventricular microinjection of SB-334867 on $\dot{V}_I$ under hypoxia during light and dark phases

Animals were placed in a plastic chamber as described in Protocol 1, and after an acclimatization period, $\dot{V}_I$ measurements were performed. Then, intracerebroventricular microinjections of vehicle or SB-334867 (5 mM or 10 mM) were administered, and the animals were submitted to hypoxia (5% O_2 and balance N_2) for 40 min. The concentration of O_2 was chosen based on previous studies of reptiles (Frische et al., 2000; Benchetrit et al., 1977).

Protocol 1c. Effect of intracerebroventricular microinjection of SB-334867 on $\dot{V}_I$ under hypercarbia during the light and dark phases

The same procedure as the previous protocol was used in this protocol, in which the hypoxic mixture was replaced with a hypercarbic mixture (5% of CO_2, 21% of O_2 and N_2 balance). After the hypercarbic exposition, the animals were exposed back to normocarbic normóxia and $\dot{V}_I$ measurements were performed again to examine the post-hypercarbic response. The concentration of CO_2 was chosen based on previous studies of reptiles (Skovgaard and Wang, 2004; Klein et al., 2002).

Protocol 2. Contribution of OX_1R and OX_2R to the ventilatory responses in green iguanas
Protocol 2a. Effect of intracerebroventricular microinjection of Almorexant on $\dot{V}_I$, under normocarbic normoxia during the light and dark phases

The same procedure of the protocol 1a was performed for this protocol but replacing the SB-334867 by Almorexant (9 mM).

Protocol 2b. Effect of intracerebroventricular microinjection of Almorexant on $\dot{V}_I$, under hypoxia during the light and dark phases

The same procedure of the protocol 1b was performed for this protocol but replacing the SB-334867 by Almorexant (9 mM).

Protocol 2c. Effects of intracerebroventricular microinjection of Almorexant on $\dot{V}_I$, under hypercarbia during the light and dark phases

The same procedure of the protocol 1c was performed for this protocol but replacing the SB-334867 by Almorexant (9 mM).
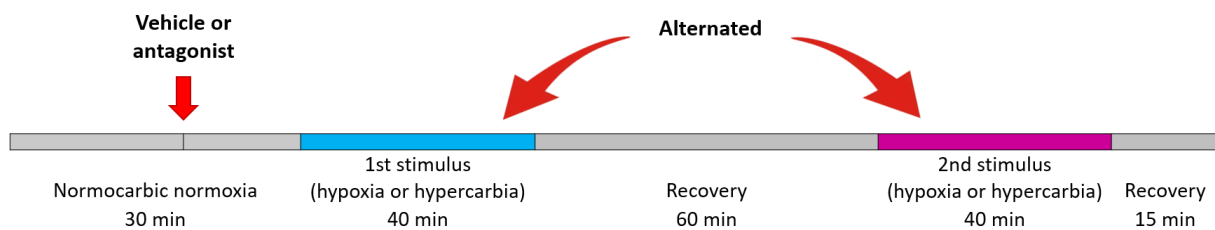

Figure 4. Scheme representing the experimental protocol.

Data analysis and statistics

All values are reported as the mean ± s.e.m. Calculations of respiratory frequency (fR), tidal volume (V_T) and ventilation ($\dot{V}_I$) were based on the last 10 minutes of the recording periods. The same thing for the non-ventilatory period (T_{NVP}), for the number of breaths per episode and for the frequency of episodes. Only for the post-hypercarbic response, the calculations were based on the first minute after the beginning of the hyperpnea (Figure 5).
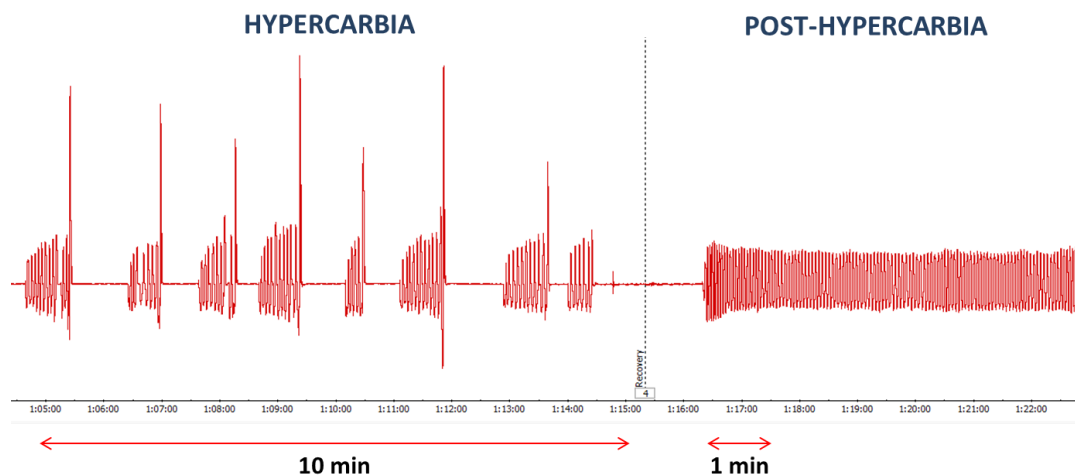
Figure 5. Recording demonstrating the times from which the data analysis calculations were made.

fR was quantified by analyzing the number of respiratory events (lung breaths) per min. V_T was obtained from the integrated area of the expired flow signal. Expired $\dot{V}_I$ was calculated as previously described by Jones (1982) as follows: $\dot{V}_I = V_T \times f_R$. Lung ventilations were identified by large pressure changes. For protocols 1a and 2a, the effects on the ventilatory variables were evaluated by one-way ANOVA with Tukey's multiple comparison test. For protocols 1b and c and 2b and c, to understand the effect of the stimuli (hypoxia or hypercarbia) it was used one-way ANOVA also with Tukey's multiple comparison test. Still, for the evaluation of the effect of the antagonists, it was used two-way repeated-measures ANOVA followed by the Bonferroni post-test to assess differences between the groups. A $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego, USA). Only the statistical differences caused by the effect of the drug are shown in the graphs, the effect of hypoxia or hypercarbia alone are described along the results.

For the analysis of the breathing pattern, inside the groups, to evaluate the effect of the stimuli, it was used one-way ANOVA with Tukey's multiple comparison test. To compare the differences among the groups, that is, effect of the antagonists on the parameters, it was used two-way ANOVA with Bonferroni's multiple comparison test. The breathing pattern parameters in the graphs are displayed as absolute values.

Localization of ORX-neurons in Iguana iguana

Figure 6 shows a photomicrograph demonstrating a slice in which we found most of the ORX-ir cells and a reptile brain illustrating the level of the transections. Figures 7 and 8 show respectively the ORX-A and ORX-B labeling that were found mainly in the periventricular hypothalamic nucleus.
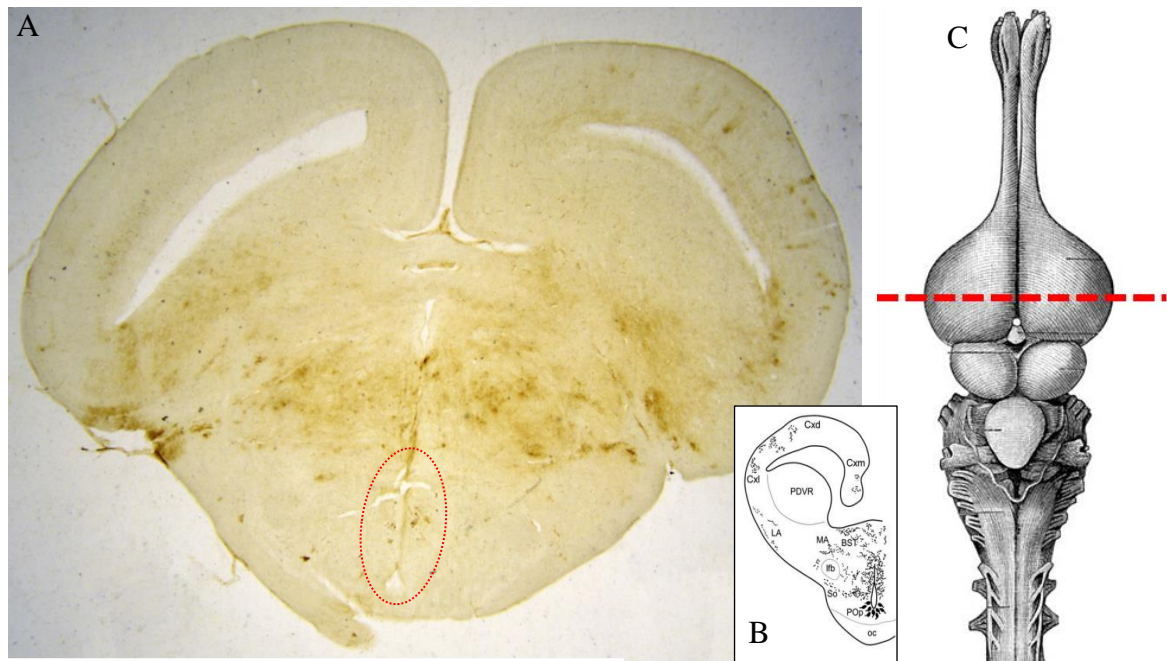

Figure 6. A photomicrograph showing one of the transections (A) where were found the most prominent group of ORX-labeling and a scheme illustrating the slice from Dominguez et al., 2009 (B). The red circle indicates the localization of the cell bodies where were found the ORX-neurons. The level of the transections in the reptile brain (C).
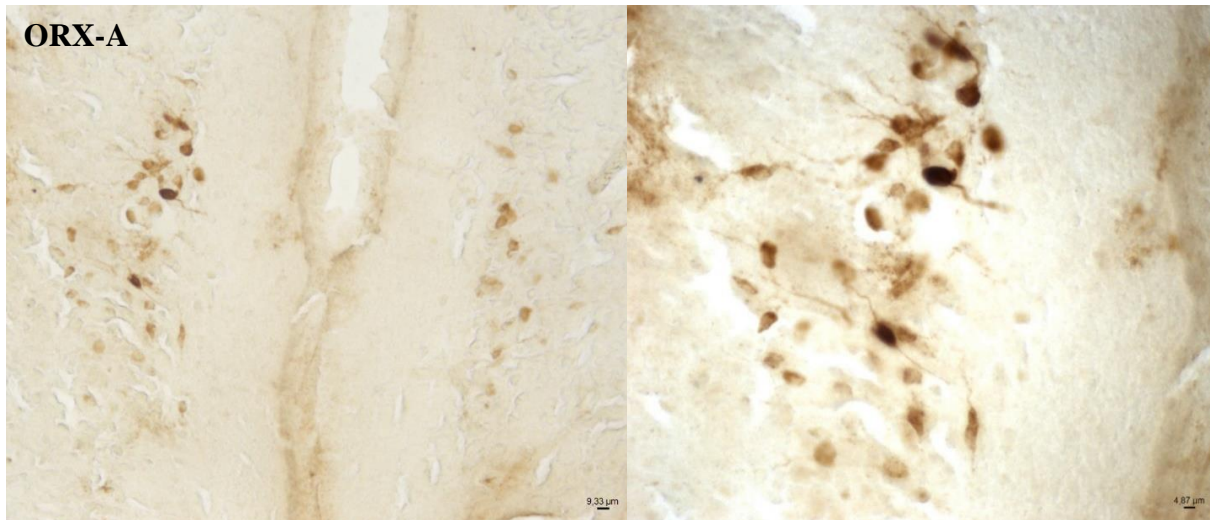
ORX-A

Figure 7. Photomicrographs of ORX-A labeling.
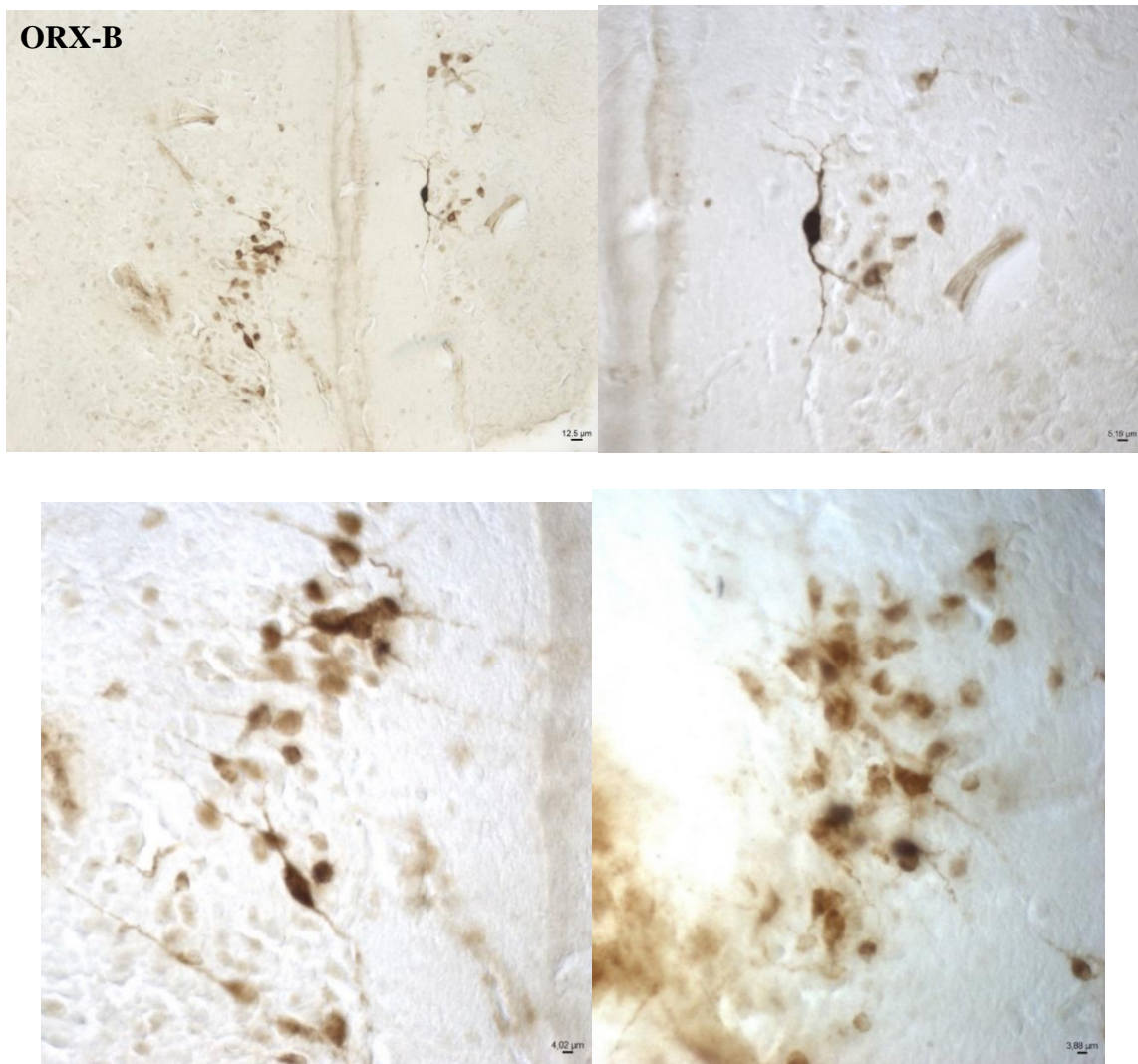
ORX-B

Figure 8. Photomicrographs showing the ORX-B labeling in detail.

ORX-A dosage

Figure 9 shows the diurnal rhythm of plasmatic ORX-A concentrations in the green iguana. The data represent average (± s.e.m.) ORX-A concentrations at 4-hour intervals for each group. The assay showed that there is a peak in the ORX-A levels at 8 a.m., in the light phase of the diurnal cycle.
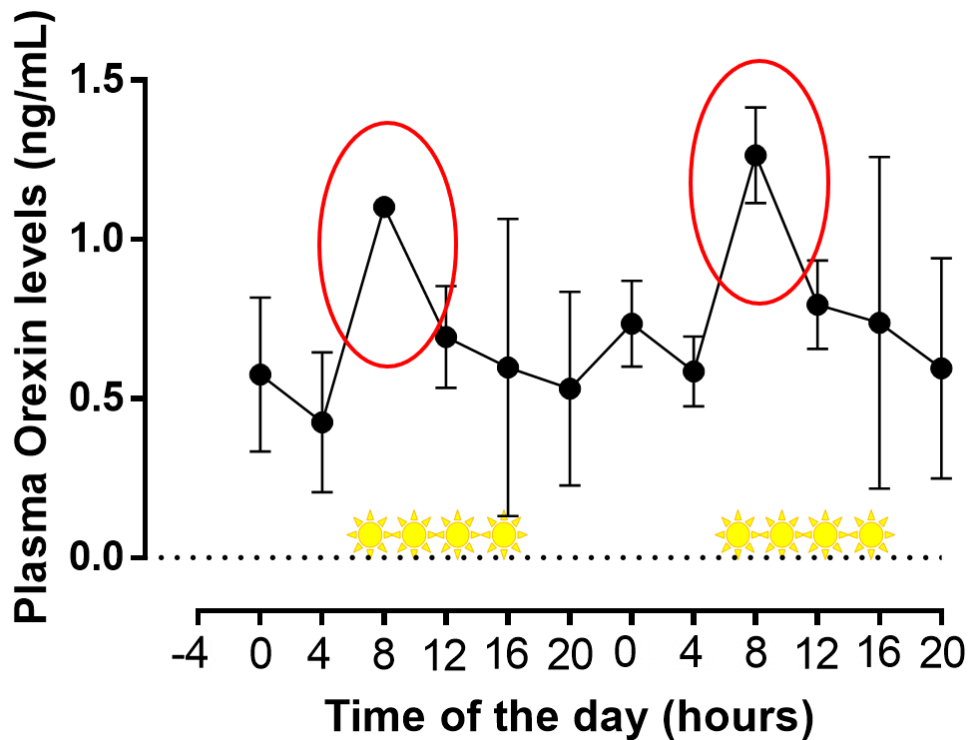

Figure 9. Daily variation of the plasma levels of ORX-A in the green iguana. The sun symbols represent the light phase of the diurnal cycle.

Breathing pattern

Before introducing the results on the participation of the ORX-receptors on ventilatory control, it is necessary to understand how *Iguana iguana* breathes in room air conditions. Below, a few representative traces showing the differences of the breathing patterns during light or dark phases are shown.

During light phase, the animals breathe continuously as shown in Figure 10, while during the dark phase the animals breathe episodically, with non-ventilatory periods intercalating with the ventilatory periods. The respiratory cycle always starts with an exhalation.
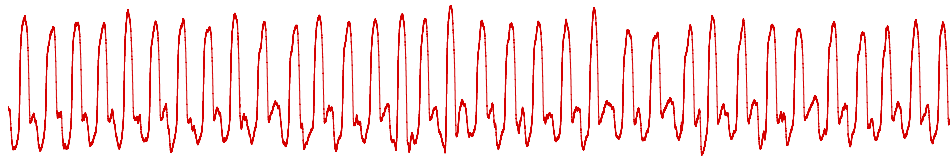

Figure 10. Representative trace from normocarbic normoxia during light phase.

Figure 11. Trace from normocarbic normoxia during dark phase.

During hypercarbia (5% CO_2), the inspired ventilation does not change. The frequency decreases and the tidal volume increases, resulting in a balanced effect that does not change the inspired ventilation. But as Figure 12 shows, there is a huge difference on the breathing pattern during the stimulus, that becomes episodic. After the hypercarbic stimulus, when the animal is exposed back to room air, then there is an increase in the inspired ventilation, and the animal breathes continuously again.

Figure 12. Representative trace from hypercarbia (5% CO_2) and recovery.

During hypoxia there is a small increase in the inspired ventilation and the animals breathe continuously.

Figure 13. Representative trace from hypoxia 5% O_2.

Ventilation

Effect of intracerebroventricular microinjection of SB-334867 on $\dot{V}_I$ under normocarbic normoxia

Figure 14 shows the fR, V_T and $\dot{V}_I$ of green iguanas microinjected with vehicle or SB-334867 (5 mM and 10 mM) in room air conditions during light and dark phases. The antagonist did not promote changes in ventilation under basal conditions during either the light or the dark phase, but the fR was significantly lower in all groups during the dark phase. (P=0.0003).

115

Figure 14. Effect of the i.c.v. injection of SB-334867 and its vehicle on fR, V_T and $\dot{V}_I$ in normocapnic normoxia in green iguanas during light or dark phases. * means different from light phase.

Effect of intracerebroventricular microinjection of SB-334867 on $\dot{V}_I$, under hypoxia during the light and dark phases

Figure 15 show the effect of the microinjection of SB-334867 on ventilation at 5% O_2 during the light and dark phases.

During light phase, hypoxia caused an increase in the inspired ventilation of 53% (P=0.0289) caused by an increase in the respiratory frequency (P=0.0299). The green iguanas did not respond to acute hypoxia during the dark phase.

The antagonist SB-334867 did not cause any changes in the O_2 chemoreflex in green iguanas during light or dark phases.

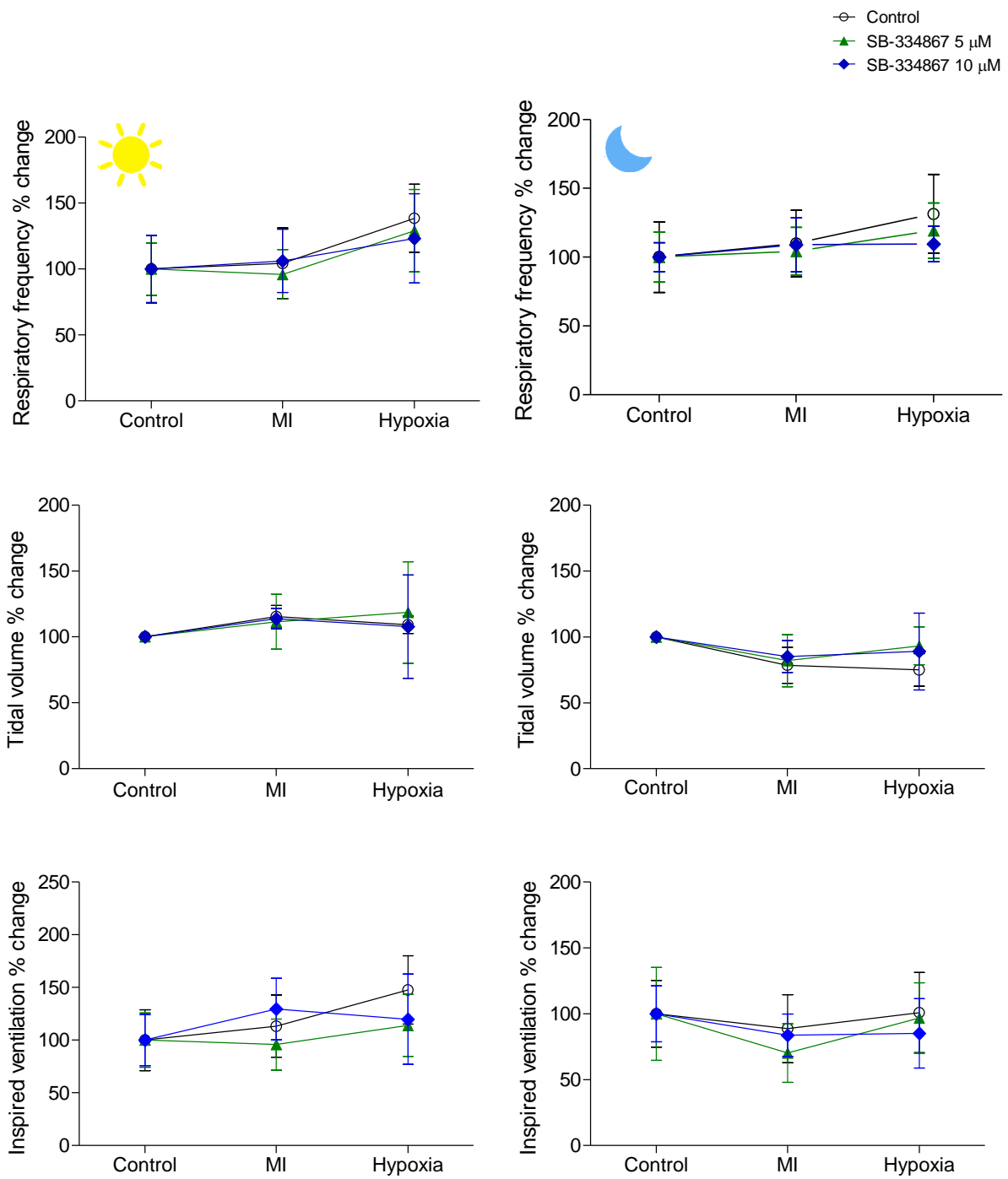**Figure 15.** Effect of the i.c.v. injection of SB-334867 and its vehicle on fR, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypoxia (5% O_2) during light or dark phases.

Effect of intracerebroventricular microinjection of SB-334867 on ventilation under hypercarbia during the light and dark phases

Figure 16 shows the effects of microinjection of SB-334867 on $\dot{V}_I$ at 5% CO_2 during the light and dark phases.

Hypercarbia only did not cause changes in the inspired ventilation but caused a post-hypercarbic response of 669.8% in relation to control (room air) during the light phase, due to changes in the respiratory frequency and in the tidal volume. For the dark phase, hypercarbia also did not cause any changes on the inspired ventilation, but on the post-hypercarbic response. The effect on the inspired ventilation was 275.1% bigger than during normocarbic normoxia and even though there were no statistical differences on the respiratory frequency or on the tidal volume, we assume that the effect was due to an additive effect of both, that alone do not present statistical difference, but when combined to generate the inspired ventilation, then an effect is seen.

Both doses of the antagonist SB-335867 caused an attenuation on the inspired ventilation after the hypercarbic stimulus (for 5 µM, P=0.0018; for 10 µM, P=0.0018) in the green iguana during the light phase. This decrease was due to changes in the tidal volume (for 5 µM, P=0.0014; for 10 µM, P=0.0015).

For the dark phase, neither dose of SB-334867 caused any changes on the hypercarbic chemoreflex in the green iguana.

Figure 16. Effect of the i.c.v. injection of SB-334867 and its vehicle on fR, V_T and $\dot{V}I$ in green iguanas exposed to acute hypercarbia (5% CO_2) during light or dark phases. * means different from vehicle.

Effect of intracerebroventricular microinjection of Almorexant on $\dot{V}_I$ under normocarbic normoxia

Figure 17 shows the fR, V_T and $\dot{V}_I$ of green iguanas microinjected with vehicle or Almorexant in room air conditions during light and dark phases. The antagonist did not promote changes in ventilation under basal conditions during either the light or the dark phase, but the fR was significantly lower during the dark phase.

Figure 17. Effect of the i.c.v. injection of Almorexant and its vehicle on fR, V_T and in $\dot{V}_I$ normocapnic normoxia in green iguanas during light and dark phases. * means different from light phase.

Effect of intracerebroventricular microinjection of Almorexant on $\dot{V}_I$, under hypoxia during the light and dark phases

Figure 18 shows the effects of microinjection of Almorexant on $\dot{V}_I$ at 5% O_2 during the light and dark phases.

Hypoxia only during light phase evoked an increase of 70.7% on the inspired ventilation during light phase, due to an increase on the respiratory frequency. The green iguanas did not respond to acute hypoxia during the dark phase.

The antagonist Almorexant did not cause any changes in the O_2 chemoreflex in green iguanas during light or dark phases.
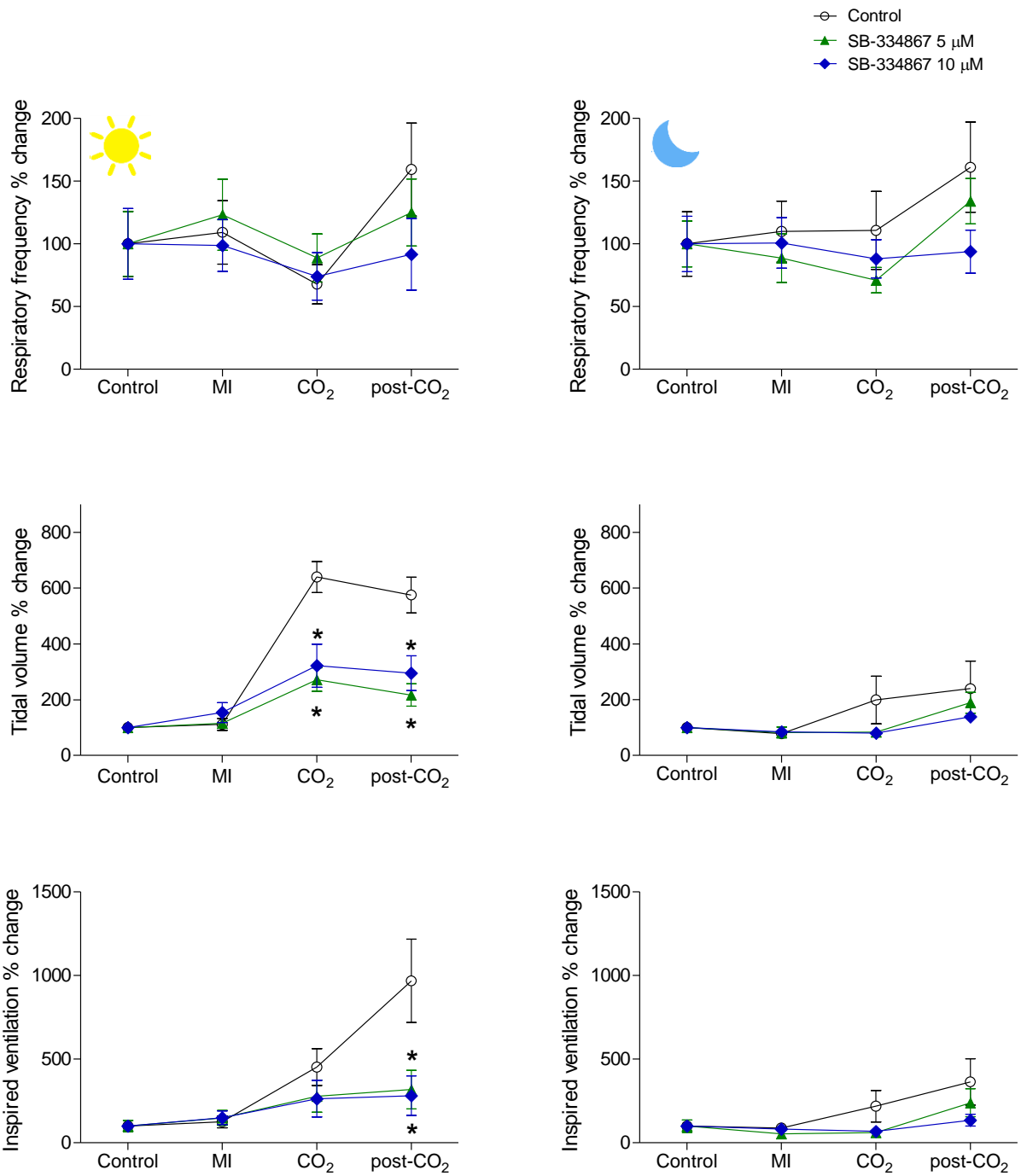
Figure 18. Effect of the i.c.v. injection of Almorexant and its vehicle on fR, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypoxia (5% O_2) during light or dark phases.

Effect of intracerebroventricular microinjection of Almorexant on ventilation under hypercarbia during the light and dark phases

Figure 19 shows the effects of microinjection of Almorexant on $\dot{V}_I$, fR and V_T at 5% CO_2 during the light and dark phases.

Hypercapnia alone during light phase did not cause any changes in the inspired ventilation during the stimulus but caused an increase of 651% in the inspired ventilation after the stimulus compared to normocarbic normoxia, due to changes in the respiratory frequency and in the tidal volume. Interestingly, during the dark phase the increase in the inspired ventilation during the post-hypercapnic response was smaller: 176.8%.

The antagonist Almorexant caused an attenuation on the inspired ventilation (P=0.0309) in the green iguana during the post-hypercarbic response during the light phase. This decrease was due to changes in the respiratory frequency (P=0.0012).

During the dark phase, Almorexant also caused as attenuation on the post-hypercarbic response (P=0.0371) due to changes in the respiratory frequency (P=0.1432).
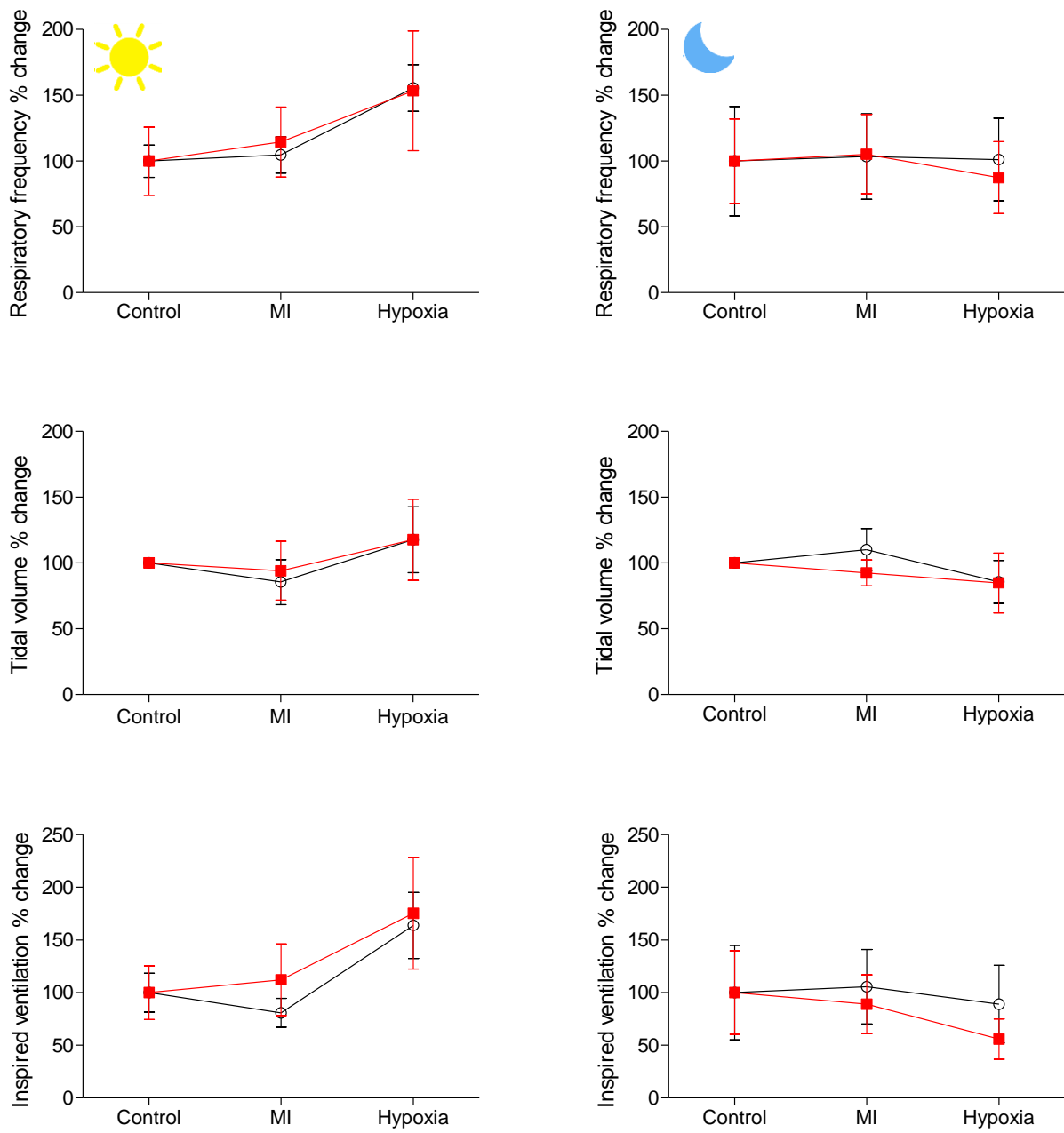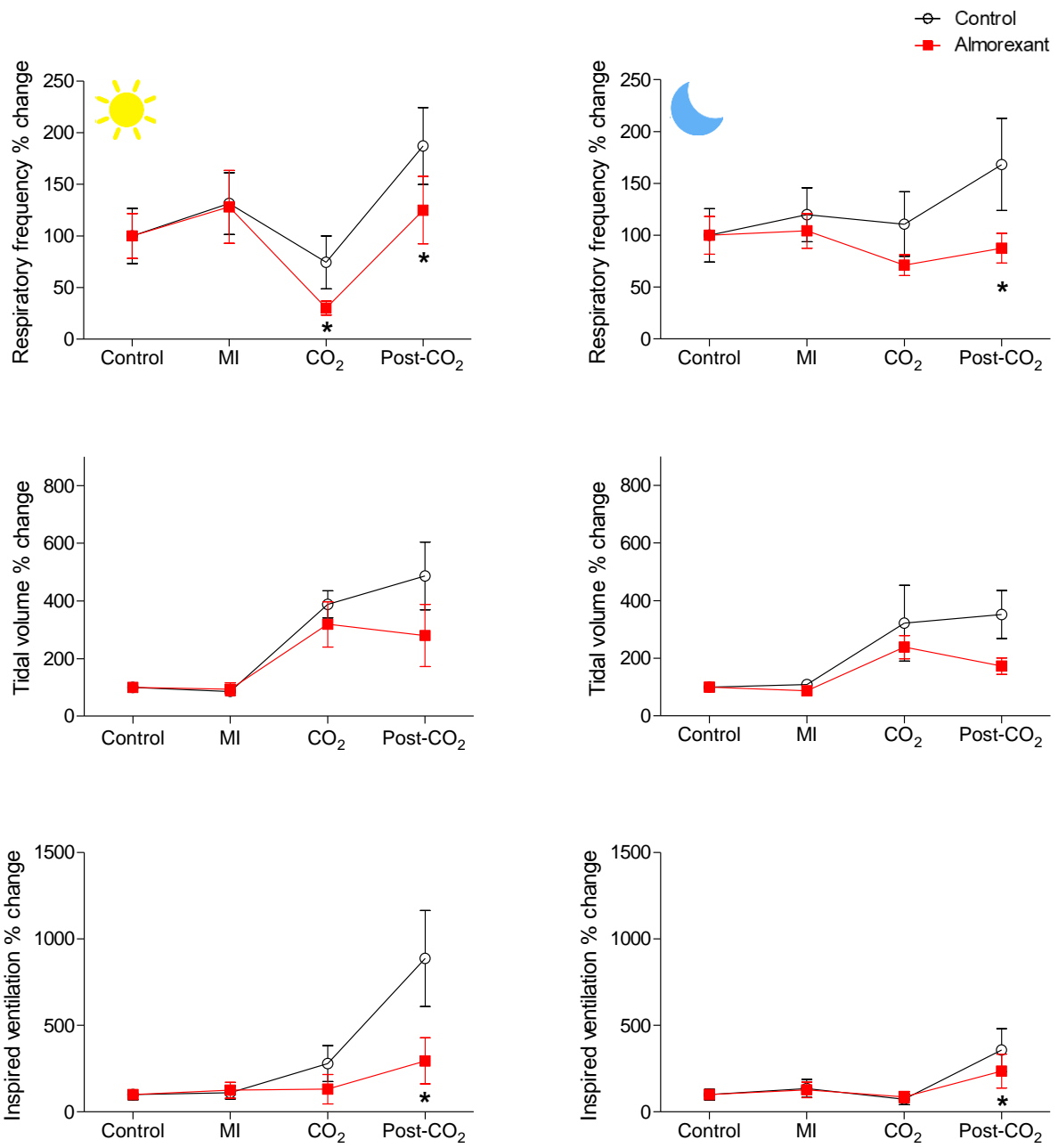
Figure 19. Effect of the i.c.v. injection of Almorexant and its vehicle on fR, V_T and $\dot{V}_I$ in green iguanas exposed to acute hypercarbia (5% CO_2) during light or dark phases. * means different from vehicle.

TREATMENT	PHASE	STIMULUS	f_R + s.e.m.		V_T + s.e.m.		$\dot{V}_I$ + s.e.m.	
No injection	Light	Normoxia	13.49	1.56	9.97	0.41	134.54	16.54
	Dark	Normoxia	9.68	0.67	9.82	0.98	95.01	20.35
Vehicle SB-334867	Light	Normoxia	13.05	2.11	10.00	2.32	130.43	16.30
		Hypoxia	20.46	1.07	10.06	0.62	205.90	16.17
		Hypercarbia	8.34	1.32	58.15	5.33	484.75	83.68
		Post-hypercarbia	20.06	3.12	51.64	4.50	1035.70	200.30
	Dark	Normoxia	10.49	1.30	7.59	0.89	79.58	24.00
		Hypoxia	14.70	1.77	7.88	1.59	115.86	28.09
		Hypercarbia	10.57	2.26	20.27	6.77	214.29	83.53
		Post-hypercarbia	15.38	1.96	23.17	7.79	356.35	86.50
SB-334867 5 µM	Light	Normoxia	15.71	2.19	9.26	1.08	145.58	30.79
		Hypoxia	19.69	3.88	10.81	2.58	212.77	39.49
		Hypercarbia	11.33	1.34	27.57	4.63	312.28	78.97
		Post-hypercarbia	15.95	1.76	22.40	4.46	357.18	98.62
	Dark	Normoxia	9.67	1.71	7.24	0.89	69.97	6.70
		Hypoxia	15.25	1.67	9.50	0.97	144.92	17.06
		Hypercarbia	7.76	0.46	10.26	2.83	79.61	26.13
		Post-hypercarbia	14.62	0.54	21.40	5.50	312.95	72.09
SB-334867 10 µM	Light	Normoxia	16.64	1.03	9.27	3.16	154.30	11.78
		Hypoxia	15.72	3.22	8.34	3.50	131.13	41.11
		Hypercarbia	12.49	1.97	31.76	8.42	396.79	147.53
		Post-hypercarbia	15.46	3.67	27.48	6.64	424.88	160.17
	Dark	Normoxia	9.55	1.20	8.76	1.22	83.68	10.25
		Hypoxia	10.86	0.97	9.47	3.28	102.79	27.78
		Hypercarbia	8.36	0.61	8.42	0.38	70.32	5.94
		Post-hypercarbia	8.90	0.86	15.64	2.80	139.20	28.65
Vehicle Almorexant	Light	Normoxia	14.98	3.20	7.96	1.10	119.28	33.83
		Hypoxia	21.64	1.59	10.61	2.28	229.63	32.25
		Hypercarbia	8.71	2.49	36.50	3.03	318.06	97.24
		Post-hypercarbia	21.91	1.33	46.12	9.71	1010.43	236.61
	Dark	Normoxia	10.71	1.02	8.48	1.20	90.80	9.36
		Hypoxia	10.47	1.09	9.06	2.12	94.87	24.86
		Hypercarbia	2.24	2.12	24.10	4.25	53.86	19.49
		Post-hypercarbia	9.28	2.88	28.43	7.74	263.78	72.47
Almorexant	Light	Normoxia	13.98	1.51	8.68	1.74	121.28	12.10
		Hypoxia	12.22	2.84	9.54	1.83	116.67	28.25
		Hypercarbia	7.60	0.46	19.10	9.29	145.10	86.52
		Post-hypercarbia	9.60	2.44	32.35	7.09	310.48	27.32
	Dark	Normoxia	9.80	0.95	8.14	0.93	79.78	9.23
		Hypoxia	7.55	1.61	7.73	1.47	58.42	11.23
		Hypercarbia	1.79	0.44	24.05	4.40	43.00	14.93
		Post-hypercarbia	7.42	0.90	15.83	2.98	117.52	46.68

Table 2. Values of f_R, V_T and $\dot{V}_I$ of *I. iguana* microinjected with vehicle, SB-334687 or Almorexant exposed to room air, acute hypoxia or hypercarbia during light or dark phases.

Effect of Almorexant and SB-334867 on the breathing pattern

Returning to the breathing pattern, the following data are about the effect of the SB-334867 or Almorexant on the non-ventilatory periods, on the number of breaths per episode and on the frequency of episodes.

<u>SB-334867 (5 and 10 μM)</u>

The effect of SB-334867 - 5 and 10 μM - on the non-ventilatory periods (T_{NVP}), on the number of breaths per episode and on the frequency of episodes in the iguanas during light and dark phases are shown in Figure 20, Figure 21 and Figure 22, respectively. As mentioned, during light phase, 5% CO_2 changed the breathing pattern from continuous to episodic, displaying non-ventilatory periods on the recordings (Figure 20 A). During dark phase in room air conditions, respiration was already episodic, thus, during hypercarbia, the non-ventilatory periods became longer (Figure 20 B), decreasing the frequency of episodes (Figure 22 B) and the number of breaths per episode (Figure 21 B). During post-hypercarbia and hypoxia, breathing was always continuous, and the pattern has not changed. Performing the i.c.v. microinjections changed the pattern making breathing less "episodic", but the injection of the vehicle evoked the same effect, so, we consider this as an effect of the injection itself, and not the antagonist or the vehicle (Figures 20, 21 and 22).

The SB-334867 has not changed the duration of the non-ventilatory periods, the number of events per episode or the frequency of episodes in room air, hypoxia or hypercarbia (and post-hypercarbia) during light or dark phases (Figures 20, 21 and 22).

A

B

Figure 20. Effect of the i.c.v. injection of SB-334867 and its vehicle on T_{NVP} in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

A

B

Figure 21. Effect of the i.c.v. injection of SB-334867 and its vehicle on the number of breaths per episode in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

A

LIGHT PHASE

B

DARK PHASE

Figure 22. Effect of the i.c.v. injection of SB-334867 and its vehicle on the frequency of episodes in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).

Almorexant

The effect of Almorexant on the non-ventilatory periods (T_{NVP}), on the number of breaths per episode and on the frequency of episodes in the iguanas during light or dark phases are shown in Figure 23, Figure 24 and Figure 25, respectively.

Different from the SB-334867, Almorexant decreased the number of the breaths per episode during the 5% CO_2 exposure during the light phase (Figure 24 A). The injection of the antagonist has not evoked any changes on the other breathing pattern parameters.
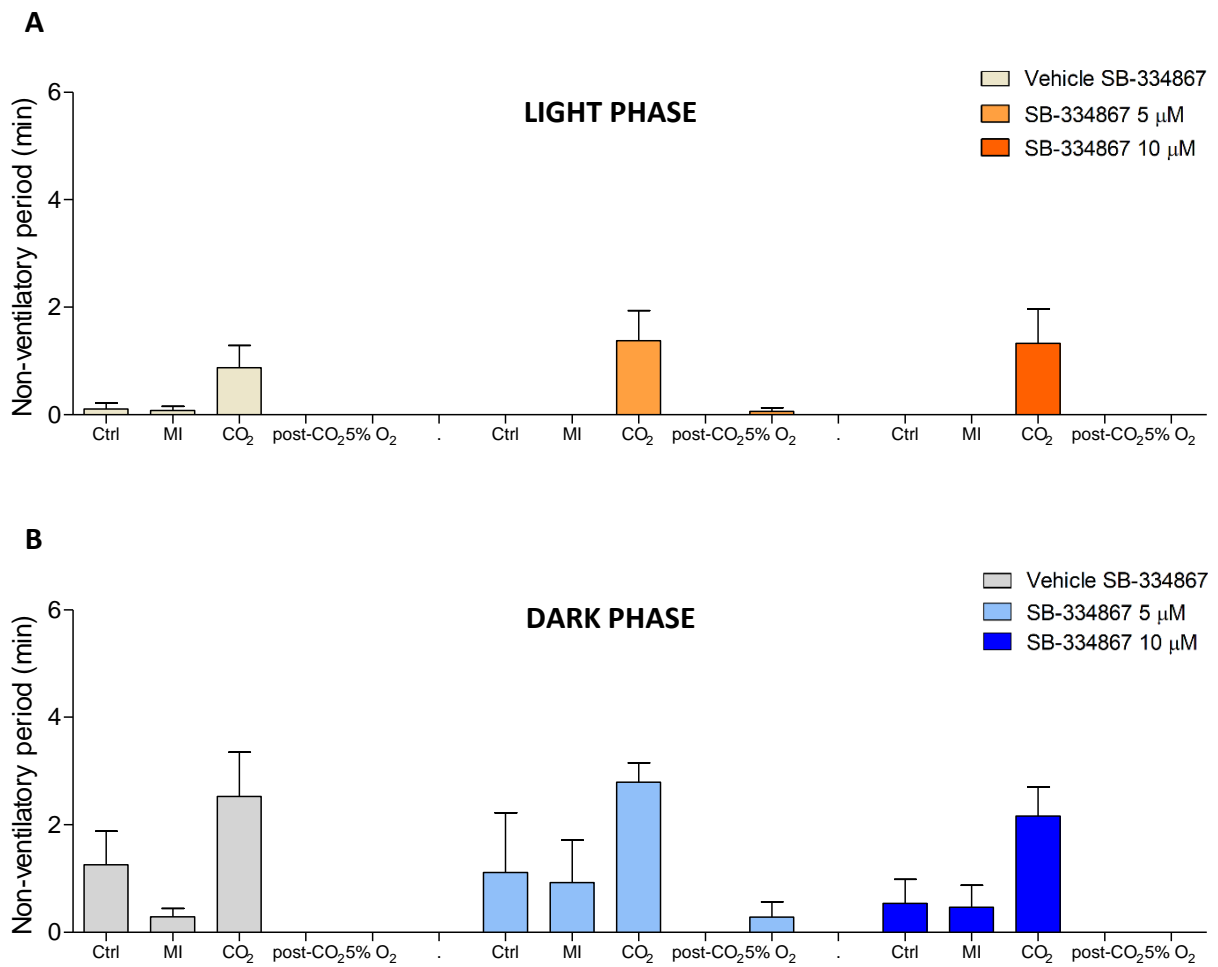

Figure 23. Effect of the i.c.v. injection of Almorexant and its vehicle on T_{NVP} in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).
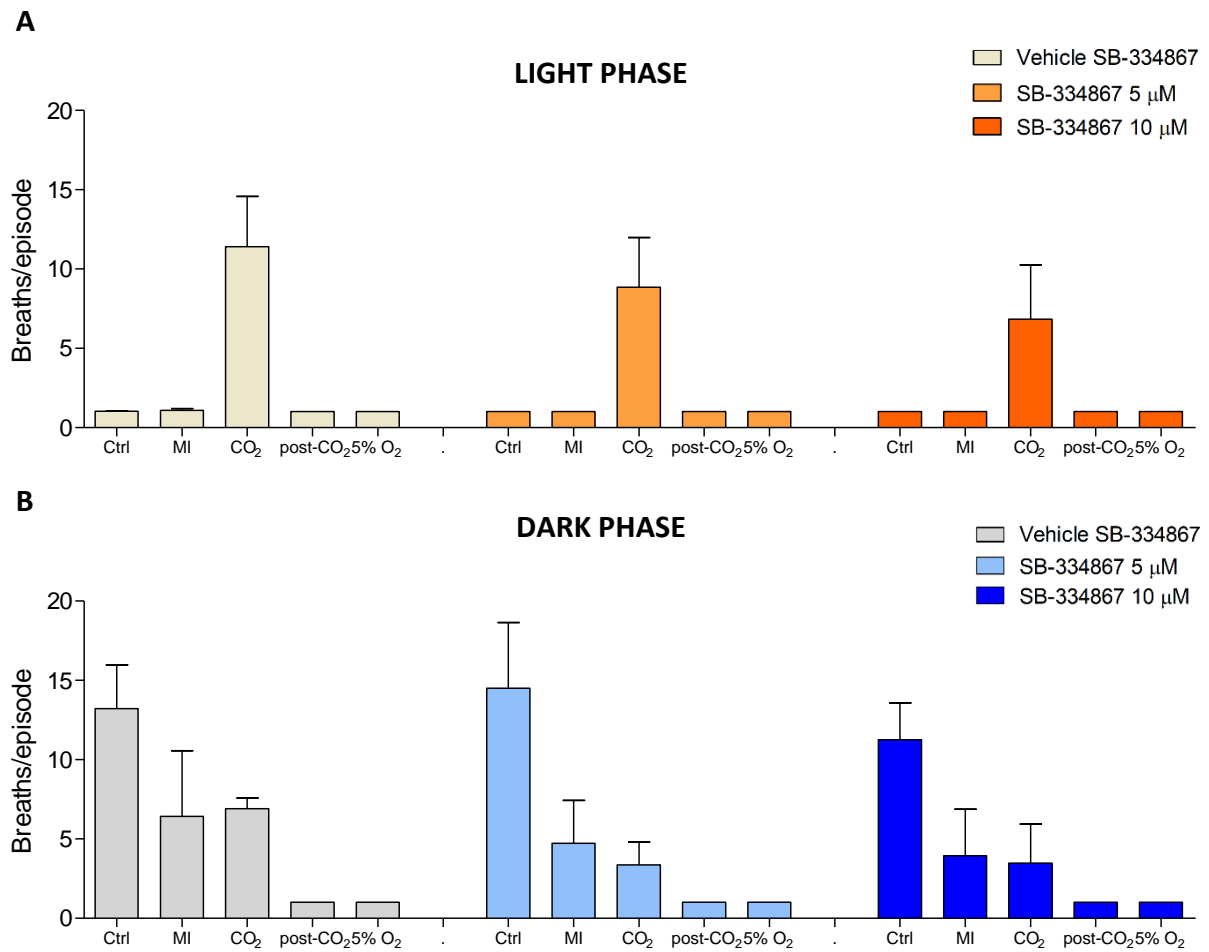
Figure 24. Effect of the i.c.v. injection of Almorexant and its vehicle on the number of breaths per episode in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (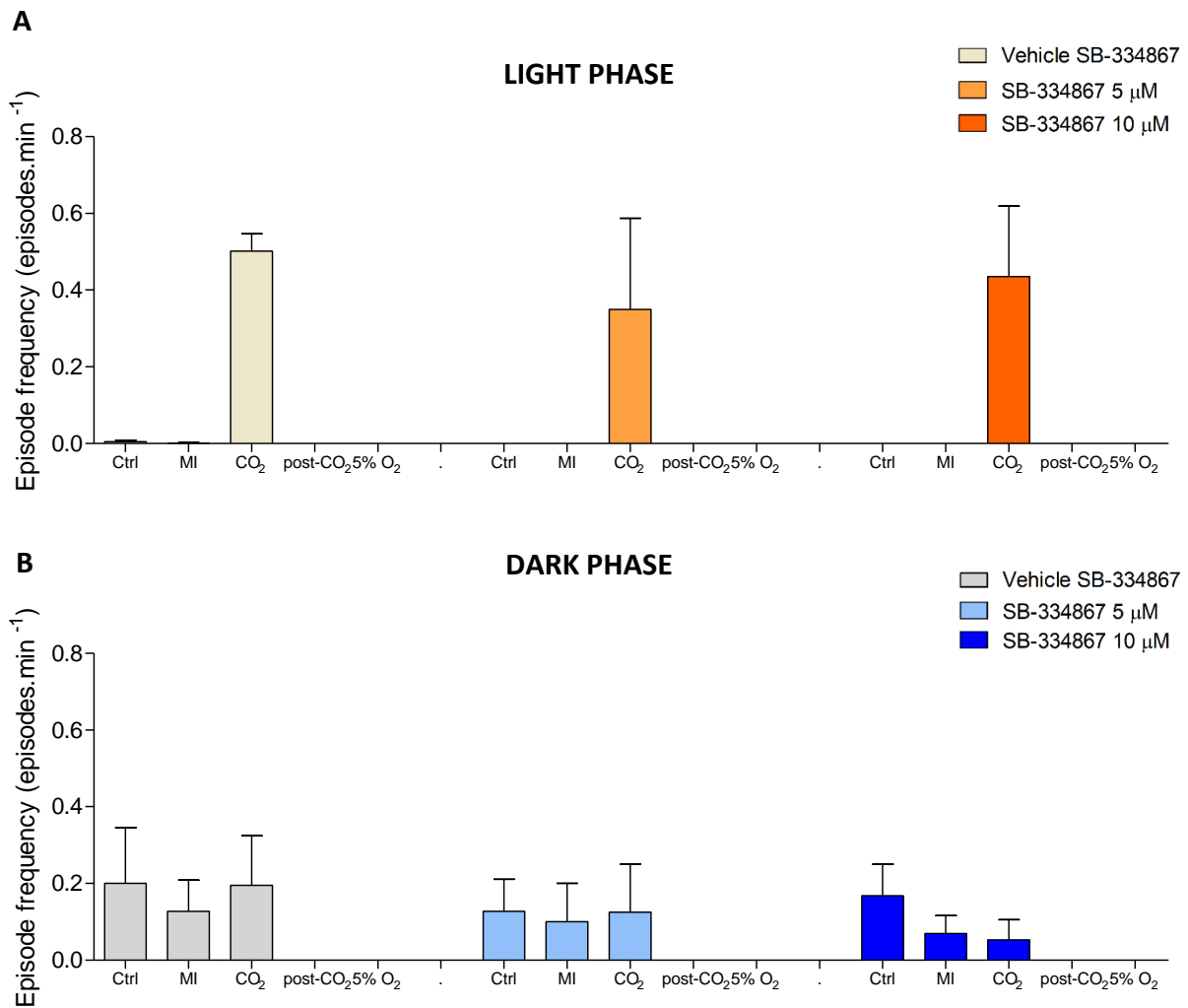5% O_2) during light (on top) or dark phase (on the bottom). * means different from correspondent stimulus of the vehicle group.

Figure 25. Effect of the i.c.v. injection of Almorexant and its vehicle on the frequency of episodes in green iguanas exposed to acute hypercarbia (5% CO_2) or acute hypoxia (5% O_2) during light (on top) or dark phase (on the bottom).
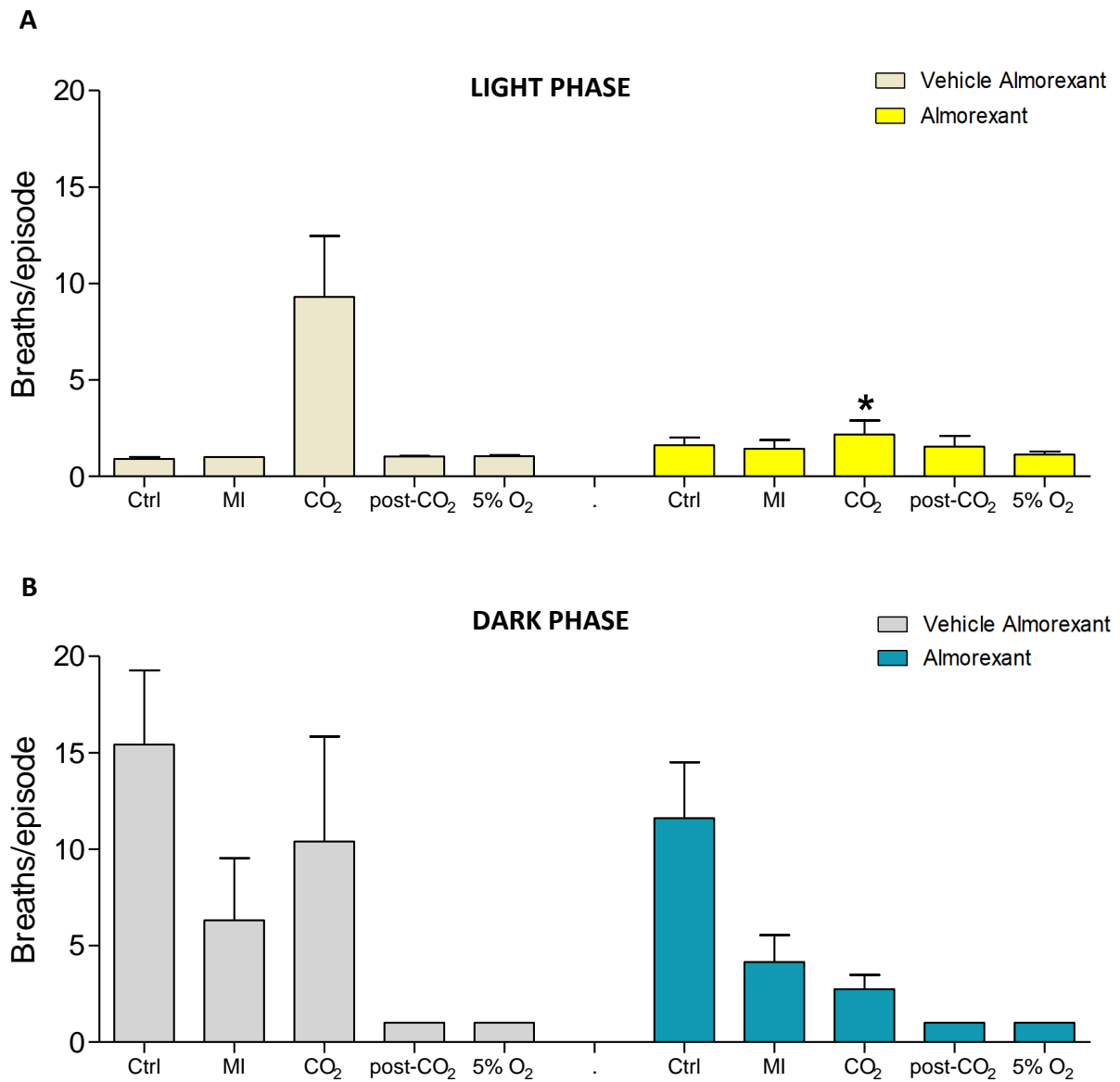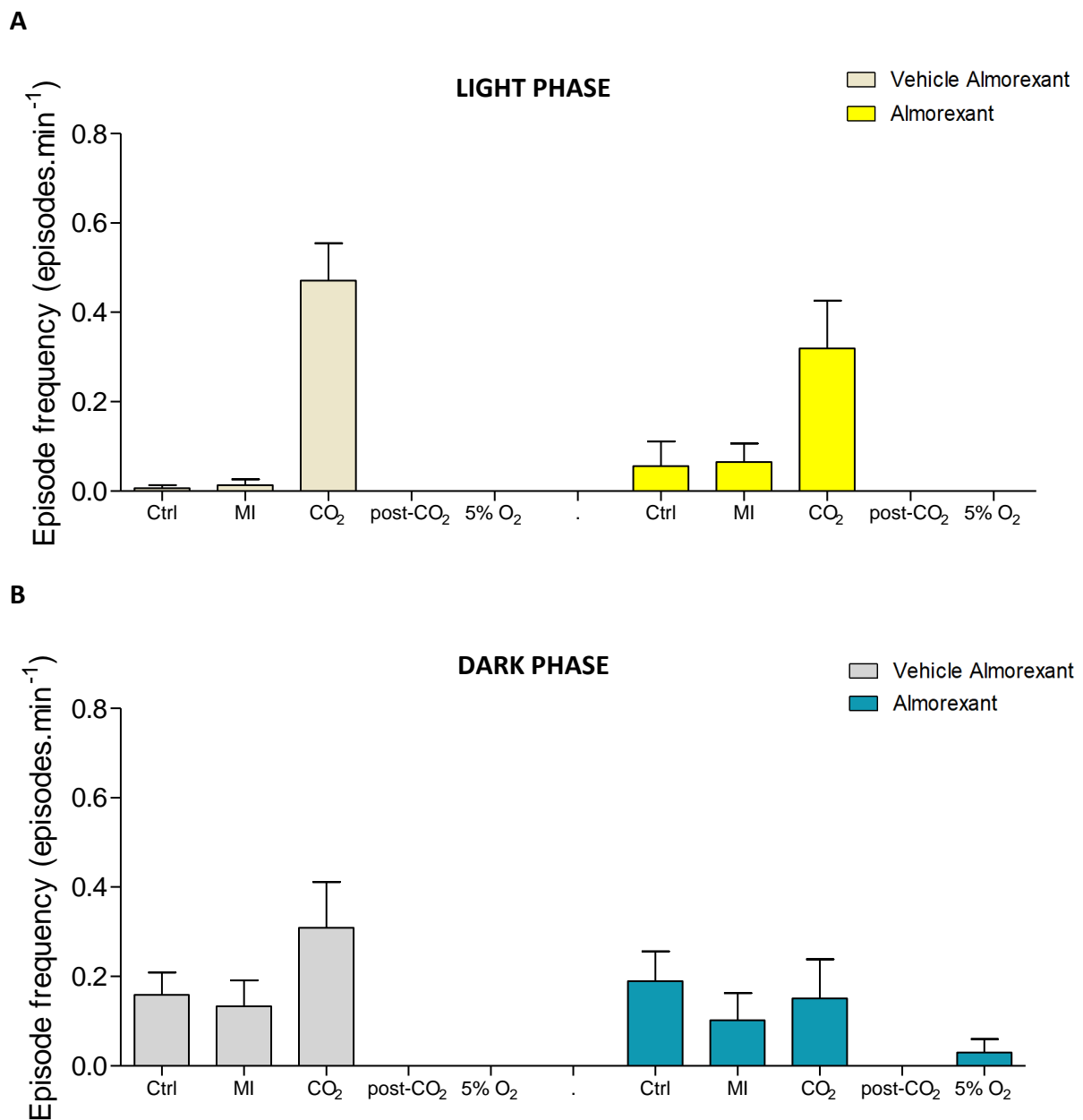
Localization of orexinergic neurons in the green iguana

According to Volkof (2012), the structures of the ORX genes, peptides and receptors appear somewhat conserved among vertebrates, suggesting that their physiological functions might also be similar. Moreover. the neuroanatomical distribution of ORX-neurons appears to be also well conserved, although little species-specific differences exist.

In our study, we found ORX-ir cells within the hypothalamus, more specifically in the periventricular hypothalamic nucleus. We could not see much innervation in the brain, but the staining on the cell bodies are strong and consistent. It is also in accordance to other studies performed in reptiles (Farrell et al., 2003; Dominguez et al., 2010). The antibodies used are made for mammals, just like the other studies of ORX-immunostaining in non-mammalian vertebrates (Farrell et al., 2003; Dominguez et al., 2010).

Only a few studies have examined the distribution of ORXs within the reptilian brain. Studies performed in the lizard *Anolis carolinensis*, in the lizard *Gekko gecko* and in the turtle *Pseudemys scripta elegans* showed ORX-ir neurons in the periventricular and the infundibular hypothalamus and ORX-ir fibers widespread distributed in the whole brain (Farrell et al., 2003; Dominguez et al., 2010). In the gecko and in the turtle studied, ORX innervation is seen in respiratory areas such as the locus coeruleus, the nucleus of the solitary tract and the raphe nuclei (Dominguez et al., 2010) - regions of the brain that have been shown to regulate sleep/wake cycles and breathing in mammals. Despite the lack of direct evidence for a role physiological of ORXs in reptiles, the presence and distribution of ORX-fibers within the reptilian brain might suggest a role for these peptides in functions like energy homeostasis, arousal and breathing, just like in mammals (Volkof, 2012).

Light/dark phase differences in ventilation and breathing pattern

We discerned differences on the respiratory parameters of the green iguanas between the light and dark phases in the present study.

On our findings, ventilation was smaller during the dark phase due to a reduction on the f_R. In the literature, in red-eared sliders, box turtles and garter snakes, nighttime reductions in ventilation under natural conditions were achieved primarily by decreasing breathing frequency, not tidal volume (Reyes and Milsom, 2009; Glass et al., 1979; Hicks and Riedesel, 1983). It contrasts with studies in mammals that show circadian rhythms in tidal volume and breathing frequency (Seifert and Mortola, 2002a; 2002b).

The reduction in breathing frequency was operated by changing the pattern from continuous to breathing in episodes during the night, leading to long non-ventilatory periods. This is consistent with the observation that the duration of the non-ventilatory periods is the primary regulated variable in the breathing pattern of reptiles (Milsom and Jones 1980). These endogenous circadian changes in ventilation and breathing pattern may serve to reduce the costs of breathing and transport (Reyes and Milsom, 2009). Further, according to the same study, day-night differences in ventilation resulted from daily changes in chemoreflex sensitivity, and not due to changes in metabolism.

Orexin and basal respiratory drive

In the current study, the microinjection of ORX antagonists (SB-334867 and Almorexant) during both phases of the diurnal cycle did not promote any changes in ventilation or breathing pattern under room air conditions in *Iguana iguana*.

Studies in mammals have shown that ORX-neurons project directly to respiratory groups in the brainstem and that these groups express ORX-receptors. The injection of ORX

into these areas stimulates respiration (Williams and Burdakov, 2008). In this regard, Zhang et al. (2005) demonstrated that the i.c.v. administration of ORX-A into the lateral ventricle in rats promotes an increase in ventilation due to changes in both tidal volume and respiratory frequency. The i.c.v. administration of SB-334867 in mice does not change ventilation in room air during either wakefulness or sleep (Deng et al. 2007). Stimulation of the lateral hypothalamus (where the ORX-neurons are located) also raises ventilation, and this response is lower in ORX-knockout animals (Kayaba et al., 2003). Shahid et al. (2011) showed an increase in minute ventilation by ORX-A injected into the rVLM (bulbo rostral ventrolateral), and this increase was due to a marked increase in phrenic amplitude. The focal antagonism of OX_1R in the RTN (Dias et al., 2009) or the rostral medullary raphe (Dias et al., 2010) does not influence spontaneous ventilation in rats. Microinjection of ORX-B into the Kolliker-Fuse nucleus in an "*in situ*" perfused brainstem preparation significantly increased the burst rate of phrenic nerve activity.

These results suggest that there is little activity of ORX-neurons at rest and therefore no activation of ORX-receptors, but when the receptors are activated, then we see different effects: or in the tidal volume, or in the respiratory frequency. This may reflect differences in the distribution of ORX-receptors and/or differences in the availability of injected ORX-A among multiple respiratory centers or the role of each area in controlling tidal volume or respiratory frequency.

Our findings, taken with other evidences in the literature, suggest that central ORX does not play a tonic respiratory role but that it may be important in specific situations, such as hypercarbia and hypoxia.

Orexin and CO$_2$ chemoreflex

Hypercarbia is a powerful stimulus for the respiratory control system, especially for terrestrial vertebrates. Central chemoreceptors are an important source of respiratory drive in and they have been established in reptiles (Hitzig and Jackson, 1978; Hitzig and Nattie, 1982; Davies and Sexton, 1987; Branco et al., 1992; Zena et al., 2016). We found that 5% CO$_2$ did not evoke any significant changes on the inspired ventilation in green iguanas during the stimulus, but an increase on the ventilatory response after the exposure. Also, the response was greater during the light phase rather than during the dark phase.

The response to hypercarbia concentrations in reptiles is variable. Apparently, high inspired CO$_2$ concentrations are tolerated well by reptiles and result in only minor increases in ventilation. However, the contributions of respiratory frequency and tidal volume may vary. Snakes and lizards show an increased tidal volume but a depressed respiratory frequency, resulting in a decreased ventilation (Nielsen, 1961; Templeton and Dawson, 1963; Glass and Johansen, 1976; Gratz, 1978). Turtles, on the other hand, increase both frequency and tidal volume in response to high CO$_2$, producing a pronounced hyperventilation (Jackson et al., 1974; Burggren et al., 1977; Glass et al., 1978). Hypercarbia may result in reduced non-ventilatory periods but may or may not increase breathing frequency or tidal volume in reptiles according to Shelton et al. (1986). In a more recent study, Trevisan-Baú et al. (2018) reported that hypercarbia significantly increased ventilation in the turtles *Trachemys scripta* and *Chelonoidis carbonarius*.

Regarding our finding about the post-hypercarbic response, that was greater during the light phase rather than during the dark phase, this agrees with other studies, like the study made by Reyes and Milsom (2009) in red-eared sliders. This study showed a reduced respiratory response to the hypoxic/hypercarbic stimulus at night compared with the day. Time

of the day has been shown to affect the ventilatory response to hypoxia, hypercapnia or both stimuli combined in mammals (Stephenson et al., 2000; Jarsky and Stephenson, 2000; Mortola and Seifert, 2002; Mortola, 2004) and birds (Woodin and Stephenson, 1998).

About the participation of ORX-receptors in the CO_2-chemoreflex, the central microinjection of SB-334867 or Almorexant caused an attenuation of the ventilatory response to the post-CO_2 exposure due to decreases in the f_R for the Almorexant, and in the V_T for the SB-334867. We observed that OX_1R antagonism affected the post-CO_2 response of iguanas only during the light phase, while the antagonism of both receptors by Almorexant promoted an attenuation in both phases. These data suggest that ORXs acting on OX_1R and OX_2R in the CNS are important modulators of the central chemoreflex in green iguanas.

Our data agree with previous data reported for mammals. In this context, prepro-orexin knockout mice (animals with deficiencies in ORXs-A and B synthesis) present a blunted CO_2 respiratory response (Deng et al., 2007) and the central administration of ORX-A or B partially restores the CO_2 ventilatory responses of these animals. Additionally, the i.c.v. administration of SB-334867 decreases the hypercapnic chemoreflex in wild-type mice. In rats, the acute antagonism of both ORX receptors by the systemic administration of Almorexant causes a 26% reduction in the CO_2 ventilatory response, but only during wakefulness in the dark/active phase, which is when the level of ORX in the central nervous system of rats is higher (Li et al., 2010). Additionally, the focal inhibition of OX_1R by the microdialysis of SB-334867 in the medullary raphe region causes a 16% reduction in the CO_2 response during wakefulness, but only during the dark/active phase (Dias et al., 2010). Moreover, SB-334867 promoted an attenuation on the ventilatory response to hypercarbia (5% CO_2) in *Rhinella* toads (that is a nocturnal animal) during dark, but not during the light phase.

The mechanisms by which ORX modulates the hypercapnic chemoreflex are poorly understood, but evidence has suggested that one of the underlying mechanisms may involve the effects of ORXs at chemoreceptor sites like the RTN and the medullary raphe (Dias et al., 2009; Dias et al., 2010) or the LC (Vicente et al., 2016).

Orexin and O_2 chemoreflex

While most animals increase pulmonary ventilation when breathing hypoxic gas mixtures (Dejours, 1975), reptiles show a variety of responses under these conditions. Apparently, most reptiles can tolerate a mild hypoxia. Acute hypoxia usually increases ventilation (Jackson, 1973; Glass and Johansen, 1976; Boyer, 1966), but decreased ventilation is also seen in the literature (Nielsen, 1962). In *Natrix rhombifera*, a species of snake, responded to hypoxia changing tidal volume and ventilatory frequency, but ventilation remained relatively unchanged (Gatz, 1978).

In the present study, we observed the increased pulmonary ventilation of the iguanas during exposure to hypoxia during the light phase, but not during the dark phase. Differences surrounding central and peripheral-chemosensing were already addressed above (Reyes and Milsom, 2008, Stephenson et al., 2000; Jarsky and Stephenson, 2000; Mortola and Seifert, 2002; Mortola, 2004) and birds (Woodin and Stephenson, 1998). In addition, the breathing pattern during hypoxia remained unchanged during vehicle or antagonists.

According to Shelton et al. (1986), the usual response to hypoxia is reducing the non-ventilatory periods and may or may not increase breathing frequency or tidal volume. Interestingly, Altland and Parker (1955) found a more episodic breathing pattern in *Terrapene carolina carolina* (a species of turtle) under normoxia that changed to a more regular breathing pattern with single breaths under hypoxic conditions.

The injection of SB-334867 or Almorexant has not caused an effect on the ventilatory response to hypoxia in our study, either during the light or the dark phase. Our results agree with the studies performed in mammals, in which the i.c.v. administration of ORX or OX_1R antagonist have no effect on the ventilatory response to hypoxia (Deng et al., 2007). Nakamura et al. (2007) have demonstrated that the response of prepro-orexin knockout mice to hypoxia does not differ compared to that of wild-type mice. These data suggest that ORX is not involved in the peripheral chemoreflex in mammals. In contrast to the results obtained in rats and mice, humans with narcolepsy–cataplexy and hypocretin deficiency as determined by a gene marker show depressed hypoxic responsiveness (Han et al., 2010); and toads from the genus *Rhinella* presented an attenuated ventilatory response to hypoxia during the light, but not during the dark phase, when microinjected with SB-334867 (Fonseca et al., 2016).

In summary. our results demonstrate that: (1) ORXs - acting on OX_1Rs and OX_2R -contribute to the hypercarbic but not to the hypoxic chemoreflexes in green iguanas; (2) these animals are diurnal and have higher ORX-A levels during early morning; (3) ORX-neurons are located in the periventricular hypothalamic nucleus in *Iguana iguana*. The present observations, taken together with other studies, indicate a considerable degree of phylogenetic conservation of the orexinergic pathway among vertebrates.

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