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Orexin in the toad *Rhinella schneideri*: The location of orexinergic neurons and the role of orexin in ventilatory responses to hypercarbia and hypoxia☆

Elisa M. Fonseca[a], Mirela B. Dias[b], Kênia C. Bícego[a], Luciane H. Gargaglioni[a,*]

[a] Department of Animal Morphology and Physiology, Sao Paulo State University-UNESP FCAV at Jaboticabal, SP, Brazil
[b] Department of Physiology, Institute of Bioscience, Sao Paulo State University-UNESP, Botucatu, SP, Brazil

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ABSTRACT

Recent reports have suggested that orexins, also known as hypocretins, play an important role in the modulation of respiratory control in mammals, but there are no data available describing the role of the orexinergic system in the peripheral and central chemoreception of non-mammalian vertebrates. Therefore, the present study was designed to examine the localization of orexin-immunoreactive neurons in the brain of toads (*Rhinella schneideri*) and to investigate the contribution of orexin receptor-1 (OX_1R) to the hypoxic and hypercarbic ventilatory responses of these animals during light and dark phases. Our results demonstrated that the orexinergic neurons of *R. schneideri* are located in the suprachiasmatic nucleus of the diencephalon. Additionally, the intracerebroventricular injection of SB-334867 (OX_1R selective antagonist) attenuated the ventilatory response to hypercarbia during the dark phase by acting on tidal volume and breathing frequency, while during the light phase, SB-334867 attenuated the ventilatory response to hypoxia by acting on tidal volume only. We conclude that in the toad *R. schneideri*, orexinergic neurons are located in the suprachiasmatic nucleus and that OX_1R contributes to hypercarbic and hypoxic chemoreflexes.

1. Introduction

Orexins, also known as hypocretins, are small neuropeptides released from hypothalamic neurons with widespread projections throughout the central nervous system. There are two subtypes of these neuropeptides, orexin A and orexin B (hypocretin 1 and hypocretin 2, respectively), which are both cleaved from the same precursor, prepro-orexin (Sakurai et al., 1998; Willie et al., 2001), and bind to two G-protein coupled receptors, orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R) (Smart et al., 1999, 2001). OX_1R is highly selective for orexin A, while OX_2R is nonselective for either orexin (de Lecea et al., 1998; Sakurai et al., 1998).

Interestingly, the amino acid sequences of orexins A and B are highly conserved across different groups of vertebrates (Alvarez and Sutcliffe, 2002). Orexin distribution has been described in all classes of vertebrates, and in most groups, orexinergic neurons are located in the hypothalamus, but they are not found exclusively within a single nucleus, they are located in various hypothalamic nuclei. Additionally, OX fibers are widespread, innervating largely similar areas. In amphibians, Singletary et al. (2005) and Galas et al. (2001) observed a single population of orexinergic neurons in the suprachiasmatic nucleus in *Hyla cinerea* and *Pelophylax ridibundus*, respectively, while Shibahara et al. (1999) demonstrated that orexin-containing neurons are localized to the ventral hypothalamus in *Xenopus*.

Orexin is known to be related to several physiological processes, such as sleep–wakefulness, feeding behavior, energy homeostasis, nociception, metabolism, the reward system and hormonal secretion (de Lecea et al., 1998; Sakurai et al., 1998). It has been shown that the orexinergic system also modulates cardiovascular and respiratory functions in rodents – rats and mice – as reviewed by Nattie and Li (2012). With regard to central chemoreflex, recent data have shown that orexinergic neurons are profoundly affected by changes in CO_2 and pH as demonstrated by *in vitro* (Williams

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* Corresponding author at: Via de acesso Paulo Donato Castellane s/n, 14870-000, Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, SP, Brazil. Tel.: +55 16 32092656; fax: +55 16 32024275.
E-mail addresses: lucihel@fcav.unesp.br, lucihel@yahoo.com.br (L.H. Gargaglioni).

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et al., 2007) and *in vivo* experiments (Sunanaga et al., 2009), which suggests that they are intrinsically chemosensitive.

Moreover, prepro-orexin-knockout mice present an attenuation of the hypercapnic chemoreflex, and this effect is partially restored by the administration of orexins A and B (Deng et al., 2007; Nakamura et al., 2007). Additionally, the intracerebroventricular injection of an OX_1R-selective antagonist (SB-334867) decreases the respiratory chemoreflex in mice (Deng et al., 2007). Functional studies in rats have suggested that the role of orexin in the CO_2 chemoreflex may be vigilance state dependent with its major effect occurring during wakefulness in the dark-active phase. The antagonism of both OX_1R and OX_2R by the oral administration of almorexant significantly decreases the hypercapnic chemoreflex only in wakefulness during the dark-active phase (Li and Nattie, 2010). Furthermore, the local acidification of the hypothalamic region in rats, which is where orexinergic neurons are located, results in increased ventilation during wakefulness but not during sleep (Li et al., 2013a,b). Finally, in conscious rats, the focal dialysis of an OX_1R antagonist at two central chemoreceptor sites, the retrotrapezoid nucleus (RTN) and the medullary raphe, results in a decreased hypercapnic ventilatory response, predominately occurring during wakefulness (Dias et al., 2009, 2010).

The vigilance state dependency of the role of orexin on the hypercapnic chemoreflex is in accordance with the fact that orexin levels, which have been measured in the cerebrospinal fluid (CSF) 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 orexinergic neurons is synchronized according to arousal states, with the highest activity taking place during active arousal (Lee et al., 2005; Mileykovskiy et al., 2005).

There are no reports of the role of orexinergic neurotransmission in central and peripheral chemoreception in amphibians. Additionally, the localization of orexinergic neurons in *Rhinella schneideri* toads has not been evaluated. Based on evidence in mammals, we hypothesized that the orexin receptor OX_1R is involved in the CO_2, but not hypoxic, chemoreflex and that the orexinergic neurons are located within the hypothalamus in toads. Therefore, we examined the localization of orexin-immunoreactive neurons in the brain of the toad *R. schneideri* and monitored ventilation and blood gases in non-anaesthetized animals exposed to normocarbic normoxia, hypercarbia and hypoxia after i.c.v. microinjections of OX_1R antagonist (SB-334867; 5 and 10 mM) during the light and dark phases.

2. Materials and methods

2.1. Animals

Fifty *R. schneideri* (Werner, 1894) toads of either sex weighing 182.1 ± 14.9 g were collected from the vicinity of the city of Jaboticabal, São Paulo, Brazil, during the rainy summer months. These animals were captured and transported in agreement with SISBIO-ICMBio (animal license 28160-1). In the laboratory the toads were maintained at 25 °C in containers with free access to water and a basking area under a 12 h light/12 h dark cycle for at least two weeks before experimentation. The animals were fed commercial carnivorous fish food, crickets or Tenebrio larvae two to three times per week. Each toad was used in only one experiment, and all experiments were performed between 8:00 AM and 5:00 PM during the light phase (inactive) or between 7:00 PM and 2:00 AM during the dark phase (active). This study was conducted in compliance with the guidelines of the Brazilian College of Animal Experimentation (COBEA) and in compliance with the approval of the local Animal Care and Use Committee (006731/12).

2.2. Surgical procedures

Animals were anesthetized in an aqueous solution of 3-aminobenzoic acid ethyl ester (MS-222; 0.3% Sigma, St. Louis, MO, USA) buffered to pH 7.8 with sodium bicarbonate. The heads of the animals were then fixed in a David Kopf stereotaxic apparatus (Model 900 Small Animal Stereotaxic, Tujunga, CA, 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 14 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 stereotaxic atlas for the toad *R. schneideri* (Hoffmann, 1973). 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 stainless-steel screws and acrylic cement. A tight-fitting stylet was kept inside the cannula to prevent occlusion and infection. The experiments were initiated at 7 days after brain surgery. One day before the experiments, a PE-50 catheter (Clay Adams, Parsippany, NJ, USA) was inserted into the iliac artery under MS-222 anesthesia for the determination of pH, HCO_3^- and blood gases. The catheter was exteriorized so blood could be collected. After stereotaxic surgery, the toads were treated with one subcutaneous injection of both prophylactic antibiotic (Enrofloxacin, Flotril®; Schering-Plough, 5.0 mg/kg, s.c.) and analgesic (Flunixin ms®; Schering-Plough, 1.0 mg/kg, s.c.) agents according to recommended doses for amphibians (Gentz, 2007; Smith, 2007).

2.3. Intracerebroventricular microinjections

Microinjections were performed according to a previous study of amphibians (Zena et al., 2013). Briefly, a dental needle (Mizzy, Inc., Cherry Hill, NJ, USA; 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 45 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 submergence in an aqueous 0.025% solution of benzocaine hydrochloride buffered to pH 7.7 with sodium bicarbonate (AVMA, 2013). Upon dissection, we observed that the dye had diffused into the periventricular tissue and spread throughout the ventricular system.

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 mock cerebral spinal fluid (mCSF, pH 7.8 at 25 °C (Branco et al., 1992) in mEq/L: 56.6 NaCl, 2.7 KCl, 0.9 $CaCl_2$, 0.45 $MgSO_4$, and 27.0 $NaHCO_3$). For the vehicle, we used a solution containing 4% DMSO and 35% (2-hydroxypropyl)-β-cyclodextrin in mCSF. The dose and method of dissolving the drug were chosen on the basis of pilot experiments and previous studies (Deng et al., 2007; Dias et al., 2009).

2.4. Ventilation measurements

Pulmonary expired ventilation ($\dot{V}_E$), tidal volume (V_T) and breathing frequency (fR) 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's snout, allowing for inspirations and expirations to be measured continuously. Inspiratory and expiratory gas flows were monitored with