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Raphe Pallidus is Not Important to Central Chemoreception in a Rat Model of Parkinson's Disease

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Abstract—Central chemoreceptors are primarily sensitive to changes in CO_2/H^+, and such changes lead to intense breathing activity. Medullary raphe and retrotrapezoid nucleus (RTN) neurons are candidates for central chemoreceptors because they are unusually pH sensitive. The pathophysiology of Parkinson's disease (PD) is related to the reduction of neurons in the substantia nigra pars compacta (SNpc) that express dopamine, although other neurons can also be degenerated in this pathology. In rodent models of PD, we showed an impairment of the hypercapnia ventilatory response due to a reduction in the number of RTN chemosensitive neurons. Here, we aimed to investigate if serotonine-expressing neurons in the Raphe pallidus/parapyramidal region (RPa/PPy) are also involved in the modulation of breathing during central chemoreception activation in a PD animal model. PD was induced in male Wistar rats with bilateral injection of 6-OHDA (6-hydroxydopamine; 24 μg/μl) into the striatum, which leads to a reduction in the catecholaminergic neurons of the SNpc by 89%. In PD animals, we noticed a reduction in the number of RPa neurons that project to the RTN, without a change in the number of hypercapnia-activated (7% CO_2) raphe neurons. The PD animals that received injection of the toxin saporin anti-SERT into the RPA/PPy region did not show a further reduction of respiratory frequency (f_R) or ventilation (V_E) at rest or during hypercapnia challenge. These experiments demonstrate that serotonergic neurons of RPa/PPy are not involved in the breathing responses induced by central chemoreceptor activation in a PD animal model. © 2017 Published by Elsevier Ltd on behalf of IBRO.

Key words: central chemoreceptors, medullary raphe, Parkinson's disease, serotonin.

INTRODUCTION

Central chemoreception is considered a mechanism responsible for stimulating breathing by increases in CO_2/H^+. A few specialized groups of cells located within the retrotrapezoid nucleus (RTN), the locus coeruleus, nucleus of the solitary tract (NTS) and medullary raphe as well as hypothalamic orexinergic neurons are considered the primary sensors (Mulkey et al., 2004; Richerson, 2004; Takakura et al., 2006; Biancardi et al., 2008; Abbott et al., 2009; Dias et al., 2009; Nattie and Li, 2009).

The serotonergic raphe neurons contribute to thermoregulatory control mechanisms. In addition, these neurons are able to modulate the respiratory network, including central chemoreception activity (Morrison, 2004; Richerson, 2004; Madden and Morrison, 2006; Hodges and Richerson, 2008). Previous studies have addressed the importance of medullary raphe that includes Raphe Magnus (RMg), Raphe Obscurus (ROb) and Raphe Pallidus/parapyramidal (RPa/PPy) to central chemoreception (Veasey et al., 1995; Wang et al., 2001; Hodges and Richerson, 2008; da Silva et al., 2013; Hawkins et al., 2014). The involvement of RMg and ROb in central chemoreception was already demonstrated in conscious animals (Dias et al., 2007; da Silva et al., 2011; Depuy et al., 2011). However, the exact role of the serotonergic RPa neurons to chemosensory control of breathing remains unclear, especially under conscious state conditions.

Parkinson's disease (PD) is a chronic and progressive movement disorder, meaning that symptoms continue and worsen over time. The disease is characterized anatomically by significant loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leading to non-motor symptoms such as neuropsychiatric, sleep and breathing disorders (Lee and Trojanowski, 2006; McDowell and Chesselet, 2012). Among the respiratory disorders, PD patients have been described to experience an increased risk of obstructive sleep apnea relative to the general population (Harmell et al., 2016; Selvaraj and Keshavamurthy, 2016). Others have reported the

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involvement of neurodegeneration in important medullary regions that control breathing and the loss of respiratory function. Experiments performed in *postmortem* human tissues and rat brains with PD showed a reduction in neurokinin-1 receptor (NK1-r) expression in the pre-Bötzinger complex (preBötC), an important brainstem region responsible for inspiratory activity (Rioux and Joyce, 1993; Benarroch et al., 2003; Tuppy et al., 2015). In addition, in the 6-hydroxydopamine animal model (6-OHDA-lesioned) of PD, a reduction of respiratory frequency at rest and during hypercapnia and a reduction in the number of the chemically-coded (i.e. phox2b) neurons of the RTN was also observed (Tuppy et al., 2015).

Previously, it was demonstrated that the integrity of aminergic neurons could contribute to the severity of motor symptoms in animal models of PD. Data from human studies have shown that striatal levels of serotonin were reduced in the brainstems of PD patients (Sarrias et al., 1990), and several hypotheses have been put forward to explain and prove the role of aminergic neurons in the pathogenesis of PD. Some studies have already shown that those neurons degenerate as part of the natural course of the disease in humans (Zarow et al., 2003; Reed et al., 2007; Del Tredici and Braak, 2012a,b) but not in animal models; the degeneration appears to occur through an unknown mechanism of aminergic protection (Fulceri et al., 2006; Natale et al., 2010). For all of the reasons, here we addressed the question if serotonergic neurons of the RPa will be involved in the chemosensory control of breathing in sham and an animal model of PD.

EXPERIMENTAL PROCEDURES

Animals

Experiments procedures and animal handling were conducted in 49 adult male Wistar rats (250–350 g at the time of experimentation). All experimental and surgical procedures conformed to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at the University of São Paulo (protocol number: 65/2015).

Injections of toxins into the striatum and raphe pallidus

The injections of 6-OHDA (Sigma, Saint Louis, MO, USA), saporin anti-SERT-SAP (Advanced Targeting Systems, San Diego, CA, 10 μg/μl) or vehicle (Saline, 0.9%) were performed while the rats were anesthetized with intraperitoneal (i.p.) administration of ketamine (Davol, São Paulo, SP, Brazil; 100 mg/kg) and xylazine (7 mg/kg). The rats were adapted in a stereotaxic apparatus (model 1760, David Kopf Instruments). Surgery was conducted using aseptic methods.

For chemical lesions of the SNpc, two injections of 6-OHDA (24 μg/μL; 0.5 μl, bilaterally) or vehicle (1 μg ascorbic acid in 1 μL of saline) were made into the caudate putamen region (CPu) at the following coordinates: (i) 0.5 mm rostral from bregma, ±2.7 mm lateral from the midline, 4.5 mm below the skull surface; and (ii) 0.0 mm from bregma, 2.7 mm lateral to the midline and 4.5 mm below the skull surface. The injections were made using pipettes with an external tip coupled to a Hamilton syringe (10 μl).

For chemical lesions of the serotonin-expressing neurons of the raphe pallidus (RPa), one injection of saporin anti-SERT-SAP (10 μg/μl) or saline was made using the following coordinates: 2.3 mm caudal from lambda, 0 mm from the midline and 8.8 mm below the skull surface. The treatments were pressure injected (Picospritzer III, Parker Hannifin) (30 nl over 3 s) through single-barrel glass pipettes (20 μm tip diameter).

After all surgeries, the animals received an injection of the antibiotic ampicillin (100 mg/kg, intramuscularly, i.m.) and the analgesic ketorolac (0.6 mg/kg, subcutaneously, s.c.). The toxin did not produce observable behavioral effects.

Physiological experiments

Breathing variables of conscious rats were measured by whole-body plethysmography method (EMKA Technologies) as previously described (Malheiros-Lima et al., 2017). The plethysmography chamber was continuously flushed with 1.5 L/min of 21% O_2 balanced with N_2 regulated by computer-driven mass flow controllers for gases (Alicat Scientific, Inc., Tucson, AZ, USA). Ambient temperature and humidified were also kept constant (23–25 °C ambient room temperature, 60–70% relative humidity, ±0.5 °C; ±5% relative humidity). The flow controllers were adjusted to 21% O_2 balanced with N_2 in normoxia and to 7% CO_2, 21% O_2 and 72% N_2 in hypercapnia.

Rectal temperature was used as a core body temperature index and was measured twice: before and at the end of the experiments. The calibration for volume was obtained by waveforms generated during each experiment by injecting the animal chamber with 20 ml of dry air, as calculated using Spike software version 7.3 (Cambridge Electronics). The ventilatory parameters measured by the plethysmography system were tidal volume (V_T, ml/kg of body weight; area under the curve during the inspiratory period), respiratory frequency (f_R, breaths/min), minute ventilation (V_E, ml/min/kg of body weight), inspiratory time (ms), and expiratory time (ms). Ventilation was calculated as the product of f_R and V_T ($V_E = f_R \times V_T$, ml/min/kg of body weight).

Hypercapnia-activated neurons

Another group of PD rats was exposed for 3 h to normoxia/normocapnia or hypercapnia breathing mixture (7% CO_2, 21% O_2, balanced with N_2) 40 or 60 days after PD induction as previously described (Silva et al., 2016a; Totola et al., 2016; Oliveira et al., 2017). After exposure to hypercapnia, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p) and perfusion-fixed.

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