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Research paper

The involvement of the pathway connecting the substantia nigra, the periaqueductal gray matter and the retrotrapezoid nucleus in breathing control in a rat model of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is characterized by a reduction in the number of dopaminergic neurons of the substantia nigra (SNpc), accompanied by motor and non-motor deficiencies such as respiratory failure. Here, our aim was to investigate possible neuronal communications between the SNpc and chemoreceptor neurons within the retrotrapezoid nucleus (RTN), in order to explain neurodegeneration and the loss of breathing function in the 6-OHDA PD animal model. Male Wistar rats received tracer injections in the SNpc, RTN and periaqueductal gray (PAG) regions to investigate the projections between those regions. The results showed that neurons of the SNpc project to the RTN by an indirect pathway that goes through the PAG region. In different groups of rats, reductions in the density of neuronal markers (NeuN) and the number of catecholaminergic varicosities in PAG, as well as reductions in the number of CO_2-activated PAG neurons with RTN projections, were observed in a 6-OHDA model of PD. Physiological experiments showed that inhibition of the PAG by bilateral injection of muscimol did not produce resting breathing disturbances but instead reduced genioglossus (GG_{EMG}) and abdominal (Abd_{EMG}) muscle activity amplitude induced by hypercapnia in control rats that were urethane-anesthetized, vagotomized, and artificially ventilated. However, in a model of PD, we found reductions in resting diaphragm muscle activity (Dia_{EMG}) and GG_{EMG} frequencies, as well as in hypercapnia-induced Dia_{EMG}, GG_{EMG} and Abd_{EMG} frequencies and GG_{EMG} and Abd_{EMG} amplitudes. Therefore, we can conclude that there is an indirect pathway between neurons of the SNpc and RTN that goes through the PAG and that there is a defect of this pathway in an animal model of PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is characterized by tremor, rigidity, bradykinesia, and gait impairment (McDowell and Chesselet, 2012). The main pathological feature of PD is the loss of dopaminergic neurons, which occurs primarily in the substantia nigra pars compacta (SNpc) (Corti et al., 2009; Fulceri et al., 2006; Lees et al., 2009). Some evidence from studies using PD animal models or patients with PD indicates that breathing disorders are present in the disease and can be associated with neurodegeneration of important neurons responsible for breathing control (Tuppy et al., 2015; Zhang et al., 2016).

A massive reduction in the number of neurons expressing the transcription factor PHOX2B in the retrotrapezoid nucleus (RTN) was observed in animals that received bilateral injections of 6-hydroxydopamine into the striatum (Tuppy et al., 2015). In those animals, reductions in resting and hypercapnic-induced respiratory frequencies were also observed (Tuppy et al., 2015). The RTN contributes to the central chemoreflex, which is a reflex characterized by activation of breathing by the elevation of the partial pressure of CO_2 (PCO_2) in the central nervous system (Feldman et al., 2003; Nattie and Li, 2009). RTN neurons are a group of glutamatergic interneurons that express the transcription factor PHOX2B and lack tyrosine hydroxylase (Amiel et al., 2003; Mulkey et al., 2007b; Stornetta et al., 2006; Weese-Mayer et al., 2005).

Based on the description above, an important open question is why there is a massive reduction of medullary neurons such as those of the RTN region in an experimental model of PD. The main aim of this study was to investigate whether there is a projection between dopaminergic neurons of the SNpc and RTN and whether there is an association

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between reduction in the number of those projections and neurodegeneration of RTN neurons since there is no evidence in the literature about SNpc-neurons projecting to medullary regions.

2. Methods

2.1. Animals

Experiments were performed in 84 adult male Wistar rats weighing between 250 and 300 g. The animals had free access to water and food and were housed in a temperature-controlled chamber at 24 °C, with a 12:12 h light/dark cycle. All experimental and surgical procedures conformed to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of São Paulo (protocol numbers: 179/2012 and 99/2014).

2.2. Surgery and anesthesia

2.2.1. Injections of 6-OHDA

For chemical lesions of SNpc, two injections of 6-OHDA (24 μg/μL; 0.5 μL, bilaterally) (6-hydroxydopamine hydrochloride, Sigma, Saint Louis, MO, USA) or vehicle (1 μg ascorbic acid in 1 μL of saline 0.9%) were made into the Caudate Putamen region (CPu) using the following coordinates: i) 0.5 mm caudal to the bregma; 3.2 mm lateral to the midline and 4.5 mm below the skull surface and ii) 0.0 mm caudal to the bregma; 2.7 mm lateral to the midline and 4.5 mm below the skull surface. All injections were performed using pipettes with an external tip coupled to a Hamilton's syringe. The dose of 6-OHDA used in the present study was selected based on the literature and previous experiments from our laboratory (Blandini et al., 2008; Falquetto et al., 2017; Oliveira et al., 2017; Tuppy et al., 2015).

After all surgeries, the rats were treated with the antibiotic ampicillin (100 mg/kg intramuscularly), and the analgesic ketorolac (0.6 mg/kg subcutaneously). The toxin did not produce observable behavioral effects.

2.2.2. Anatomical experiments

Retrograde and anterograde tracer injections were made while the rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (7 mg/kg) administered intraperitoneally (i.p.). The animals were placed in a stereotaxic frame (Model 1760, David Kopf Instruments) and the surgery used standard aseptic methods. After surgery, the rats were treated with the antibiotic ampicillin [100 mg/kg, intramuscular (i.m.)] and the analgesic ketorolac [0.6 mg/kg, subcutaneous (s.c.)]. Different groups of four rats each received injections of one of the following: i) 2% FluorGold (FG, Fluorochrome, Inc., Englewood, USA) in sterile saline in the left RTN, to retrogradely label neurons that innervate RTN in three groups of animals: naïve, vehicle into CPu, and 6-OHDA into CPu; ii) anterograde tracer biotinylated dextran amine (BDA; lysine-fixable, MW 10000; 10% *w/v* in 10 mM phosphate buffer, pH 7.4; Molecular Probes, Eugene, OR, USA) into the SNpc, to anterogradely label varicosities from the SNpc; iii) 2% FG in sterile saline in the left PAG, to retrogradely label neurons that innervate PAG and iv) BDA into the PAG, to anterogradely label varicosities from PAG. The tracers were pressure injected (30 nL in 5 s) unilaterally through single-barreled glass pipettes (20 μm tip diameter). The coordinates to reach the RTN were 8.8 mm below the dorsal surface of the cerebellum, 1.7–1.8 mm lateral to the midline and 2.6–2.8 mm caudal to the lambda. The coordinates to reach PAG were 5.3 mm below the dorsal surface of the cerebellum, 0.6 mm lateral to the midline and 6.8 mm caudal to the lambda. The coordinates for the SNpc were 7.8 mm below the dorsal surface of the cerebellum, 2.3 mm lateral to the midline and 5.3 mm caudal to the lambda. All those coordinates were adjusted to reach the region of interest after Paxinos and Watson (Paxinos and Watson, 1998). Seven to ten days following tracer application, the rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and immediately transcardially perfused with fixative.

2.3. Physiological experiments

2.3.1. Awake animals

Measurements of pulmonary ventilation (V_E) were performed using the whole-body plethysmography method (EMKA Technologies). Freely moving rats were housed in a 5-L plethysmography chamber with room air for 45–60 min before starting to record the ventilatory parameters. The plethysmography chamber was continuously flushed at a rate of 1.5 L/min, regulated by computer-driven mass flow controllers for O_2, N_2, and CO_2 (Alicat Scientific, Inc., Tucson, AZ, USA). The flow controllers were adjusted to 21% O_2 balanced with N_2 for normoxic conditions, and 7% CO_2, 21% O_2 and 72% N_2 in hypercapnic conditions. Hypercapnia was induced by titrating CO_2 into the respiratory mixture up to a level of 7% for 15 min. Ventilatory parameter measurements were made during the last 2 min before exposure to the stimulus and during the 2 min period at the end of hypercapnic stimulus, when breathing stabilized. Ambient temperature (23–25 °C) and humidity (60–70%) were continuously recorded inside the plethysmography chamber and used to calculate the tidal volume (V_T). V_T was calculated using the formula described by Malan (1973) and used in previous studies from our laboratory (Tuppy et al., 2015; Oliveira et al., 2017). Rectal temperature was used as a core body temperature index. The ventilatory parameters measured by the plethysmography system were V_T (ml/kg), respiratory frequency (f_R, bpm) and V_E (ml/min/kg).

2.3.2. Anesthetized animals

Surgical procedures and experimental protocols were similar to those described previously (Takakura et al., 2011; Takakura and Moreira, 2011; Takakura et al., 2006). Briefly, general anesthesia was induced with inhalation of 5% isoflurane in 100% oxygen. The rats received a tracheostomy, femoral vein cannulation for the administration of fluids and drugs, and removal of the occipital plate to insert a recording electrode into the medulla oblongata via a dorsal transcerebellar approach. Artificial ventilation with 1.5% isoflurane in 100% oxygen was maintained throughout the surgery. A bilateral vagotomy was performed distal to the carotid bifurcation, as described previously (Guyenet et al., 2005). Bipolar electrodes were coupled to record the activity of the diaphragm (Dia_{EMG}), genioglossus (GG_{EMG}) and abdominal (Abd_{EMG}) muscles. The rats were ventilated with 100% oxygen throughout the experiment. Rectal temperature (maintained at 37 °C) was also monitored throughout the experiment.

On completion of surgical procedures, isoflurane was gradually replaced with urethane (1.2 g/kg i.v. over 30 min). After injection of the intravenous anesthetic, the anesthesia level was monitored by testing for the absence of a withdrawal response and a lack of AP changes upon a firm paw pinch. Activation of the peripheral chemoreflex was achieved by intravenous bolus injections of potassium cyanide (KCN; 40 μg/kg i.v.), and activation of the central chemoreflex was done via hypercapnia (10% of CO_2).

All analog data (end-expiratory CO_2, EMG activities and AP) were stored on a computer via a micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed using version 5 of the Spike 2 software (CED). Integrated electromyography activity ($\int$ EMG) was obtained after rectification and smoothing (τ = 0.003 s) (Mulkey et al., 2007a; Oliveira et al., 2016; Takakura et al., 2014).

2.3.3. Drugs and intraparenchymal injections

Muscimol, an agonist for γ-aminobutyric acid A (GABA-A) receptors, was diluted in 2 mM of sterile saline (pH 7.4) and was pressure injected (Picospritzer III, Parker Hannifin Corp, USA) (30 nL in 3 s) through single-barrel glass pipettes (20 μm tip diameter). Injections into the PAG were made 5.3 mm below the dorsal surface of the cerebellum, 0.6 mm lateral to the midline and 6.8 mm caudal to the