

Effect of 6-OHDA on hypercapnic ventilatory response in the rat model of Parkinson's disease

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Hypercapnic ventilatory response in Parkinson's disease model

Summary

Breathing impairments, such as an alteration in breathing pattern, dyspnoea, and sleep apnoea, are common health deficits recognised in Parkinson's disease (PD). The mechanism that underlies these disturbances, however, remains unclear. We investigated the effect of the unilateral damage to the rat nigrostriatal pathway on the central ventilatory response to hypercapnia, evoked by administering 6-hydroxydopamine (6-OHDA) into the right medial forebrain bundle (MFB).

The respiratory experiments were carried out in conscious animals in the plethysmography chamber. The ventilatory parameters were studied in normocapnic and hyperoxic hypercapnia before and 14 days after the neurotoxin injection.

Lesion with the 6-OHDA produced an increased tidal volume during normoxia. The magnified response of tidal volume and a decrease of breathing frequency to hypercapnia were observed in comparison to the pre-lesion and sham controls. Changes in both respiratory parameters resulted in an increase of minute ventilation of the response to CO₂ by 28% in comparison to the pre-lesion state at 60 s.

Our results demonstrate that rats with implemented unilateral PD model presented an altered respiratory pattern most often during a ventilatory response to hypercapnia. Preserved noradrenaline and specific changes in dopamine and serotonin characteristic for this model could be responsible for the pattern of breathing observed during hypercapnia.

Keywords: Parkinson's disease, 6-OHDA, rat model, hypercapnia, respiratory pattern

Introduction

The respiratory response to hypercapnia is primarily regulated by sensory feedback from central respiratory chemoreceptors and, to a lesser extent, from peripheral chemoreceptors. Only one-third of the response in the normoxic condition occurs via stimulation of the peripheral carotid body chemoreceptors (Forster *et al.* 2008; Teran *et al.* 2010).

The respiratory chemoreflex modulates ventilation in response to deviations in arterial and brain PaCO₂, mainly through the detection of changes in pH (Guyenet *et al.* 2010). CO₂ in physiological conditions is rapidly hydrated to carbonic acid and dissociates to bicarbonate and proton, which immediately entails an increase in H⁺ ion concentration and a decrease in pH (Nattie, 1999). In the central nervous system, hypercapnia reduces the pH of brain extracellular fluid, producing an increase in minute ventilation to maintain normal CO₂ levels in the blood (Guyenet *et al.* 2010; Nattie and Li, 2009). Central chemosensors located in the brainstem and several chemosensitive areas connected to the respiratory network are considered to play a role in CO₂ chemoreception: medullary raphe serotonergic neurons, noradrenergic neurons of locus coeruleus (LC), nucleus of the solitary tract (NTS) and the neurons of retrotrapezoid nucleus (RTN) (Biancardi *et al.* 2008; Dean *et al.* 1989; Erlichman *et al.* 2009; Guyenet and Bayliss, 2015; Kumar *et al.* 2015; Nattie and Li, 2012; Richerson, 2004; Ruffault *et al.* 2015; Teran *et al.* 2010).

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. The main pathological characteristic of PD is dopaminergic cell death in substantia nigra pars compacta (SNpc) and degeneration of other cell groups like noradrenergic and serotonergic neurons (Huot *et al.* 2011; Titova *et al.* 2016; Zarow *et al.* 2003). PD is a progressive disease whose incidence increases with age (Dauer and Przedborski, 2003; Lees *et al.* 2009). The disorder is manifested by motor and non-motor deficits. The main motor symptoms, such as

tremor, muscle rigidity, akinesia and postural instability are due to degeneration of the basal ganglia, controlling the motor system. Non-motor symptoms include deficits in cognition and memory and a disordered emotional state.

Breathing impairments, like alteration in breathing pattern, tachypnoea, dyspnoea, decreased respiratory pressure and sleep apnoea are common health deficits recognised in PD patients (Arnulf *et al.* 2002; Frazao *et al.* 2014; Guedes *et al.* 2012; Kumar *et al.* 2007). Disturbed ventilation and chronic hypoxia or hypercapnic episodes may worsen the quality of life and increase general morbidity. Studies on respiration in PD are scarce and the results are inconclusive. There are very few data describing ventilatory impairment and the decreased ability to generate stimulatory response to hypercapnic stimulus in PD patients (Onodera *et al.* 2000; Serebrovskaya *et al.* 1998); further, the results are confounding. Seccombe *et al.* (2011) observed reduction, Onodera *et al.* (2000) normal and Feinsilver *et al.* (1986) increased reaction to increased PaCO₂.

In the bilateral model of PD, 6-hydroxydopamine (6-OHDA) administration into the rat striatum diminished response to normoxic hypercapnia, which was restored to control value after 60 days post-lesion, was described (Tuppy *et al.* 2015; Oliveira *et al.* 2017, 2018; Lima *et al.* 2018). The mechanism underlying ventilatory impairment in hypercapnic response and the question of whether central or peripheral chemoreceptors participated in the effect remain unresolved.

We hypothesise that the respiratory effects obtained in different PD models may vary and can strictly depend on the experimental conditions applied. The current study was focused on analysing disturbances in the central chemoreception in a unilateral model of PD induced with the administration of 6-OHDA to the medial forebrain bundle (MFB), imitating the early stage of Parkinson's disease. The animals were pre-treated with desipramine to preserve noradrenergic neurons and to make lesion more selective. Hyperoxic hypercapnia was applied

to attenuate activity of the carotid body chemoreceptors and generate only the central respiratory response to CO₂ stimulus (Fitzgerald and Dehghani, 1982; Sinski *et al.* 2014).

Methods and materials

Animals and experimental protocol

Prior to the study, approval of animal use was granted by the Fourth Local Ethics Committee for Animal Experimentation (Warsaw, Poland). The experiments were performed in accordance with the guidelines of EU Directive 2010/63/EU for animal experiments. A total of 13 conscious male adult Wistar rats, weighing 240–260 g (10–12 weeks old), were used. The animals were kept individually on a 12-hr light/dark cycle in room temperature, with conventional food and water *ad libitum*.

Ventilatory response to hypercapnia and a behavioural test were investigated in rats implemented with the 6-OHDA PD model and in a respective sham control group before and two weeks after operating.

Two groups of animals were studied:

1. 6-OHDA group with unilateral 6-OHDA injection into the medial forebrain bundle (n=7)
2. Sham control group with unilateral vehicle injection into the MFB (n=6)

6-OHDA unilateral model

Rats were anaesthetised with an intraperitoneal injection of thiopentalum natrium (Sandoz GmbH, Austria) at a dose of 90 mg kg⁻¹ and positioned in a stereotaxic instrument (Digital Lab Standard Stereotaxic Stoelting, USA). To prevent the uptake of 6-OHDA by noradrenergic nerve terminals, 30 min before the operation rats received an i.p. administration of desipramine hydrochloride (25 mg kg⁻¹, Sigma Aldrich, Poland). After skin incision, the skull was trephined with a dental drill in specific stereotaxic coordinates according to the

Paxinos and Watson Atlas (2007). Vehicle or 6-hydroxydopamine hydrochloride (20 µg dissolved in 0.9% NaCl containing 0.1% ascorbic acid (Sigma Aldrich, Poland)) at a volume of 5µl was injected in two locations of the right MFB with a sterile Hamilton microsyringe with rate of 1 µl min⁻¹ (Andrzejewski *et al.* 2017a). After injection, the needle was left in the brain for 5 min to prevent the solution from flowing backward and was then slowly retracted. Stereotaxic coordinates for the first injection site were: antero-posterior, bregma: -2.2 mm, lateral: 1.5 mm right of the midline, ventral dura: -7.8 mm, and incisor bar: -3.5 mm, and second site: antero-posterior, bregma: -4.4 mm, lateral: 1.5 mm right of the midline, ventral dura: -7.9 mm, and incisor bar: -3.5 mm. After the operation, the rats were left to recover under standard laboratory conditions, and with unlimited access to food and water. Two weeks later, cylinder and hypercapnic tests were performed.

Ventilation Measurements

Ventilation and its response to acute hypercapnia were investigated in a whole body rodent plethysmograph (WBP, model PLY 3223, Buxco Electronics, USA). The system is composed of two chambers: recording and reference. The pressure fluctuations in the experimental chamber, created by the inspiration and expiration of the animal, are proportional to respiratory flow. The calibration for volume was obtained during each experiment by an injection of 1 mL of air into the animal chamber. The pressure signal was amplified, filtered, recorded, and analysed with data analysis software (Biosystem XA for Windows, SFT3410 230 ver. 2.9; Buxco Electronics, Wilmington, NC) generating tidal volume (V_T , ml) and breathing frequency (f , breaths min⁻¹). Tidal volume was calculated using the approach of Epstein *et al.* (Epstein *et al.* 1980). Minute ventilation (V_E , ml·min⁻¹, BTPS) was determined as a product of tidal volume and breathing frequency. V_T and V_E were normalised to body weight (ml kg⁻¹ and ml kg⁻¹ min⁻¹, respectively). All experiments were performed at room temperature (24–26 °C).

Each rat was placed in the chamber (4.7 l) and left for 30 min of adjustment, while flushing with a fraction of atmospheric air at 2.5 l min^{-1} to prevent CO_2 accumulation. Acute hypercapnia was achieved by a rapid flushing of a gas mixture containing 7% of CO_2 in O_2 . Ventilation and its responses to inspired hypercapnia before and after implementation of the PD model were registered.

After 30 minutes of adaptation by the rats to breathing the chamber air, pulmonary ventilation was taken as the baseline level of ventilation and recorded during 1 min before the introduction of hypercapnia. Ventilation during 3 min of hypercapnia and 1 min after switching to the air breathing was recorded. The period of 30 s breathing preceding hypercapnia was calculated as a control for normocapnic breathing. The period of hypercapnic breathing was divided into 30 s time periods and analysed.

Behavioural study

Behavioural experiments were performed to reassure the effectiveness of 6-OHDA injections using the cylinder test as previously described (Andrzejewski *et al.* 2017a). The cylinder test to assess forelimb asymmetry was performed before and after unilateral MFB lesion. The rat was placed in a transparent cylinder 20 cm in diameter and 30 cm in height. The number of each forepaw contacts to the cylinder wall was recorded and counted for a period of 5 min. The number of each forepaw, as well as both limbs' simultaneous contact with the cylinder wall during landing and rearing, was taken into consideration.

TH immunohistochemistry

After ventilatory and behavioural tests, rats were anaesthetised with an i.p. injection of urethane and α -chloralose (750 mg kg^{-1} and 150 mg kg^{-1} , respectively, Sigma-Aldrich, Poland). For brain tissue fixation, rats were perfused transcardially with 100 ml of phosphate buffered saline (PBS), followed by 100 ml of 4% paraformaldehyde in PBS. The brains were

removed and postfixed in ice-cold paraformaldehyde for 4 hr and next cryoprotected for 24 hr in a 20% sucrose solution at 4 °C. A series of 20 µm sections were cut on a cryotome (Leica CM1850 UV, Germany) in the frontal plane. The slices in the vicinity of the trace of the needle were placed on glasses for tyrosine hydroxylase (TH) immunostaining. Non-specific binding was blocked with 3% normal goat serum (Sigma Chemicals, Australia). Any endogenous peroxidase activity was silenced by peroxidase block (0.03% hydrogen peroxide). Afterwards, specimens were incubated with monoclonal anti-tyrosine hydroxylase antibody produced in mice (1:500, Sigma Chemicals, Australia), followed by two sequential incubations: one with secondary monoclonal anti-mouse antibody conjugated with a labelled polymer horseradish peroxidase (1:200, Dako, USA) and one with DAB solution for staining (Dako, USA). The procedure was performed in accordance with the manufacturer's instructions. The sections were observed under a light microscope (Nikon, Japan), employing 40x and 100x magnification. TH-positive neurons were counted on brain slices of 6-OHDA lesioned rats and the extent of dopaminergic cells decrease was expressed as a percentage of cell loss in the right SNpc compared to the left contralateral one.

Statistical analysis

All experimental data are presented as mean \pm SEM. The data were analysed by two-way ANOVA, followed by repeated measurements with defined time points (prior to and after hypercapnia) with surgical status (before and after lesion) as a between condition factor. Differences between individual time points and experimental conditions were evaluated using the Newman-Keuls *post-hoc test*. In all cases $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using STATISTICA (StatSoft, Poland).

Results

Tyrosine hydroxylase (TH) immunohistochemistry

Fig. 1 demonstrates a typical photomicrograph showing a coronal brain section through the SNpc, immunostained for tyrosine hydroxylase. Significant TH-positive cell loss in the SNpc of the injected right hemisphere was confirmed. Cell number decreased from 244 ± 9 in the contralateral SNpc to 19 ± 3 ($p < 0.001$, $n=6$) in the ipsilateral 6-OHDA lesioned one. The extent of dopaminergic neuronal loss was 92%.

Normocapnic breathing and respiratory response to hypercapnia in sham operated rats

Injection of vehicle into the rat MFB did not affect either normocapnic breathing or respiratory response to hypercapnia. The values of all respiratory parameters were alike before and two weeks after sham lesion (data not shown).

Normocapnic breathing and respiratory response to hypercapnia in 6-OHDA treated rats

Ventilatory response to hypercapnia of PD rats was compared to its control value before the lesion (Fig. 2ABC) and to the values of sham lesioned animals two weeks after operating (Fig. 2DEF).

During normocapnic breathing, two weeks after unilateral 6-OHDA administration, minute ventilation and frequency of breathing were not changed significantly in comparison to control parameters before lesion and that of sham operated rats. However, the average value of normocapnic tidal volume in 6-OHDA treated rats was higher more than 10% in comparison to pre-lesion state and to sham operated animals (two-way ANOVA, $p < 0.05$, Fig. 2CF).

Hypercapnic exposure increased all ventilatory parameters in both 6-OHDA and sham rats. Following neurotoxin treatment, however, frequency of breathing increased to a lesser degree,

whereas tidal volume increased to a higher level. These transformations in the hypercapnic response of tidal volume and respiratory rate had no effect on the magnitude of minute ventilation response to hypercapnia (Fig. 2D). It attained similar levels as in the sham control group and significantly increased at 60 s of hypercapnic exposure in a 6-OHDA state in comparison to its pre-lesion control (Fig. 2A).

Behavioural tests

In the cylinder test, rats lesioned with a 6-OHDA injection demonstrated a decreased motor activity in comparison to the sham operated animals. After a neurotoxin injection into the right MFB, the animals preferred to use the right ipsilateral forepaw, while no such preference was observed in the sham control group (Fig. 3).

Discussion

This study has demonstrated an alteration in the respiratory response to hypercapnia in the 6-OHDA unilateral animal model of PD. After 6-OHDA lesion to the MFB, rats breathed with increased tidal volume during normocapnia, while hypercapnia evoked changes in both tidal volume and frequency of breathing. There was a shift in the magnitude of the response of respiratory parameters to hypercapnia in the 6-OHDA group. An increase in frequency of breathing was reduced, while tidal volume increased to a higher level. In consequence, the hypercapnic response of minute ventilation was nearly unaltered in the 6-OHDA group compared to the response in the sham control group. All animals subjected to unilateral 6-OHDA administration exhibited substantial loss of dopaminergic neurons in the right ipsilateral substantia nigra and characteristic asymmetry in motor activity, which confirmed dopamine depletion.

In the scarce human studies reporting respiratory response to hypercapnia, Onodera *et al.* (2000) described unchanged ventilation and no significant differences in the sensitivity to hypercapnia. The similarity to the results of our study might be linked with the choice of patients in an early stage of the disease. Our unilateral model with MFB injection imitates an early stage of idiopathic PD, where unilateral dopamine deficit has been observed (Djalldetti *et al.* 2006; Hobson *et al.* 2012). It is of note, however, that patients with PD were taking their regular dopaminergic medication, which might have influenced the results obtained.

In the animal study focusing on the consequences of bilateral 6-OHDA lesion on the hypercapnic respiratory response, decrease in minute ventilation caused by diminished frequency of breathing was noted 40 days after lesion, with restoration after 60 days (Oliviera *et al.* 2017; Tuppy *et al.* 2015). The authors observed a diminished respiratory rate already in normocapnic breathing. We also noted a decreased response of frequency of breathing, however only during hypercapnia, yet it was accompanied by an increased tidal volume, which was also slightly elevated in normocapnic breathing. Dissimilar experimental conditions of the present study, such as unilateral lesion and the possibility of compensation from the intact hemisphere, the use of desipramine sparing noradrenergic neurons, and hypercapnic tests performed 14 days after operation, might have contributed to somewhat different results. Besides, we exposed rats to CO₂ stimulus, which lasted only 3 min while in the above-mentioned studies it lasted 3 hours. We also applied hyperoxic hypercapnia inhibiting peripheral chemoreceptors, which indicates that only central chemoreceptors accounted for the altered hypercapnic ventilatory response. Tuppy *et al.* (2015) attributed a mild reduction in the respiratory frequency response to hypercapnia in PD rats to the selective cell loss of phox2b neurons in the RTN region, responsible for chemical drive. In the present study, we did not investigate degeneration of chemoreceptive neurons, yet two weeks after lesion seems to be insufficient time to evoke such changes. In our previous study performed

to the same model, we have demonstrated that the dopamine level in the brainstem was not altered, confirming that neurotoxin injection to the MFB did not affect dopaminergic neurons in this region (Andrzejewski *et al.* 2017b; 2016). We also demonstrated that in the same unilateral MFB model noradrenaline content was preserved in the striatum and brainstem, yet serotonin level was decreased on both sides: over 30% in the striatum and 20% in the brainstem (Andrzejewski *et al.* 2017b). Therefore, we propose that specific depletion of serotonin and preservation of noradrenaline in the brainstem might be responsible for the characteristic pattern of respiratory response during hypercapnia in unilaterally lesioned rats.

It has been demonstrated in the latest study by Oliveira *et al.* (2018) that although serotonergic neurons of raphe pallidus projecting to RTN are reduced in the rat model of PD, it does not concern hypercapnia activated raphe neurons. Yet, stimulated with CO₂, raphe pallidus serotonergic neurons were not critical for respiratory response to hypercapnia in animals with implemented PD model and their sham controls. Unfortunately, the authors did not examine the contribution of raphe magnus to CO₂ stimulated ventilatory response, although its engagement in chemosensory control has been previously demonstrated (Brust *et al.* 2014).

Serotonin has been evidenced as playing an important role in the regulation of hypercapnic ventilatory response. Mice deprived of serotonergic neurons in the brain had a reduced respiratory response to hypercapnia due to a decrease in the frequency of breathing in comparison to the wild-type animals (Hodges *et al.* 2008). Destruction of serotonergic raphe nuclei in rats attenuated ventilation in response to hypercapnia due to a decrease in both parameters V_T and f (Nattie *et al.* 2004). In contrast, the excess of endogenous serotonin, induced by chronic micro dialysis to raphe nuclei of selective serotonin reuptake inhibitor, increased respiratory response to CO₂ (Taylor *et al.* 2004).

We could speculate that tidal volume increase in response to hypercapnia in our 6-OHDA model could have been the effect of attenuated inhibitory effect of DA on respiration, which was previously demonstrated and discussed (Andrzejewski *et al.* 2016; Fallert *et al.* 1979; Guner *et al.* 2002; Sabol and Ward, 1987), yet diminished response of frequency of breathing could be related to serotonin deficit. It is worthy of note that the same minute ventilation with lower respiratory rate and increased tidal volume present in our lesioned rats could result in higher alveolar ventilation and more efficient gas exchange than in sham group.

Another study by Oliveira *et al.* (2017) points to the important role of locus coeruleus and its catecholaminergic neurons in compensation of the loss of RTN neurons, which resulted in mild respiratory rate reduction in response to hypercapnia in the bilateral 6-OHDA model of PD. As mentioned before, in 6-OHDA MFB model the level of noradrenaline has been preserved by desipramine pre-treatment (Andrzejewski *et al.* 2017b). Thus, it is possible that intact noradrenergic neurons might be responsible for the compensation for the changes in dopamine and serotonin levels, and for the increase in tidal volume response during hypercapnia, and may balance the respiratory rate decline. In fact, Biancardi *et al.* (2008) reported that destruction of noradrenergic neurons with 6-OHDA administration in the locus coeruleus reduced the respiratory response to hypercapnia, mainly due to the depression of the tidal volume response. A plausible explanation of respiratory changes present during response to CO₂ in our study could be degeneration of hypothalamic orexinergic neurons, which can be involved in central chemoreception (Kuwaki, 2010). In fact, such a decrease in the number of these neurons has been noted in a similar MFB model with two site injections with a higher volume of 6 µl of 6-OHDA per site (Cui *et al.* 2010). However, since the first signs of the decrease were noticed more than 21 days post-lesion, we are hesitant to expect degeneration of orexinergic neurons in our study after only 14 days.

Our results demonstrated that rats with an implemented unilateral PD model presented an altered respiratory pattern during ventilatory response to hypercapnia as early as two weeks after lesion. This response was mediated solely by central chemoreceptors. In the MFB model only one hemisphere was lesioned, thus, the contralateral hemisphere could evoke a compensatory effect in normocapnic breathing, insufficient in a hypercapnic condition. Preserved noradrenaline and specific changes in dopamine and serotonin in the MFB model, which we have demonstrated in our earlier experiments (Andrzejewski *et al.* 2016, 2017b), could be responsible for the hypercapnic pattern of breathing. Future studies focusing on the regulation of breathing in Parkinson's disease should carefully select an appropriate animal model of PD and take into consideration all of its characteristics and drawbacks.

Conflict of interest statement

None to declare (valid for all the authors).

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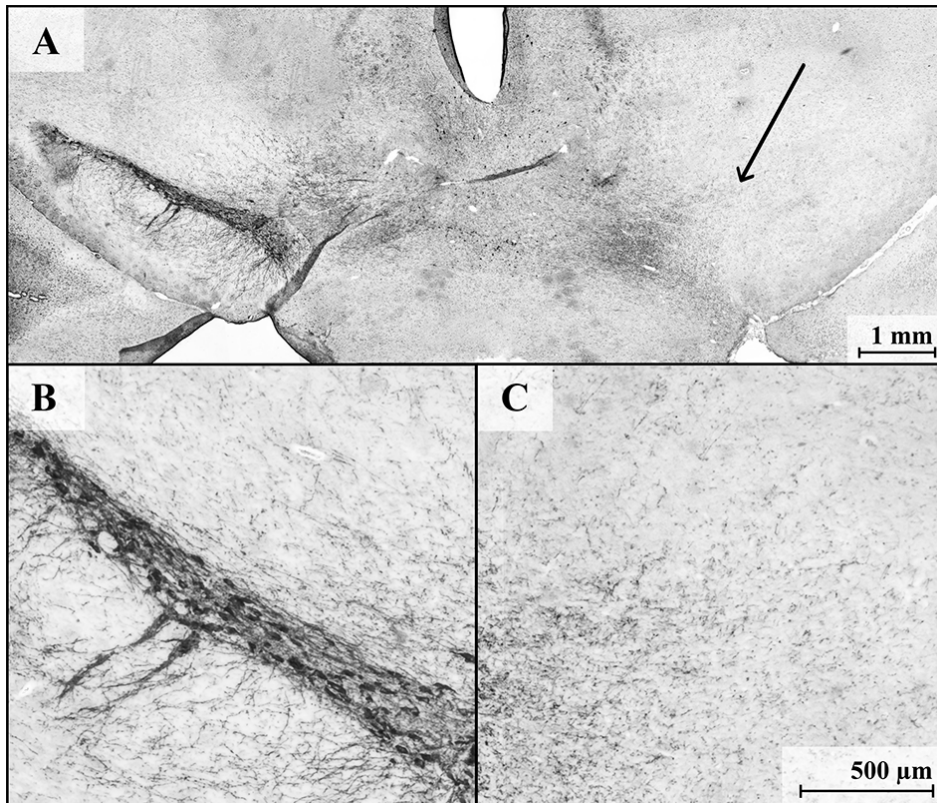


Fig. 1. Depletion of TH-immunoreactive neurones in the SNpc induced with unilateral MFB injection of 6-OHDA; an arrow indicates the side of lesion (A). Intact (B) and lesioned SNpc (C); magnification x 40 and 100.

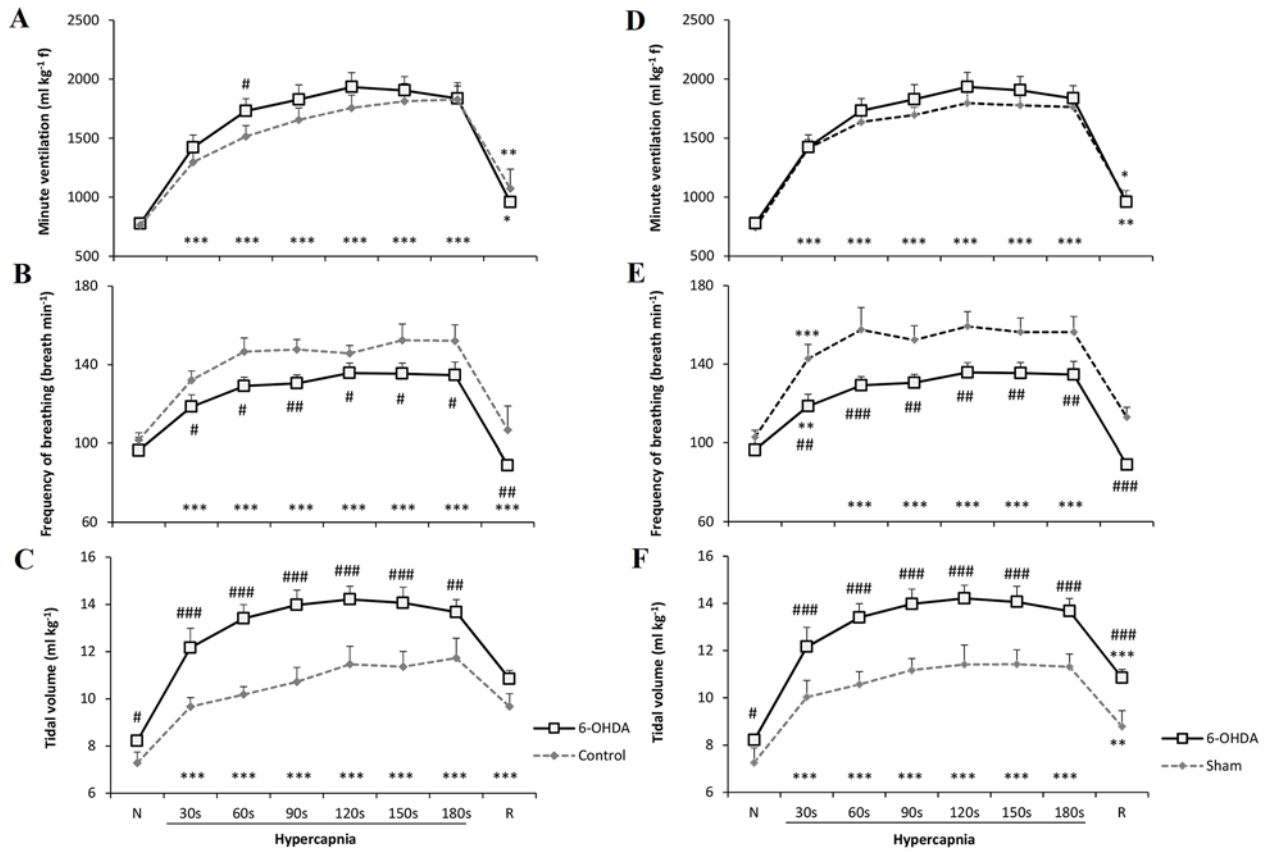


Fig. 2. Minute ventilation, respiratory rate and tidal volume during normocapnic breathing (N) and hypercapnic ventilatory response after 6-OHDA injection to MFB. Comparison with the effects before lesion with 6-OHDA (A,B,C) and with the effects in sham operated group after vehicle injection to MFB (D,E,F). All values are means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus the respective pre-hypercapnic baseline value; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, versus the corresponding pre-lesion or sham values (n=6-7).

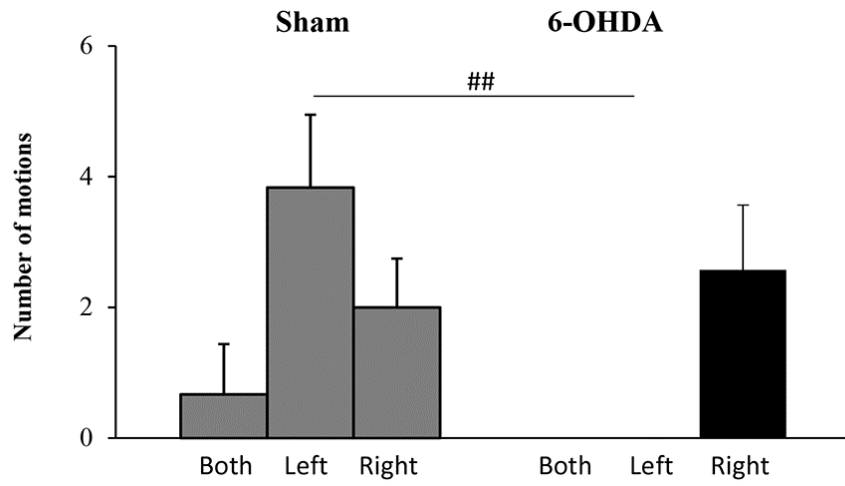


Fig. 3. Effects of 6-OHDA administration on frequency of using ipsilateral and contralateral forepaws in comparison to sham rats. All values are means \pm SEM. $^{##}p < 0.01$, versus the corresponding value between 6-OHDA and sham operated rats (n=6-7).