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Chronic impairment of the vagus nerve function leads to inhibition of dopamine but not serotonin neurons in rat brain structures

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Abstract:
Background: Recent clinical studies have shown that the dorsal motor nucleus of the vagus nerve is one of the brain areas that are the earliest affected by α-synuclein and Lewy body pathology in Parkinson's disease. This observation raises the question: how the vagus nerve dysfunction affects the dopamine system in the brain?
Methods: The rats underwent surgical implantation of the microchip (MC) in the abdominal region of the vagus. In this study, we examined the effect of chronic, unilateral electrical stimulation of the left nerve vagus, of two different types: low-frequency (MCL) and physiological stimulation (MCPh) on the dopamine and serotonin metabolism determined by high-pressure chromatography with electrochemical detection in rat brain structures.
Results: MCL electrical stimulation of the left nerve vagus in contrast to MCPh stimulation, produced a significant inhibition of dopamine system in rat brain structures. *Ex vivo* biochemical experiments clearly suggest that MCL opposite to MCPh impaired the function of dopamine system similarly to vagotomy.
Conclusion: We suggest a close relationship between the peripheral vagus nerve impairment and the inhibition of dopamine system in the brain structures. This is the first report of such relationship which may suggest that mental changes (pro-depressive) could occur in the first stage of Parkinson's disease far ahead of motor impairment.

Key words:
vagus nerve, electrical stimulation, dopamine metabolism, serotonin metabolism, brain structures, rat

Introduction

Parkinson's disease (PD) is a frequent neurodegenerative disease of old age. The loss of dopamine in the nigrostriatal neurons as well as the presence of α-synuclein-positive intracytoplasmic inclusions known as Lewy bodies (LBs) in dopaminergic cells of the substantia nigra and in other affected regions of the central nervous system have been observed in PD. In recent

years, there has been increasing recognition of the features of PD including its non-motor symptoms. The common non-motor symptoms include gastrointestinal disturbances and depression, which may precede motor signs [1, 8]. In addition, retrospective studies indicate that affective symptoms may be manifested as the first symptoms of PD many years before motor signs [10, 14, 27]. Also, recent experimental studies support the hypothesis that small damage functions in the limbic dopamine system in rat may result in depressive symptoms in the forced swimming test without changes in motor function [18]. Some authors demonstrated the involvement of dopamine and serotonin systems in depressive disorders in parkinsonian patients [17, 27]. Moreover, autopsy-based studies of people who died at different stages of the disease also suggest that the brain region known as the dorsal motor nucleus of the vagus, which is connected with the gastrointestinal system, is affected very early in the disease [5]. Recent clinical trials [29] indicate that the vagus nerve may be pathologically altered in PD patients. Postmortem immunohistochemical studies have found that dorsal motor nucleus is one of the brain areas that are the earliest affected by α-synuclein and Lewy body pathology in PD. It is important to mention also that vagus nerve stimulation (VNS) is a novel therapy for the control of epilepsy, and several studies, including two large double-blind randomized clinical trials have confirmed the efficacy of VNS in different types of epilepsy [3, 9, 16]. Similarly, studies in animal models have shown that VNS reduces seizure activity induced by maximal electroshock or pentylenetetrazol by increasing the noradrenaline release, which could be a biomarker for the efficacy of vagus nerve stimulation in a model of limbic seizures in the rat [26, 31, 35].

However, it should be mentioned that the mechanism of action of VNS in epilepsy, depression or its function in the early stage of Parkinson's disease is currently unknown, although significance of the state of activity of the vagus nerve for the function of central monoaminergic systems has been raised. Since the dorsal motor nucleus of the vagus nerve can be considered the true point of departure of the disease process, we evaluated the effect of vagus nerve stimulation on dopaminergic system. In the light of these observations the question arises: whether and how the vagus nerve dysfunction may affect the neurotransmitter activity in the brain.

The aim of this study was to examine the effect of chronic, electrical VNS of two different types by implanted microchips (MC): unilateral microchip stimulation of low-frequency (MCL) and unilateral physiological stimulation (MCPh) on the activity of dopamine and serotonin systems in rat brain structures. Additionally, we also compared the neurochemical effects of VNS with vagotomy in the rat.

Materials and Methods

Animals

The experiments were performed on male Wistar rats (220–260 g) during the light phase of the light-dark cycle (between 8:00 – 15:00) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval (no. 62/2010) of the Jagiellonian University Bioethical Committee as compliant with the Polish Law (21 August 1997). All experiments were carried out on intact animals.

Microchip electrical stimulation

The rats underwent surgical implantation of the MC in the abdominal region of the vagus (MCs were produced by the Institute of Electron Technology, Kraków, Poland). The 1 cm-diameter silicon-coated (RTV 3140, Dow Corning) and battery driven microchip (signal period: 20 s signal duration: 0.1 s; amplitude 200 mV) was connected to the subdiaphragmatic part of the left vagus nerve (VN). After 12 h of food deprivation and under general anesthesia with pentobarbital intraperitoneally (Vetbutal 25 mg/ kg of body mass, Biowet, Puławy, Poland), the rats were placed on their backs and a 3 cm long incision was made in the abdomen. The liver was carefully moved to the right to expose the esophagus and the left VN was localized subdiaphragmatically. The MC's silver electrodes were brought into the abdominal cavity to connect with the vagus by wrapping their unisolated wires around the nerve with cathode and anode positioned 0.5 cm from each other [4, 38]. Next, the subcutaneous pocket was formed by the skin and underlining fascia where the microchip was placed. Finally,

the abdominal muscles and the skin were sutured with surgical silk. After closing the wounds, the rats were placed in the cages with food and water *ad libitum*.

Subdiaphragmatic vagotomy

After 12 h of food deprivation and under general anesthesia with pentobarbital intraperitoneally (Vetbutal 25 mg/kg of body mass), the rats were placed on their backs and a 3 cm long incision was made in the abdomen. The liver was carefully moved to the right side to expose the esophagus. Then both right and left trunks of the vagus nerve were exposed and small fragments were dissected. The abdominal muscles and the skin were then sutured with surgical silk, and the rats were placed in the cages with food and water *ad libitum*.

The effectiveness of the vagotomy was assessed by *post-mortem* stomach weights. At the end of the experiment (14 days after surgery), the rats were decapitated with the guillotine, the brain was rapidly removed and appropriate structures were isolated, and additionally their stomachs were removed and weighed. Only rats in which the stomach weight increased to more than 4% of the body weights were considered to be properly vagotomized and were included in the data presented.

Groups tested

The rats were divided into 4 experimental groups. 1) Control group – underwent sham-laparatomy; 2) MCPh – unilateral physiological microchip stimulation (0.5 ms, 2 mA, 1 Hz) of the left vagus nerve; 3) MCL – unilateral microchip stimulation of low-frequency (current pulse 10 ms, 200 mV, 0.05 Hz) of the left vagus nerve; 4) VAGO – vagotomy group where the right and left trunks of the vagus were cut subdiaphragmatically.

Ex vivo biochemical experiment

After two weeks of the left VN stimulation by different electrical pulses generated by MC, the rats were decapitated with a guillotine, the brain was rapidly removed to an ice-chilled glass plate and the appropriate brain structures (frontal cortex, FCx; striatum, STR; nucleus accumbens, NAc; ventral tegmental area, VTA; substantia nigra, SN) were removed. Isolation correctness of VTA is verified by dopamine concentration in this structures. The samples in which the average error is greater than 45 to 50% of dopamine concentration are not taken for statistical calculations.

The structures were collected and stored in dry ice (–70°C) until used for biochemical assays. The content of dopamine (DA) and its metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) and serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) were assayed by means of high-performance liquid chromatography (HPLC) with electrochemical detection. The chromatograph (Hewlett-Packard 1050) was equipped with Hypersil BDS-C18 column (3 μm).

Data analysis

The results from e*x vivo* biochemical experiments were analyzed by means of a one-way analysis of variance, followed when appropriate by *post-hoc* Duncan test. Dopamine system activity was assessed from the ratio of the extraneuronal metabolite concentration, 3-methoxytyramine to intraneuronal metabolite concentration, 3,4-dihydroxyphenylacetic acid and expressed as the catabolic rate index: ([3-MT]/[DOPAC]) × 100. The rate of serotonin metabolism was expressed as the ratio: the intraneuronal metabolite concentration, 5-hydroxyindoleacetic acid to serotonin – ([5-HIAA]/[5-HT]) × 100. The indices were calculated using the concentrations from individual tissue samples as described before [2].

Results

The effect of chronic unilateral VNS (MCL, microchip low-frequency-stimulation; MCPh, physiological-stimulation) and vagotomy (VAGO) on dopamine system in the brain structures (Tab. 1 and 2)

The one-way ANOVA showed no effect of chronic VNS and vagotomy on dopamine concentration in all investigated brain structures. At the same time, the statistical analysis revealed a significant effect of MCL and VAGO on the level of an extraneuronal metabolite of dopamine, 3-MT in the following mesolimbic structures: VTA $F3/14 = 2.54$, $p < 0.025$; nucleus accumbens $F3/32 = 4.13$, $p < 0.013$; frontal cortex $F3/32 = 5.47$, $p < 0.0037$ as well as in the striatum

Tab. 1. The effect of unilateral microchip electrical stimulation (MC) of the left vagus nerve and vagotomy (VAGO) on the level of dopamine and its metabolites in the mesolimbic and mesocortical structures of the rat brain

Treatment	n	Dopamine	DOPAC	3-MT	HVA
		Ventral tegmental area			
Control	4	2697 ± 940	684 ± 180	59 ± 28	343 ± 70
MCPh	4	2140 ± 442	551 ± 79	58 ± 15	284 ± 33
MCL	5	1821 ± 479	503 ± 109	13 ± 8^{*+}	273 ± 51
VAGO	5	2023 ± 519	502 ± 86	18 ± 13^{*+}	266 ± 49
F		F3/14 = 0.37	F3/14 = 0.52	F3/14 = 2.54 p < 0.025	F3/14 = 0.42
		Nucleus accumbens			
Control	8	12007 ± 443	1862 ± 84	202 ± 19	713 ± 56
MCPh	8	11161 ± 400	1690 ± 79	212 ± 18	731 ± 53
MCL	10	11549 ± 292	1753 ± 71	133 ± 16^{*++}	681 ± 38
VAGO	10	10799 ± 608	1666 ± 112	149 ± 20^{*+}	656 ± 53
F		F3/32 = 1.70	F3/32 = 0.89	F3/32 = 4.13 p < 0.013	F3/32 = 0.44
		Frontal cortex			
Control	8	675 ± 59	147 ± 8	18 ± 2	106 ± 5
MCPh	8	651 ± 61	141 ± 9	19 ± 1	101 ± 7
MCL	10	623 ± 50	115 ± 6^{*+}	11 ± 1^{**++}	87 ± 5
VAGO	10	649 ± 39	135 ± 8	13 ± 1^{*+}	96 ± 6
F		F3/32 = 0.17	F3/32 = 3.31 p < 0.032	F3/32 = 5.47 p < 0.0037	F3/32 = 2.09

After two weeks of unilateral stimulation of the left vagus nerve by different electrical pulses generated by microchips (MC), the rats were decapitated and appropriate brain structures were removed. The concentration of dopamine and its metabolites: DOPAC, 3-MT and HVA were assayed by HPLC with electrochemical detection. Control group – sham-laparatomy, MCPh – a microchip physiological stimulation of the left vagus nerve, MCL – a microchip low-frequency stimulation of the left vagus nerve, VAGO – vagotomy group where the right and left trunks of the vagus were cut subdiaphragmatically. The data are the means ± SEM. The results were analyzed by means of a one-way ANOVA, followed when appropriate, by *post-hoc* Duncan test. Statistical significance: * $p < 0.05$, ** $p < 0.01$ *vs.* control group; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ *vs.* MCPh group

F3/32 = 5.35, $p < 0.004$ (Tab. 1 and 2). The Duncan *post-hoc* test indicated that MCL similarly to VAGO strongly decreased the level of 3-MT compared to control and MCPh groups. Additionally, MCL produced the decrease in DOPAC level in the frontal cortex F3/32 = 3.31, $p < 0.032$ (Tab. 1).

The activity of dopamine system expressed as catabolic rate index: ([3-MT]/[DOPAC]) in the mesolimbic structures and in the striatum was also significantly decreased by MCL and VAGO compared to control and MCPh groups (Fig. 1 and 2).

The effect of chronic unilateral VNS (MCL, microchip low-frequency-stimulation; MCPh, physiological-stimulation) and vagotomy (VAGO) on serotonin system in the brain structures (Tab. 3 and 4)

The one-way ANOVA demonstrated no effect of chronic VNS and vagotomy on serotonin level in all investigated brain structures. The concentration of serotonin metabolite, 5-HIAA was not changed except for the frontal cortex where MCL produced a significant fall of its level, F3/32 = 4.69, $p < 0.0079$ (Tab. 3).

Tab. 2. The effect of unilateral microchip electrical stimulation (MC) of the left vagus nerve and vagotomy (VAGO) on the level of dopamine and its metabolites in the extrapyramidal structures of the rat brain

Treatment	n	Dopamine	DOPAC	3-MT	HVA
		Substantia nigra			
Control	8	1094 ± 80	204 ± 18	37 ± 27	77 ± 7
MCPh	8	1075 ± 96	206 ± 21	41 ± 3	77 ± 9
MCL	10	1235 ± 108	237 ± 19	37 ± 5	77 ± 6
VAGO	10	1189 ± 125	222 ± 21	37 ± 3	72 ± 6
F		F3/32 = 0.50	F3/32 = 0.59	F3/32 = 0.25	F3/32 = 0.13
		Striatum			
Control	8	12620 ± 387	1490 ± 47	365 ± 25	891 ± 42
MCPh	8	12669 ± 396	1477 ± 29	379 ± 14	894 ± 41
MCL	10	11999 ± 496	1463 ± 61	279 ± 22^{**++}	801 ± 38
VAGO	10	13470 ± 423	1580 ± 45	321 ± 15^{+}	907 ± 17
F		F3/32 = 2.09	F3/32 = 2.09	F3/32 = 5.35 p < 0.004	F3/32 = 2.13

For explanations see Table 1. Statistical significance: * p < 0.05, ** p < 0.01 *vs.* control group; $^{+}$ p < 0.05, $^{++}$ p < 0.01 *vs.* MCPh group

The activity of serotonin system expressed as catabolic rate index: ([5-HIAA]/[5-HT]) was not changed by chronic VNS and VAGO in the mesolimbic and extrapyramidal structures (data not shown).

Discussion

The main finding reported here is that MCL of the left vagus nerve in the rat produced a dopamine system inhibition in different brain structures containing both the dopamine cell bodies and nerve endings (VTA, NAc, FCx, STR, Tabs. 1 and 2 and Figs. 1, 2). Indeed, so clear and widely current changes seem to be specific to the dopamine neurons. However, we observed the decrease of 5-HIAA level only after MCL stimulation but not after vagotomy and limited to one structure – the frontal cortex (Tab. 3). The obtained biochemical data clearly suggest that MCL in contrast to MCPh impaired the function of dopamine system similarly to vagotomy. The neurochemical effects of vagotomy (VAGO group) and chronic unilateral VNS (MCL group) were almost identical and demonstrated a significant inhibition of dopamine neurons in all the investigated brain structures. In contrast to MCL, physiological stimulation (MCPh group) of the vagus nerve produced no change in dopamine and serotonin metabolism and its effect was similar to the data obtained from the control group. The present data have shown for the first time a close and important relationship between the state of the peripheral vagus nerve activity and the function of dopamine system in the brain. Earlier extensive *postmortem* immunohistochemical studies [5] demonstrated that the brain region known as the dorsal motor nucleus of the vagus, which is connected to the gastrointestinal systems, was affected very early in PD. If so, early damage to the vagus nerve function can cause a some type of affective disorders associated with the inhibition of dopamine system in the brain much earlier than the motor symptoms appeared. From enteric nervous system the process may gain access to the lower brainstem *via* the vagal nerve and then ascend through the basal mid and forebrain until it reaches the cerebral cortex [5, 12].

The question arises whether PD starts outside the brain. Our present study showed that there were strong links between the activity of the peripheral vagus nerve and cerebral dopamine function. Studies in humans and in animal models suggest that this gastrointestinal connection is not an accident, and that the disease may progress in intestinal nerve cells even

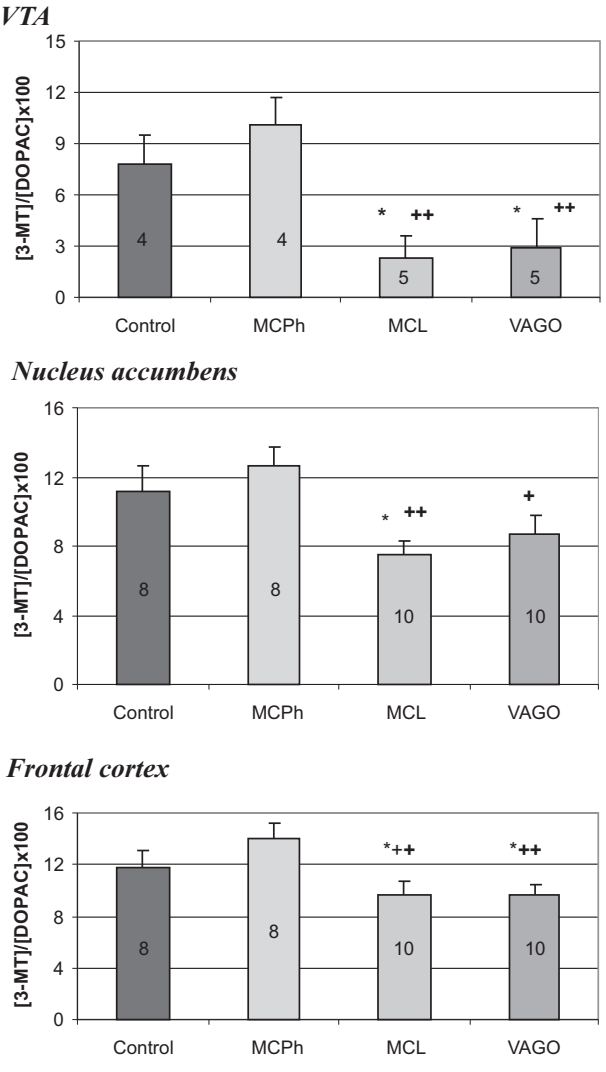

Fig. 1. The effect of various electrical microchip stimulations of the left vagus nerve in comparison with vagotomy on the activity of dopamine system in the mesolimbic brain structures of the rat expressed as an index of dopamine release: [3-MT]/[DOPAC]. After two weeks of the left VNS by different electrical pulses generated by MC the rats were decapitated with a guillotine and appropriate brain structures (VTA, nucleus accumbens, frontal cortex) were removed. The data are the means ± SEM. The indices were calculated using the concentrations in individual tissue samples (n = from 8 to 10), except for VTA (n = 4 to 5). The results were analyzed by means of one-way ANOVA, followed when appropriate by *post-hoc* Duncan test. Statistical significance: * $p < 0.05$ *vs.* control group; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ *vs.* MCPh group

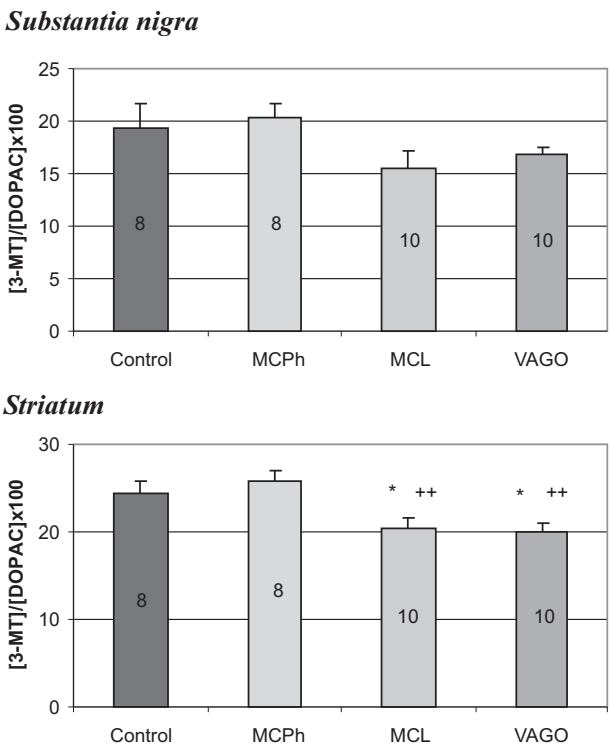

Fig. 2. The effect of various electrical microchip stimulations of the left vagus nerve in comparison with vagotomy on the activity of dopamine system in the extrapyramidal brain structures of the rat expressed as an index of dopamine release: [3-MT]/[DOPAC]. After two weeks of the left VNS by different electrical pulses generated by MC the rats were decapitated with a guillotine and appropriate brain structures (substantia nigra and striatum) were removed. The data are the means ± SEM. The indices were calculated using the concentrations in individual tissue samples (n = from 8 to 10). The results were analyzed by means of one-way ANOVA, followed when appropriate by *post-hoc* Duncan's test. Statistical significance: * $p < 0.05$ *vs.* control group; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ *vs.* MCPh group

before it reaches the brain [6, 19]. Dysfunction of the autonomic nervous system is a poorly recognized but important aspect of the basal ganglia disease. Clinical and experimental evidences support the involvement of dopamine structures in the brain in the regulation of some autonomic functions. In PD which is the best studied disease, the most frequent autonomic dysfunctions are those affecting the gastrointestinal, cardiovascular, urinary, and thermoregulatory systems [8, 13, 15, 28, 32, 33]. Gastrointestinal manifestations, reflect a failure of the sympathetic nervous system, according to experimental and clinical observations. The experimental observations support the assumption that central dopamine receptors are involved in many autonomic system disorders [11, 25]. The observations coming from experimental studies suggest that the extrapyramidal dopamine system could be implicated also in the regulation of the cardiovascular system function. It was demonstrated that electrical or chemical stimulation of the substantia nigra pars compacta in rats enhanced dopamine release in the STR and elicited proportional hypertension and tachycardia [23]. This effect was blocked by intrastriatal microinjection of haloperidol or the striatal lesion [22, 36]. Another relationship between central dopamine

Tab. 3. The effect of unilateral microchip electrical stimulation (MC) of the left vagus nerve and vagotomy (VAGO) on the level of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in mesolimbic and mesocortical structures of the rat brain

Treatment	n	5-HT	5-HIAA
		Ventral tegmental area	
Control	4	518 ± 65	743 ± 129
MCPh	4	490 ± 108	623 ± 138
MCL	5	588 ± 73	686 ± 90
VAGO	5	507 ± 36	670 ± 67
F		F3/14 = 0.37	F3/14 = 0.20
		Nucleus accumbens	
Control	8	520 ± 78	293 ± 15
MCPh	8	478 ± 45	295 ± 22
MCL	10	490 ± 18	261 ± 16
VAGO	10	475 ± 43	255 ± 16
F		F3/32 = 2.08	F3/32 = 1.70
		Frontal cortex	
Control	8	471 ± 17	250 ± 8
MCPh	8	496 ± 29	269 ± 8
MCL	10	467 ± 12	214 ± 14*++
VAGO	10	510 ± 14	261 ± 12
F		F3/32 = 0.79	F3/32 = 4.69 p < 0.0079

After two weeks of unilateral stimulation of the left vagus nerve by different electrical pulses generated by microchips (MC) the rats were decapitated and appropriate brain structures were removed. The concentrations of serotonin and its metabolite, 5-HIAA were assayed by HPLC with electrochemical detection. Control group – sham-laparatomy, MCPh – a microchip physiological stimulation the left vagus nerve, MCL – a microchip low-frequency stimulation the left vagus nerve, VAGO – vagotomy group where the right and left trunks of the vagus were cut subdiaphragmatically. The data are the means ± SEM. The results were analyzed by means of a one-way ANOVA, followed when appropriate, by *post-hoc* Duncan test. Statistical significance: * $p < 0.05$, *vs.* control group; ++ $p < 0.01$ *vs.* MCPh group

Tab. 4. The effect of unilateral microchip electrical stimulation (MC) of the left vagus nerve and vagotomy (VAGO) on the level of serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in extrapyramidal structures of the rat brain

Treatment	n	5-HT	5-HIAA
		Substantia nigra	
Control	8	819 ± 43	392 ± 17
MCPh	8	835 ± 68	437 ± 33
MCL	10	839 ± 63	399 ± 30
VAGO	10	759 ± 20	363 ± 13
F		F3/32 = 0.56	F3/32 = 1.96
		Striatum	
Control	8	378 ± 14	395 ± 8
MCPh	8	404 ± 15	463 ± 20
MCL	10	404 ± 16	428 ± 24
VAGO	10	397 ± 18	439 ± 13
F		F3/32 = 0.51	F3/32 = 2.27

For explanation see Table 3

system and autonomic nervous system is connected with the bladder function. Clinical studies have indicated that neurogenic bladder dysfunction may occur in patients with extrapyramidal disease, particularly parkinsonism, however, this type of disturbance is relatively rare [30]. Summarizing, we should take into account that the autonomic nervous system dysfunction is a pervasive problem in PD.

The second important finding of this study is that the applied parameters of the left vagus stimulation in the MCL group evoked functional vagotomy as the brain changes in the latter were almost identical to those observed in the VAGO group. Our results indicate that the central effects of chronic vagal neuromodulation are not self-evident. The chosen low-frequency parameters are based on our previous studies [20, 21]. However, until now we did not recognize the central biochemical effects of the vagus pacing. It was quite surprising for us that a low-frequency stimulation of a peripheral nerve could evoke functional vagotomy. It is widely accepted that the activity of a nerve can be modulated by applying different parameters of pacing [7]. To achieve reduced signaling in a nerve it should be paced with electric pulses at high rate or at voltage that substantially exceeds normal traffic. As a result, the nerve is "overpaced", runs out of neurotransmitter substance and transmits less stimuli. Alternatively, a relatively high voltage potential can be applied to the nerve to create a blockade. This method is known as "voltage clamping". The vagus in our experiment was paced with relatively low frequency and voltage similar to gastric mechanoreceptor bursts. Because the procedure of implantation of both types of MC (low-frequency and physiological one) was performed in identical manner and conditions, we can exclude the damage of the vagus during MC implantation since the MCPh rats represented almost the same brain metabolism profile as the control (intact) group. Additionally, recordings of the vagus activity in the rats with low-frequency

MC revealed the presence of higher-rate impulses in the nerve than in the control group without MC (unpublished data). The "physiological" parameters of neuromodulation were obtained from sophisticated analysis of vagal afferent discharges in normal rats [37]. We intended to mimic low level physiological activity of the vagus what was confirmed by dopamine and serotonin metabolism.

The present study pointed out clearly the close relationship between the peripheral VN function and the dopamine release in the brain structures especially in the mesolimbic (VTA, NAc) and mesocortical (FCx) systems, which may implicate serious psychological consequences in the first stage of PD. Moreover, such non-motor symptoms as anxiety, depression and cognitive impairment are common and may be present as the first symptom of PD many years before motor signs [24]. Correspondingly, the activation of dopamine system by reversible monoamine oxidase inhibitors, dopamine receptors agonists or levodopa/carbidopa is recommended for the initial treatment of PD [34].

Summing up the literature from clinical research and taking into account our present results, it seems that the onset of PD may be associated with neurodegeneration and impairment of the vagus nerve function, which leads mainly to the inhibition of mesolimbic and mesocortical dopamine system in the brain. As a consequence of functional inhibition of the limbic dopamine system, mental disorders may follow. Such an approach may be important from clinical point of view and creates many possibilities of pharmacological intervention by using neuroprotective/dopaminergic drugs in the early stages of PD to slow down the neurodegenerative process and to improve the dopamine system function in the brain.

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