PERIPHERAL MECHANISMS OF INTESTINAL DYSMOTILITY IN RATS WITH SALSOLINOL INDUCED EXPERIMENTAL PARKINSON'S DISEASE.

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Gastrointestinal dysmotility in Parkinson's disease (PD) has been attributed in part to peripheral neurotoxine action. Our purpose was the evaluation of the salsolinol effect on intramuscular interstitial cells of Cajal (ICC), duodenal myoelectrical activity (DMA) and vagal afferent activity (VAA) in rats with experimental PD. Twenty rats were divided into 2 equal groups. Experimental PD was produced in one group by 3 weeks of the intraperitoneal salsolinol injections (50 mg/kg/day), whereas the 2-nd group served as control. DMA and VAA were recorded in both groups during fasting and stepwise - gastric distension (GD) of 10 ml. Subsequently fragments of duodenum were removed and intramuscular ICC were assessed as c-Kit antigen percentage in the duodenal muscular zone. Analyses of the fasting DMA and VAA recordings didn't reveal differences between the compared groups. During GD increase of DMA dominant frequency (p=0.04) and VAA frequency (p<0.01) was observed in the controls whereas in the salsolinol group both parameters remained unchanged. Image analysis of duodenum revealed decreased c-Kit expression in the salsolinol-injected animals (p=0.05). The results of our study may suggest the direct effect of salsolinol on both ICC and neuronal pathways of gastro-duodenal reflexes.

Key words: Parkinson's disease, salsolinol, myoelectrical activity, vagal afferent activity, interstitial cells of Cajal

INTRODUCTION

Gastrointestinal (GI) dysmotility develops frequently in course of Parkinson's disease (PD) and appears in several segments of the upper and
lower GI tract. Throat and oesophagus weakness and discoordination bring about clinical manifestation of dysphagia and threaten with aspiration induced respiratory complications. Gastroparesis inhibits gastric emptying and causes dyspeptic symptoms. Intestinal dysmotility delays intestinal transit leading to constipation (1-3). Delayed gastric emptying and intestinal dysmotility are among the principal PD related GI abnormalities however their pathophysiology hasn't been precisely described so far. Previous studies didn't reveal changes of the peripheral mechanisms of motility control like vagal nerves and / or enteric nervous system (ENS) (4,5). In the current study we focused our attention on peripheral elements of brain-gut axis in experimental PD. We examined the effect of salsolinol (1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline) on the intramuscular interstitial cells of Cajal (ICCs) expression as well as short (ENS) and long (vago-vagal) gastro-duodenal reflexes. We used salsolinol for its evidenced role as one of main neurotoxins, which affect the substantia nigra (SN) neurones (6-8). Regarding previous studies increased PD risk is produced by increased activity of R-salsolinol-N-methyltransferase, the enzyme that transforms salsolinol to N-methyl-R-salsolinol (NMRS). Naoi et al. observed high NMRS activity in cerebrospinal fluid of PD patients. Moreover they noticed NMRS induced mitochondrial impairment and apoptosis of SN neurones (9,10). In spite of those observations we cannot exclude that salsolinol and / or its methyl derivative can also exert direct impairing effect on peripheral mechanisms involved in GI regulation. In our study we hypothesised that chronic peripheral administration of salsolinol would change fasting intestinal c-Kit expression, duodenal myoelectrical activity (DMA) and vagal afferent activity (VAA) and DMA / VAA response to gastric distension (GD).

MATERIAL AND METHODS

Twenty male rats (Wistar, Poland) weighing 200g were used for the study. The animals were housed in cages (5 animals per one) at constant temperature and 12 hours light / dark periods. Food and water were accessible ad libitum. The rats were divided into 2 equal groups. One group was subjected to intraperitoneal (i.p.) salsolinol injections (50 mg/kg/day) for 21 days (11) whereas the second group received saline and served as the control. In both groups DMA and vagal afferent activity (VAA) were measured during fasting and after GD. After the recordings intramuscular ICCs expression was assessed in the removed duodenum fragments. All experimental protocols were approved by Local Bioethical Committee of the Polish Board of Scientific Investigations in Krakow.

Surgical procedures

Placement of electrodes in duodenal muscular layer

Electrode placement in the animal's small intestine was performed four days before the DMA recordings. The animals were anaesthetised using mixed solution of ketamine (100 mg/kg) and xylazyn (2 mg/kg) (Biowet, Poland). During the surgery a thermostatically controlled heating pad
maintained rectal temperature at 37 ± 1°C. After opening the abdomen and uncovering the small intestine 3 electrodes were implanted in the intestinal muscular layer. The most proximal electrode was installed in the duodenum 1 cm distally to the pylorus and the others at distance of 5 and 10 cm. The proximal and distal electrodes were positive whereas the middle one was negative and served as a reference. Such combinations allowed obtaining 2 leads with the electrical recording shifted one to another. The proximal ends of the wires ran subcutaneously to the sub-scapular region where they were externalized.

**Placement of gastric fistulae**

The procedure was carried out simultaneously to the intramuscular electrodes implantation. The small stainless steel fistulae were implanted on the larger curvature of the rats' stomachs in the border of corpus and fundus and fixed with sutures Monosof 5-0 (Tyco Healthcare, USA). Subsequently the abdominal wall was closed using the suture Monosof 4-0. After the procedure the rats were allowed to recover for 10-15 days.

**Placement of the vagal electrodes**

The procedure was performed under anaesthesia with pentobarbital (Vetbutal, Biowet) - 0.25 mg/kg. The left vagus nerve was uncovered on the animal neck, isolated from the cervical artery and cut as proximally possible for access to the distal nerve trunk of 1.5 cm length. The cuff electrode consisted of silver wire of diameter 75 µm (A-M Systems, Carlsborg, USA) and elastic tube of 1,5 mm diameter and of 1,5 cm length was installed on the distal end of the nerve trunk accordingly to the data previously published (12). In order to avoid postoperative damage response a latency period of 15 min. before the commencement of the recording was applied.

**DMA evaluation**

Myoelectrical activity of the duodenum was recorded using the implanted electrodes. For the fasting recordings the animals were fasted with free access to water 18 hrs. before the study. Prior to the experiment rats were placed in the Bollman cages and the gastric fistulae were flushed with warm (37°C) 0.9 % NaCl solution (13). Latex balloon was introduced into stomach via the fistula and remained not distended for 1 hr. to allow for stabilisation. After 60-minutes of the fasting recording the balloon was filled up to 10-ml. The selected volume was within physiological capacity of the rat stomach after meal consumption. The DMA recording was continued for another 10 min (Fig.1). Frequencies of the migrating myoelectrical complexes (MMC) were assessed within the 1-hr. fasting recordings. Both fasting and GD DMA were subjected to spectral analysis based on fast Fourier transformation (FFT) by application of the Spike Histogram (ADInstruments, Australia) software. The applied spectral analysis allowed for evaluation of the dominant frequency (DF), which is defined as frequency of potentials that dominates in the analysed recording.

**VAA evaluation**

VAA recordings were performed in the rats anaesthetised with pentobarbital using the electrodes implanted on the left vagus nerve. The initial 1 h. of fasting recording was followed by 10 min recording during GD (Fig. 2 A, B). The potentials were amplified by the BIO Amp (ADInstruments) amplifier and analysed spectrally using the Spike Histogram (ADInstruments) software within 1-hr. fasting and 10-min GD periods. The frequency of the potentials recorded in the vagal afferent fibres and the VAA response to GD were compared in both groups. After the
procedure animals were sacrificed by overdosage of anaesthetics and fragments of duodenum were removed for the assessment of the intramuscular c-Kit expression.

**Intramuscular c-Kit assessment**

Intramuscular ICCs were revealed as expression of tyrosine kinase receptor (c-Kit antigen), a well-known ICCs marker in the GI wall. The removed fragments of duodenum were fixed in alcohol and then immersed in paraffin. Five mm thick slices, oriented to contain longitudinal sections of the intestinal wall, were deparaffinated and processed using the rabbit monoclonal antibody anti-CD117 (c-Kit Antibody C-19, Santa Cruz Biotechnology, USA) and the dye set En Vision (DAKO Corporation, USA) for presentation of the c-Kit antigens. Percentage of c-Kit antigen expression in the muscular zone was evaluated using an optical microscope Axiophot (Zeiss, Germany), interfaced with software used for morphometric determinations (14).

**Statistical analysis**

The results were performed as mean values ± standard deviations (SD). Data obtained from the same group of animals were subjected to the "t"-Student test for small groups of images. The "t"-Student test for two populations of images was applied for the analysis of the DMA and VAA results obtained for the 2 compared groups of rats. ANOVA analysis of variance was used for comparison of c-Kit antigen expression. For each test p<0.05 was considered as statistically significant.
RESULTS

Effect of salsolinol on DMA

Analysis of the fasting DMA recordings revealed slightly but not significantly higher mean slow wave frequency in the salsolinol group (0.62 ± 0.2 vs. 0.44 ± 0.2 · 10^{-4} Hz, p=0.1) however, both values remained within the physiological ranges. Comparison of the fasting duodenal MMC frequency also did not show significant differences between the control and the salsolinol rats (14 ± 2.2 vs. 12 ± 1.7 · 10^{-4} Hz, p=0.06).

Gastric distension of 10 ml revealed different DMA response in both compared groups of animals. In the salsolinol receiving group mean DMA frequency decreased by 14%, which wasn't significant change (0.62 ± 0.2 vs. 0.53 ± 0.2 · 10^{-4} Hz, p=0.1) whereas in the controls the DMA frequency increased significantly by 43% (0.44 ± 0.2 vs. 0.63 ± 0.03 · 10^{-4} Hz, p=0.04) (Fig. 3).

Simultaneously in the controls SD of DMA frequency decreased during GD, which might have reflected decrease of DF dispersion in this group. In the salsolinol rats SD of DMA remained unchanged, which also suggested lack of the GD effect on the gastro-intestinal reflexes.

Fig. 2. Vagal afferent activity (VAA) recorded in left vagus nerve of the fasted control rat (A) and the fasted rat with experimental Parkinson's disease (B). [1] - gastric distension (10 ml), [2] - end of gastric distension.
Effect of salsolinol on VAA

Comparison of the fasting VAA didn't show significant differences of the recorded potential frequency between the compared groups (0.29 ± 0.1 vs. 0.32 ± 0.2 Hz). However the response of the vagal afferent fibres to GD differed in both compared groups. In the control group the frequency of the recorded potentials increased during GD (0.29 ± 0.1 vs. 0.86 ± 0.2 Hz, p<0.01) whereas in the salsolinol injected group remained unchanged (0.29 ± 0.1 vs. 0.37 ± 0.3 Hz, p=0.4) (Fig. 4).

Effect of salsolinol on duodenal c-Kit

Microscopic observation of the examined duodenum fragments didn't show visible changes of intramuscular c-Kit antigen expression between the salsolinol-injected and the control group. However the applied analysis of variance revealed a slightly smaller mean percentage of c-Kit expression in the duodenal muscular zone of the salsolinol-injected rats (4.7 ± 1.2 vs. 5.8 ± 2.4 %, p=0.05) (Fig. 5).

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**Fig. 3.** Dominant frequency of the duodenal myoelectrical activity (DMA) recorded in the rats with experimental Parkinson's disease and the control rats before and during gastric distension.

**Fig. 4.** Frequency of the vagal afferent activity recorded in the rats with experimental Parkinson's disease and the control rats before and during gastric distension.
Gastrointestinal symptoms frequently observed in the PD patients are explained as the direct PD effect on mechanisms that control and co-ordinate GI motility. Previous clinical studies revealed positive correlation between the intensity of dysmotility symptoms and the duration or severity of disease, which might have suggested progressive process of degeneration of the ENS structures in the PD patients (15). Therefore exploration of pathophysiology of gut dysmotility seems to be of great importance for better PD management. Previous papers suggested that PD related GI dysmotility could derive from dysfunction of the ENS neurones. Krygowska-Wajs et al. (16) studied gastric emptying and gastric myoelectrical activity in patients with advanced PD and observed coexistence of delayed gastric emptying and both fasting and postprandial dysrhythmia. Similarly Chen et al. (17) noticed impaired fasting basic electrical rhythm (BER) of the stomach and its abnormal response to the test meal in the PD patients. Those results remain in agreement with the previous ones obtained by Kaneoke and Soykan (4,18) and seem to be the effect of either abnormal BER generation by ICCs or impaired regulatory function of the ENS neurones. However they don't allow excluding the involvement of central mechanisms based on the dopaminergic neurones located in the medullary periventricular area (19).

Peripheral mechanisms of the intestinal dysmotility could have been due to histopathological findings in the ENS neurones of the PD patients. In the middle eighties Qualman et al. (3) found pathological Levy's bodies in the oesophageal myenteric plexus of PD patients with dysphagia or achalasia. Simultaneously Wakabayashi et al. (5) observed Levy's bodies in neurones of the Auerbach's and the Meissner's plexus and Kupski et al. (20) described presence of Levy's bodies in the colonic ganglia of PD patients. The authors stated that those degenerative changes in the ENS neurones could have been responsible for the observed
dysphagia, dysmotility and constipation. However it hasn't been clarified if those changes could have directly influenced myoelectrical activity.

In our study we have considered the direct effect of peripherally given salsolinol on both function of the intramuscular ICCs and reflex mechanisms located within the intestinal wall or the vagal nerves. We have evaluated the effect of experimental PD on the fasting and postprandial DMA and VAA however both electrical activities recorded in the duodenal musculature and in the vagal afferent fibres are produced by the intestinal ICCs. For the study we used dose of salsolinol that was similar to doses used by the other authors and described as sufficient for development of the experimental PD (11). The animals of both groups didn't present significant differences of fasting DMA and VAA however reaction to GD was different. The differences revealed by stomach distension could have reflected the influence of salsolinol mostly on the reflex regulation of myoelectrical activity. In the control rats GD brought about increase of the DMA mean frequency by about half. In the animals previously injected with salsolinol we did not observe increase but rather decrease of the DMA frequency. Similarly behaved the activity recorded in the afferent fibres of the left vagus nerves. Vagal afferent fibres serve as the afferent branch of the long vago-vagal reflex and their activity depends on the mechanoreceptor sensitivity of the intestinal wall (21,22). Therefore, it could be hypothesised that the DMA changes produced by the peripheral effect of salsolinol may coexist with similar changes of the recorded VAA. In our study a significant three-time increase of the mean vagal potential frequency observed in the controls during GD wasn't accompanied by the comparable changes in the group administered with salsolinol. Our study showed that salsolinol affected not only the short gastro-intestinal reflexes, located within ENS, but also the long vago-vagal reflexes. Our observations suggested stronger depression of the gastro-intestinal or the vago-vagal reflexes than the fasting duodenal myoelectrical activity. Only the activation of the reflex response by GD revealed dramatic differences of the neuronal or muscular electrical activity between the compared groups.

However our results don't depreciate the possible role of ICCs dysfunction in the observed neuronal or myoelectrical changes. ICCs are the elements of the intestinal wall that generate and control the muscular BER therefore changes of their activity might play an essential role in the PD related dysmotility (23,24). In our study, according to the previous experiments, we used the expression of the tyrosine kinase (c-Kit) receptor as the marker of the ICCs activity in the duodenum wall (25). It has been previously evidenced that c-Kit blockade in the intestinal wall causes loss of capability to generate BER and ICCs transformation onto the typical muscular cells (26). In our experiment we observed slight decrease of c-Kit expression, which remained on the border of significance, after 3 weeks of peripheral salsolinol administration. Regarding the results of the applied image analysis we cannot exclude the influence of salsolinol on the functional ICCs changes. We suppose that the applied schema of the neurotoxin
administration resulted in discrete changes of the intramuscular ICCs function, manifested only after the mechanical stimulation of their electrical output.

In summary, our results suggest that neurotoxins may induce changes of the intestinal c-Kit expression and generation of myoelectrical activity. However mechanisms of fasting intestinal myoelectrical activity probably do not serve as the main targets for the neurotoxine action. Significant changes of the DMA and VAA response to GD suggest that the impairment of the gastro-intestinal reflexes encoded within ENS and / or vago-vagal reflexes are the principal effect of salsolinol. Disturbed ENS and vago-vagal regulatory function can produce delayed gastric emptying, intestinal dysmotility and slow intestinal transit in the PD patients.

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