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Expanded insights into the neural component of the activity-based anorexia animal model — morphological changes in the enteric nervous system and altered pain perception

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Abstract: Anorexia nervosa (AN) is an eating disorder characterized by distinct etiopathogenetic concepts that are gradually being linked together to unravel the dominant pathophysiological pathways underlying the disease. Excessive food restrictions, often accompanied by over-exercise and undertaken to lose weight, lead to the development of numerous complications. The biological concept of neurohormonal dysfunction in AN seems incomplete without demonstrating or excluding the role of the enteric nervous system (ENS). Using an animal model of activity-based anorexia (ABA), we conducted the preliminary assessment of the ENS structure. Here we show, in preparations stained by immunohistochemistry with anti-ChAT, anti-NOS, anti-PGP 9.5, anti-c-fos, and anti-TH antibodies, a lower density of cholinergic and nitrergic nerve fibers as well as reduced neuronal activity in myenteric plexus. Such structural and functional damage to the ENS may be responsible for a number of gastrointestinal symptoms that worsen the course of the disease. In addition, we expanded the study to address the unresolved issue of mechanical and thermal pain sensitivity in AN. The Von Frey and hot plate tests revealed, that in ABA animals, the pain threshold for mechanical stimulus decreases while for thermal increases. In this way, we have significantly supplemented the background of AN with potentially observable nervous system changes which may influence the evolution of the therapeutic approach in the future.

Keywords: anorexia nervosa, eating disorders, activity-based anorexia, animal model, enteric nervous system, gut-brain axis, pain perception, pain sensitivity.

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Introduction

Anorexia nervosa (AN) is an eating disorder which has a tremendous influence on one's health. According to DSM-5 criteria, it is characterized by extreme food intake restriction, low body weight, distorted body image and fear of gaining weight [1]. Due to malnutrition which affects almost every body organ, AN can lead to numerous health consequences including metabolic, cardiovascular, gastrointestinal, endocrine, and neurologic abnormalities like hypoglycemia, prolongation of the QTc interval, slowed gastric emptying, amenorrhea or cerebral atrophy [2–5]. It has the highest mortality rate among all psychiatric illnesses [2, 6–9]. Furthermore, studies suggest that the mortality rate is higher among men than women [8, 10, 11]. Because of its multidimensional character, despite being a significant social issue and years of research, the etiology of the disorder remains unsettled. However, genetic and environmental factors are thought to play a role in the development of the disease. The heritability of AN is estimated to range from 48% to 74% [12].

A number of gastrointestinal (GI) complications, such as bloating, constipation or early satiety, may be the first physical symptom of the disease [13–15]. Underlying disruption of intestinal homeostasis can be the aftermath of malnutrition leading to myopathy or electrolyte imbalance. However, impaired gut motility, which is highly dependent on the proper functioning of the enteric nervous system (ENS), may also exacerbate the typical spectrum of AN symptoms [16]. ENS is a network of ganglions organized in a form of two plexuses — submucosal and myenteric, embedded in the gut wall. Over half a billion neurons account for a complex enteric circuitry capable of autonomic activity as well as an interplay with the central nervous system in governing the functional integrity of the GI tract. Extrinsic primary afferent neurons (EPANs) provide information about mechanical and chemical stimuli in the gut to the central nervous system, which interacts with the ENS via sympathetic and parasympathetic pathways. Both neuronal components are further influenced by the microbiota (both developmentally and functionally), which is known to be significantly affected by AN in terms of its diversity and composition. Moreover, disorders of gut-brain interaction (DGBI), such as gastroparesis, functional dyspepsia, functional constipation, and irritable bowel syndrome, are commonly seen in anorectic patients [17]. However, given that DGBIs are diagnosed based on symptomatic criteria there is still a lot of uncertainty about the causal relationship with eating disorders, the overlap between the two diseases, or the similarity of symptoms [18]. As in many GI diseases, the exact role of ENS in the etiology of AN symptomatology has yet to be determined despite its undeniable contribution. AN is also characterized by alterations in the perception of different stimuli, including smell, taste or interoception [19–21]. Furthermore, there is some evidence that pain sensitivity is altered in eating disorders. Regarding thermal stimuli, several studies have indicated elevated pain thresholds in subjects with AN

[22–28]. Although it is hypothesized that lower skin temperature and endocrine abnormalities may play a significant role in that, the precise underlying mechanism of this phenomenon remains unclear. As for mechanical stimuli, studies have not been conclusive on whether the pain threshold is altered [27–29]. Thus, this study aimed to conduct a preliminary assessment of the ENS, which, due to its complexity, is gaining considerable interest in anorectic patients together with an analysis of pain threshold, an unresolved issue in AN. An activity-based rat model of anorexia (ABA), which is a reliable AN animal model [30, 31], was applied in our study.

Materials and Methods

Animals

Wistar female rats weighing 174–222 g (mean body weight: 198.7 ± 14 g; Jagiellonian University Medical College Animal Laboratory, Krakow, Poland) were acquired for the experiment. Upon arrival, the animals were housed for a week under controlled conditions —12 h light/12 h dark cycle and temperature of $22 \pm 2^\circ\text{C}$. Transparent cages were placed adjacent to each other to provide sight, acoustic, and odor contact. All cages contained suitable bedding materials and environmental enrichment. Animals were fed with standard dry chow: protein 25%, fat 8%, carbohydrates 67%, metabolizable energy 2.86 kcal/g (Labofeed B, Kcynia, Poland). Tap water was always available ad libitum.

The study has been complied with all the relevant national regulations and institutional policies for the care and use of animals after approval and under the supervision of the local Animal Welfare Committee of Jagiellonian University (protocol number 331/2019).

Experimental design

After an initial acclimatization period, female rats ($n = 24$) were randomly assigned to one of two groups:

1. Activity-based anorexia group (ABA group) — animals placed in cages with unlimited access to a running wheel (Tecniplast 2154F0105 Activity Cage System for Rats). The feeding schedule was restricted to 1.5 h per day. Food was distributed at the end of the light phase ($n = 18$).
2. Control group — animals placed in two separate standard cages with an ad libitum feeding schedule ($n = 6$).

Pain assessment for each animal subjected to the ABA group was performed twice — on the day the experiment started and on the last day of modeling (Fig. 1). To ensure that food availability was time-restricted according to the experimental design,

A GENERAL TIMELINE OF THE ABA EXPERIMENT

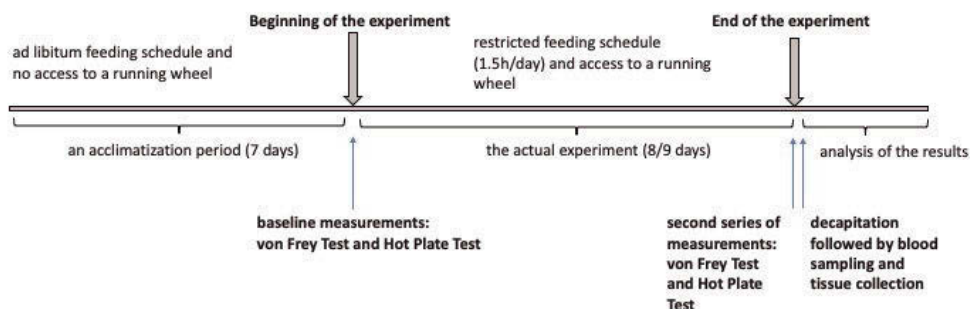


Fig. 1. The general timeline of the ABA protocol used in the study.

the cage bedding was refreshed immediately after the feeding, and the bottoms of cages were additionally wiped to remove all remaining food crumbs and dust.

The general health status of the experimental animals was evaluated daily during handling and by observing their in-cage behavior. At the same time, the handling of animals was kept to a minimum since ABA is an example of a biobehavioral phenomenon, and it was critical to minimize the amount of unpredictable stress and maximize comfort for experimental animals. Body weight (g) and physical activity were monitored once a day before feeding between 4 p.m. and 6 p.m. The physical activity of an animal was determined based on the number of turns in the wheel during 24 hours. The experiment was discontinued and animals were euthanized when the body weight loss exceeded 25% or humane endpoint criteria were met. ABA modeling was carried out in a series of 6 individuals each. Due to different rates of weight loss, one series was terminated on day 8 and the other two on day 9. Animals were first anesthetized with an injection of ketamine/xylazine and then euthanized by decapitation.

Biochemical analysis

Blood samples from the jugular vessels (trunk blood) were collected in plastic tubes immediately after decapitation and incubated for at least 30 min at 4°C to induce clot formation. After centrifugation at 1500× g for 20 min at 4°C (Megafuge 1.0R, Heraeus Instruments, Germany), serum samples were separated and kept frozen in small volumes at -20°C until further analysis. Samples were thawed immediately before the assays. All measurements were performed in duplicate.

Pain assessment methods

Baseline measurements were taken on the same day that ABA induction began. The second series of measurements were taken when the modeling was completed based on the criteria described above. First, an electronic Von Frey test was performed, followed by a hot plate test 4 hours apart.

The Von Frey Test

The electronic Von Frey test was performed using a Dynamic Plantar Aesthesiometer (Dynamic Plantar Aesthesiometer 37450; Ugo Basile, Italy). The device comprises a movable touch-stimulator, a microprocessor, a large testing surface (perforated metal platform) and a modular animal enclosure offering 6 spaces. To assess rat hind paw mechanical threshold, rats were first acclimatized to the plastic box with a metal wire mesh table for 15 min. The cessation of exploratory and grooming behavior was determined by the researcher and represented the end of the habituation period. The mechanical stimulus was delivered to the plantar surface of the hind paw from below the floor of the test chamber by an automated testing device operated by the researcher. A steel rod of diameter 0.5 mm was pushed against the hind paw with an ascending force of 0 to 50 g over a period of 50 s at a rate of 1 g/s. When the animal withdrew its hind paw, the mechanical stimulus automatically stopped, and the force at which the animal withdrew its paw was recorded to the nearest 0.1 g. Animals were subjected to 4–6 consecutive trials with at least 3–5 min intervals between the trials and the results were then averaged.

The Hot Plate Test

The hot plate test was performed using a hot plate equipped with a transparent plexiglass enclosure with a matching cover (Hot/cold plate 3510; Ugo Basile, Italy). Each measurement was preceded by an acclimatization period of approximately 15–20 minutes. Each animal was placed in a separate cage to acclimatize to the new environment. As with the Von Frey test, the end of the habituation period was determined by the experimenter and occurred when exploration and grooming behaviors ceased. Animals were individually placed on a hot plate preheated to 50°C. From this point, the time until the characteristic nociceptive response, which is the licking of the hind paw (can be combined with vocalization), was measured using a stopwatch. The result is a latency time and is specified in seconds. When the described reaction was observed, the animal was immediately removed from the hot plate to prevent skin damage and placed back in a separate cage. The hot plate surface was cleaned with 20% ethanol after each rat.

Histopathological analysis

Tissue processing

Fresh fragments of the proximal part of jejunum were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, routinely processed, and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine histology

The sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin (HE) to evaluate the gross tissue organization. Masson trichrome staining was performed to detect collagen deposits.

Immunofluorescence

After deparaffinization and rehydration, slides were incubated for 30 min in PBS with the appropriate normal serum and 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) at room temperature. This step was followed by overnight incubation at 4°C in a solution of PBS with the appropriate normal serum containing a primary antibody (or a mixture of primary antibodies) and 0.3% Triton X-100. After five washes (10 min each) in PBS, the specimens were incubated for 1 h at room temperature with a secondary antibody (or a mixture of secondary antibodies) diluted in PBS with the appropriate normal serum and 0.3% Triton X-100. Finally, the slides were washed in two changes (10 min each) of PBS and cover-slipped with a fluorescence mounting medium (Dako, Glostrup, Denmark). Some slides have 4', 6-diamidino-2-phenylindole (DAPI) nuclear counterstain from UltraCruz® Aqueous Mounting Medium with DAPI (catalog number sc-24941). Labeled specimens were analyzed immediately. Primary and secondary antisera used are summarized in Table 1.

Microscopic examination

All slides were examined using a BX43 Olympus epifluorescence microscope equipped with an Olympus DP74 digital CCD camera and CellSense software (Olympus Corporation). Digital images were collected at 200 or 400× magnification. The qualitative analysis of cells and nerve fibers was provided in 10 consecutive high-power fields of vision (400×) using Multiscan 18.03 (CSS, Warsaw, Poland), a computer-based image analysis system. To avoid bias, two independent specialists assessed all the samples (each blind to the other). Nerve cells and fibers were evaluated based on their morphology and specific immunomarkers positivity. PGP-, iNOS-, and ChAT-immunoreactivity was evaluated to assess the presence and distribution of different populations and subtypes of enteric nerves [32, 33].

Table 1. Type, sources, and dilution of antibodies. ChAT — choline acetyltransferase, PGP — P-glycoprotein, NOS — nitric oxide synthase, cFOS — phosphoprotein FOS, TH — tyrosine hydroxylase

Antibody	Catalog Number and Company	Dilution
Primary Antibodies		
Monoclonal mouse anti-ChAT	sc-55557, Santa Cruz, Dallas, Texas, USA	1:100
Polyclonal rabbit anti-PGP	Z5116, Dako, Glostrup, Denmark	1:100
Polyclonal mouse anti-NOS	sc-7271, Santa Cruz, Dallas, Texas, USA	1:100
Polyclonal rabbit anti-c-fos	sc-52, Santa Cruz, Dallas, Texas, USA	1:500
Polyclonal rabbit anti-TH	AB438RA01, Clone Cloud, USA	1:150
Secondary Antibodies		
Alexa Fluor 594 Goat Anti-Mouse	115-585-146, Jackson ImmunoResearch, Ely, UK	1:800
Alexa Fluor 488 Goat Anti-Rabbit	111-545-144, Jackson ImmunoResearch, Ely, UK	1:800
Alexa Fluor 488 Goat Anti-Mouse	115-545-146, Jackson ImmunoResearch, Ely, UK	1:800

Statistical analysis

Numerical results are presented in mean form with standard deviation (SD). The two-tailed paired or independent-samples Student's t-test were used to compare differences between two dependent groups. A one-way repeated measures ANOVA was performed in the ABA group to determine if there were statistically significant differences in body weight, food intake and wheel revolutions over the course of the experiment. If the assumption of sphericity was violated, as assessed by Mauchly's test of sphericity, a Greenhouse-Geisser correction was applied, followed by post hoc analysis with Bonferroni adjustment. The differences when $P < 0.05$ were considered statistically significant. The calculations were performed using IBM SPSS Statistics for Mac, Version 28.0. Armonk, NY: IBM Corp (licensed to Jagiellonian University).

Results

Reduction of body weight in relation to food consumption and physical activity

During the experiment, the weight of the rats in the ABA group decreased significantly over 8 days ($F(2.505, 42.584) = 284.923, P < .001$, post hoc analysis with a Bonferroni adjustment). The average body weight of the rats at the beginning of the study was 198.49 ± 3.77 g, and the final weight was about 164.89 ± 4.39 g, a reduction of 16.92%. Of the initial 18 animals, in 12 individuals, modeling was conducted for 9 days based on the termination criteria of the experiment. Their average weight loss was 18.12% (Fig. 2).

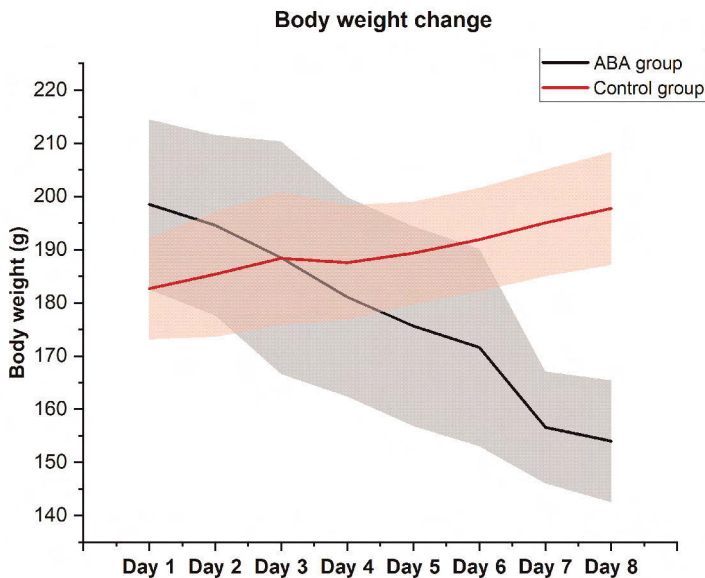


Fig. 2. Change in body weight in ABA and control animals during the experiment. Results are presented in grams as mean \pm SD.

The rodents in the ABA group ate less than the control group, but the portions consumed were increasingly larger on subsequent days of the experiment ($F(4, 68) = 21.22, P < .001$). This may be related to the increasing energy demand resulting from increased physical activity. Despite the increasing amount of food consumed daily, their energy requirements were not met.

Despite the conditions of time-limited access to food, the physical activity of animals in the ABA group was increasingly higher on successive days of the experiment ($F(1.869, 26.165) = 25.109, P < .001$). On the first day, the average number of revolutions in the running wheel was 3587.5 ± 1287 and on the last day, it already reached 27150 ± 7481 (Fig. 3).

Biochemical parameters

The mean results from measurements of biochemical parameters, their standard deviations, minimum and maximum values, and medians for the experimental and control groups are shown in Table 2. Concentrations of glucose, total calcium, alanine aminotransferase (ALT) and all lipid metabolism parameters were significantly lower in the serum of ABA rats compared to the control group. Given the small size of the control group, the low statistical power for albumin, total protein and AST levels determines the low reliability in interpreting the results for these parameters.

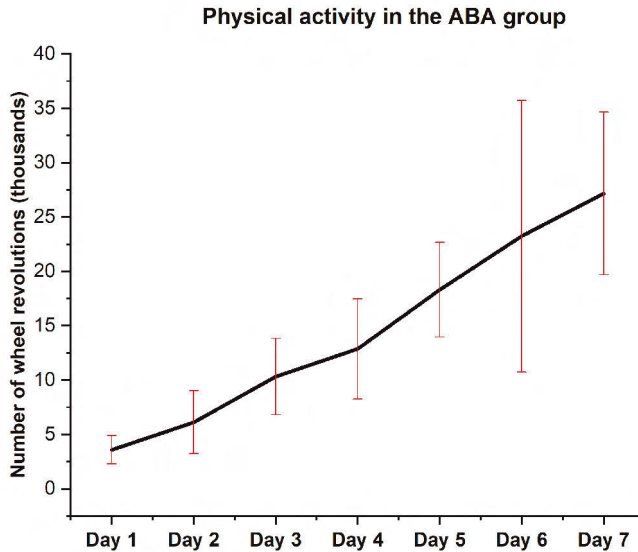


Fig. 3. Physical activity in the running wheel of animals in the ABA group. Results are presented as mean \pm SD.

Table 2. Biochemical analysis of the blood samples collected from 24 rats. Statistically significant difference between groups marked as: * — $p < 0.05$, ** — $p < 0.001$ (independent-samples t-test).

Parameter	Control group			ABA group		
	Mean \pm SEM	SD	Median (Min, Max)	Mean \pm SEM	SD	Median (Min, Max)
ALB (g/dL)	46.17 \pm 1.13	2.77	44.95 (43.9, 49.9)	45.92 \pm 0.64	2.62	45.3 (41.9, 51.3)
TP (g/dL)	60.75 \pm 1.05	2.58	59.75 (57.9, 64.3)	57.76 \pm 0.79	3.26	56.7 (52.8, 64.2)
ALT (U/L)	62.98 \pm 2.92	7.14	62.5 (54.9, 71.3)	22.39 \pm 0.84**	3.45	21.5 (17, 29)
AST (U/L)	169.5 \pm 13.43	32.9	183 (117, 199)	145.05 \pm 7.51	30.98	138 (87.9, 224)
Ca total (mmol/L)	2.58 \pm 0.03	0.07	2.57 (2.5, 2.68)	2.3 \pm 0.01**	0.06	2.31 (2.22, 2.42)
K (mmol/L)	5.04 \pm 0.11	0.27	5.1 (4.64, 5.32)	4.51 \pm 0.07*	0.3	4.53 (4.09, 4.94)
Na (mmol/L)	139.07 \pm 0.44	1.09	138.95 (137.5, 140.4)	141.95 \pm 0.34**	1.4	142.2 (138.9, 144.2)
GLC (mmol/L)	8.81 \pm 0.37	0.9	8.65 (7.46, 9.83)	4.78 \pm 0.25**	1.02	4.39 (3.38, 6.45)

Table 2. cont.

Parameter	Control group			ABA group		
	Mean \pm SEM	SD	Median (Min, Max)	Mean \pm SEM	SD	Median (Min, Max)
CHOL (mmol/L)	1.65 \pm 0.63	0.15	1.65 (1.44, 1.9)	0.98 \pm 0.04**	0.17	1.01 (0.63, 1.3)
HDL (mmol/L)	1.22 \pm 0.06	0.15	1.2 (1.06, 1.49)	0.68 \pm 0.03**	0.12	0.7 (0.46, 0.87)
LDL (mmol/L)	0.14 \pm 0.003	0.01	0.15 (0.13, 0.15)	0.11 \pm 0.004**	0.02	0.11 (0.08, 0.15)
TG (mmol/L)	2.07 \pm 0.25	0.6	2.12 (1.12, 2.99)	0.41 \pm 0.02**	0.07	0.4 (0.29, 0.53)

Histopathological examination of the intestinal tissue

In HE-stained preparations, a reduced thickness of the intestinal wall and mucosa was observed in rats of the ABA group (Fig. 4A) compared to the control animals (Fig. 4B). In addition, better organization in all layers of the intestinal wall in the control group

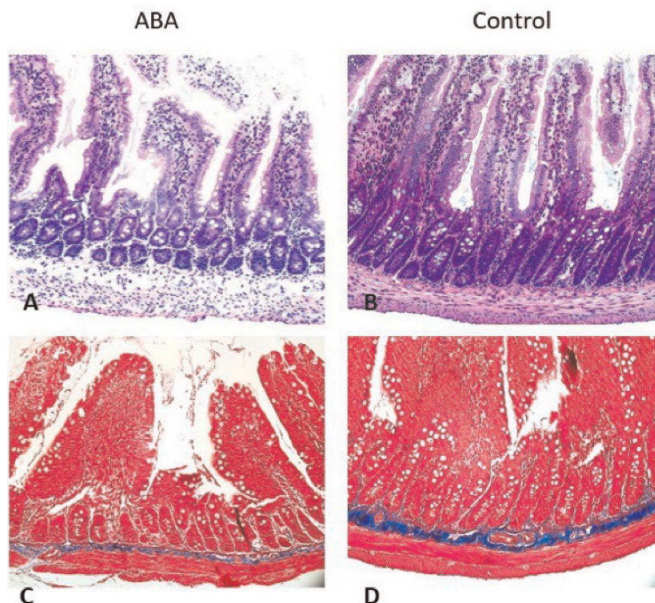


Fig. 4. Tissue samples of the small intestine from the ABA group (A, C) and the control group (B, D). HE staining (A, B) illustrates the gross tissue organization in both observed groups, while Masson Trichrome staining (C, D) — the ratio between different kinds of tissue within the intestinal wall. Muscle fibers are red, collagen — blue, and nuclei — black. Original magnification: 200 \times .

was also apparent. On the other hand, in tissues stained by the Trichrome-Masson method, no significant changes in structure were seen between the anorectic (Fig. 4C) and control animals (Fig. 4D).

Although gross morphology of the jejunal cross-sections did not show any significant inflammatory tissue alterations in ABA rats in comparison to control animals, sections from the ABA group (including unstained formalin-fixed paraffin-embedded sections) were characterized by high autofluorescence emission in blue and green spectra, noted in all layers of the intestinal wall, which could be an indication of pathology and can be seen in representative pictures (Fig. 5–8).

Decreased PGP 9.5-immunoreactivity (Fig. 6) in both enteric plexuses was observed in the samples from the ABA group in comparison with the control ones. PGP 9.5, also known as ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1), is a cytoplasmic

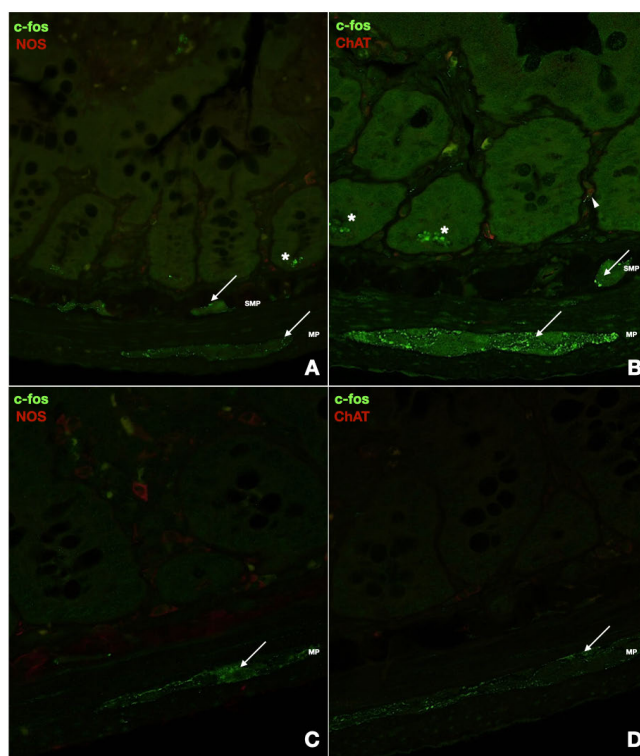


Fig. 5. Representative pictures of cross-sectioned jejunal samples obtained from ABA (A, B) and control (C, D) groups co-stained with antibodies against *c-fos* and either NOS (left panel) or ChAT (right panel). Original magnification 400× (objective 40×, NA 0.75). *C-fos* immunoreactivity is marked with arrows (enteric ganglia), arrow heads (activated cholinergic neurons), and asterisks (intestinal crypts). Abbreviations: *c-fos* — a protein product of a proto-oncogene used as a marker for neuronal activity, ChAT — choline acetyltransferase, MP — myenteric plexus, NOS — nitric oxide synthase, SMP — submucosal plexus.

protein widely used to visualize different populations and subtypes of nerves, including neurons of the enteric nervous system. Enteric ganglia in ABA rats were also found to be noticeably smaller and rounder.

C-fos-immunoreactivity (Fig. 5) was present in both myenteric and submucosal ganglia, yet the expression was variable between the experimental groups. Expression of the proto-oncogene product, c-fos, used as a marker of changes in neuronal activity, signals activity-dependent shifts in gene expression and may reflect adaptation of enteric nerves to external denervation [34]. What is more, double staining with antibodies against markers for nitrergic and cholinergic neurons revealed modest co-immunoreactivity suggesting that other neuronal subpopulations (or other cell types) are activated in the enteric ganglia. Interestingly, c-fos-immunoreactivity was also found at the base of the intestinal crypts.

NOS-immunoreactivity (Fig. 6) was detected in neuronal and non-neuronal cells, which was revealed by a double-staining with a neuronal marker PGP 9.5. Pictures from both blue and green spectra were added due to variable autofluorescence. Nitric oxide synthase (NOS) is used as a marker of nitrergic neurons. Nitrergic enteric neurons are key players in the descending inhibitory reflex of intestinal peristalsis. A decreased intensity of luminescence (including a lower density of enteric nitrergic neurons) was noted in the samples from the ABA group in comparison with the control samples.

ChAT-immunoreactivity (Fig. 7) was also detected in neuronal and non-neuronal cells, which was revealed by a double-staining with a neuronal marker PGP 9.5. Pictures from both blue and green spectra were added due to variable autofluorescence.

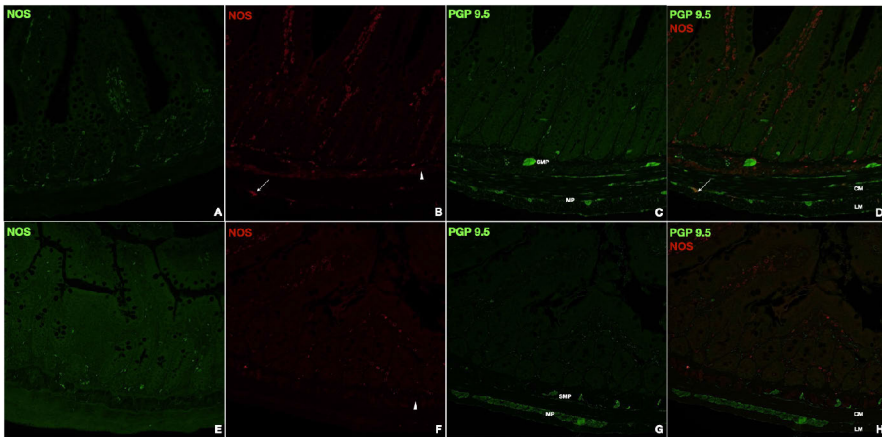


Fig. 6. Representative pictures of cross-sectioned jejunal samples obtained from control (A, B, C, D) and ABA (E, F, G, H) groups stained with antibodies against NOS (A, E) or NOS and PGP 9.5 (B, C, D, F, G, H). Original magnification 200× (objective 20×, NA 0.5). Arrows mark nitrergic neurons while arrowheads mark submucosal autofluorescence. Abbreviations: CM — circular muscle layer, LM — longitudinal muscle layer, MP — myenteric plexus, NOS — nitric oxide synthase, PGP 9.5 — a neuronal marker for neuronal activity, SMP — submucosal plexus.

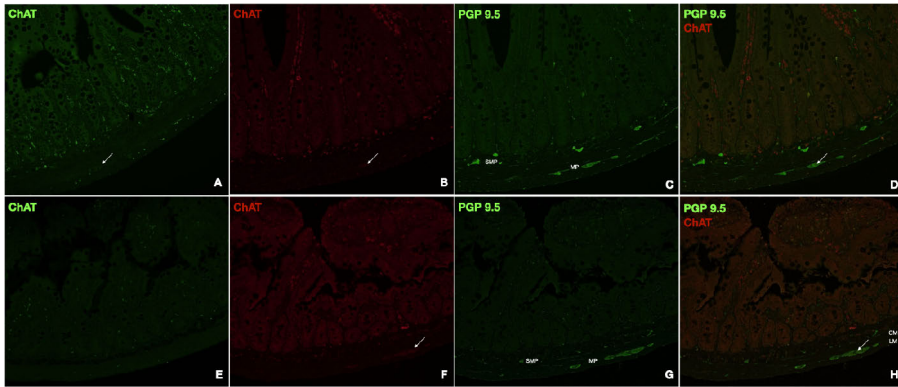


Fig. 7. Representative pictures of cross-sectioned jejunal samples obtained from control (A, B, C, D) and ABA (E, F, G, H) groups stained with antibodies against ChAT (A, E) or ChAT and PGP 9.5 (B, C, D, F, G, H). Original magnification 200× (objective 20×, NA 0.5). Arrows mark cholinergic neurons. Abbreviations: ChAT — choline acetyltransferase, CM — circular muscle layer, LM — longitudinal muscle layer, MP — myenteric plexus, PGP 9.5 — protein gene product 9.5, a pan-neuronal marker, SMP — submucosal plexus.

cence. Choline acetyltransferase (ChAT), the synthesizing enzyme for acetylcholine, is the definitive marker for cholinergic neurons. A decreased intensity of luminescence (including a lower density of enteric cholinergic neurons) was noted in the samples from the ABA group in comparison with the control samples.

TH-immunoreactivity (Fig. 8) was especially detected in the intestinal epithelium and enteric ganglia. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis and is used as a marker of catecholaminergic neurons, including dopaminergic ones. However, no differences were seen between the experimental groups, apart from an increased epithelial TH-immunoreactivity in the intestinal crypts in the ABA group.

Pain perception

A paired-sample t-test was used to evaluate pain thresholds measured by the Von Frey and hot plate tests. A significant reduction in latency time of 7.47 seconds was observed in the Von Frey test ($P = 0.001$; Fig. 9). The mean reaction time in the baseline measurement was $28.92 (\pm 1.69)$ seconds, while in the second measurement it reached $21.45 (\pm 0.86)$ seconds. In the hot plate test, there was a significant increase ($P = 0.013$) in latency time in the second measurement (86.78 ± 7.58 seconds) compared to the baseline measurement (69.28 ± 5.77 seconds) of 17.42 seconds (Fig. 10). There was no statistically significant difference between the two measurements in the control group in any of the tests performed ($P = 0.099$ for the Von Frey test and $P = 0.439$ for the hot plate test).

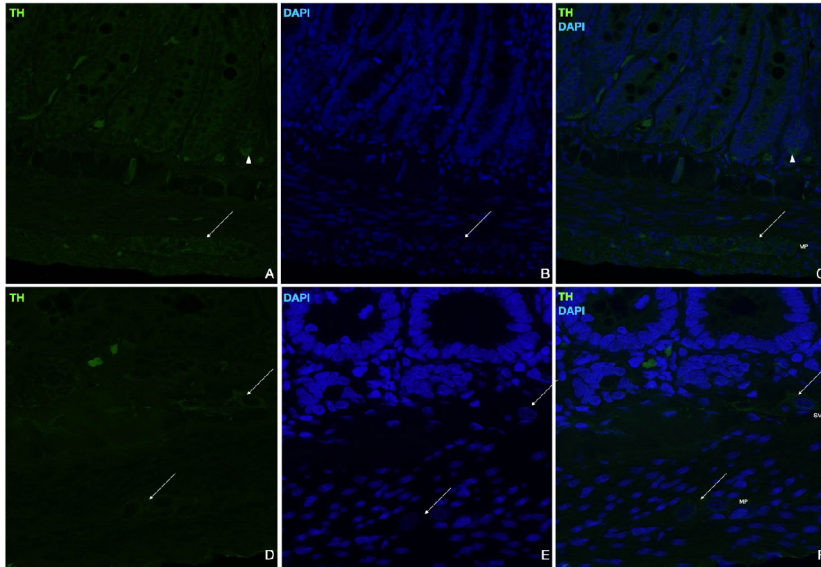


Fig. 8. Representative pictures of TH-stained and DAPI-counterstained cross-sectioned jejunal samples obtained from ABA (A, B, C) or control (D, E, F) groups. Original magnification 200× (objective 20×, NA 0.50). TH-immunoreactivity is marked with arrows (enteric neurons) and arrowheads (intestinal crypts). Abbreviations: MP — myenteric plexus, SMP — submucosal plexus, TH — tyrosine hydroxylase.

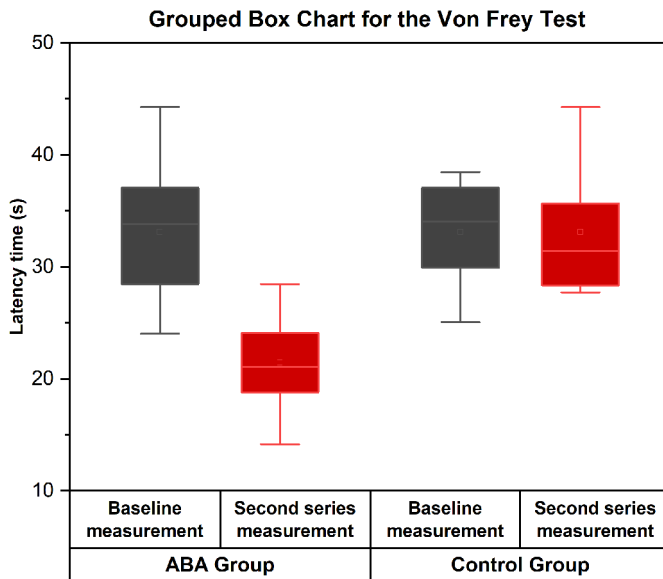


Fig. 9. Box plot for mean latency time in the ABA and control groups for the Von Frey test. The baseline measurements serve as reference measurements.

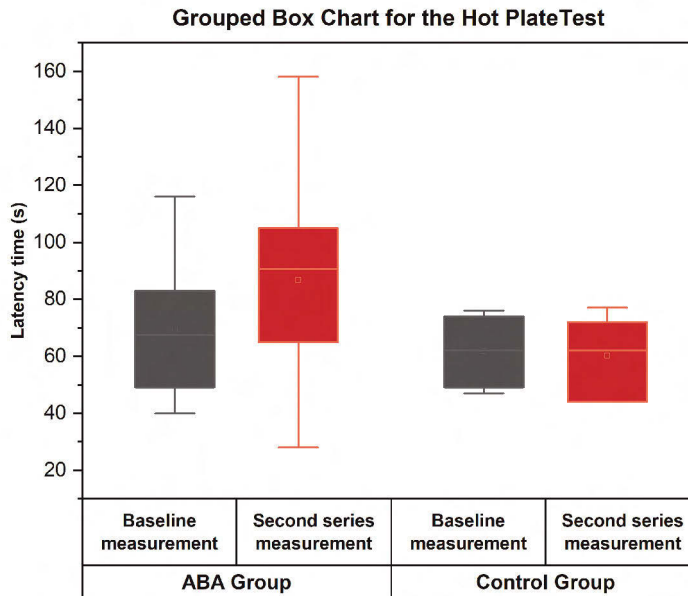


Fig. 10. Box plot for mean latency time in the ABA and control groups for the hot plate test. The baseline measurements serve as reference measurements.

Discussion

The nutritional status of an individual can be monitored by many different biochemical parameters. In our study, we focused on indicators of liver function, proteins, carbohydrates, lipid metabolism as well as electrolyte profile. The elevation of liver enzymes in AN patients, especially in groups with BMI values lower than 10–13, has been mentioned in several human studies [35–38]. The main proposed mechanism is liver damage due to starvation-induced autophagy, although initially, this process may be protective, it induces acute liver failure during prolonged starvation [35, 39, 40]. However, in our study, we did not observe elevated liver enzymes among the ABA rodents. We suggest that this is due to insufficient weight loss to cause liver damage. Other factors that may be responsible for the elevation of liver enzymes that are not present in the animal model are a reaction to refeeding [35, 38] and the adverse effects of antidepressants [41].

Physical activity can have a twofold impact on the level of ALT in humans. Some studies have shown that excessive exercise causes an increase in ALT levels [42, 43], while other scientific reports suggest that regular mild physical activity lowers the ALT level [44, 45]. In our study, the statistically significant lower level of ALT in the ABA group could have been caused by additional physical exercise, however, it is not consistent with our previous research on the animal model, which showed that the

liver enzymes were negatively affected by increased physical activity [30]. The ambiguous correlation between physical exercise and ALT levels could be the reason behind the different results in our studies.

Serum albumin level does not correlate with the BMI of AN patients [46, 47]. Even extremely malnourished individuals with this disease typically maintain an albumin level within the normal range. The exact biological mechanism behind this phenomenon has not yet been verified [48, 49]. Several human studies confirmed that individuals with AN have decreased total serum protein levels compared to healthy control [41, 50]. In our study, rats from the experimental and control groups had a similar albumin level, while rats from the ABA group had a slightly lower total protein level than the healthy control, although this difference was not statistically significant. The results of our study on protein metabolism were consistent with what has been observed in humans so far.

Several studies have shown a reduction in fasting glucose levels in patients with AN compared to control [41, 51, 52]. Furthermore, hypoglycemia is a common finding in severely malnourished patients with AN [53]. There are a few proposed mechanisms including excessive exercise, depletion of liver glycogen, ineffective gluconeogenesis and issues with glucagon secretion [54]. In our experimental group, the glucose level was significantly lower than in the control group, and severe hypoglycemia was also observed in ABA rats — this is consistent with our previous study on the ABA model [30]. It is well established that hypoglycemia in ABA rodents is caused by food restriction, whereas in exercised controls the glucose level is not significantly altered [55–57].

Typically AN patients have elevated concentrations of total cholesterol. Some studies also confirmed elevated levels of LDL and HDL among these individuals. The biological mechanism behind these changes is very complex and may be due to accelerated cholesterol metabolism, reduced catabolism and low concentrations of T3 [30, 49]. In our study, ABA rats had significantly reduced levels of all four parameters of the lipid profile (total cholesterol, HDL, LDL, TG) compared to the control group. This is the opposite of what is observed in people with AN. However, a low concentration of each of the components of the lipid profile is a common finding in the state of severe malnutrition. In our previous study on the ABA model we confirmed that both physical activity and food restriction significantly lower the level of triglycerides. Regarding total cholesterol, we observed that exercise alone significantly decreases its level, while restricted feeding alone is related to a non-significant decrease [30]. However, the impact of food restriction on lowering the total cholesterol level in rats has been confirmed in the literature [58].

Hyponatremia, hypokalemia, and hypocalcemia are common findings among subjects with AN in human studies [4, 49, 53]. In our study, we observed lower levels of potassium and total calcium in the experimental group — these results are con-

sistent with those reports. Semistarvation has been found to be a factor that causes a decrease in calcium levels in rats [59]. In contradiction to human studies, we noted a slight increase in sodium level — this may support the theory that the main reason for a lower sodium level in AN patients is usually dilutional hyponatremia due to excessive water digestion, which is sometimes deliberate to increase weight prior to weighing [49], a behavior not present in rats. In humans, there is also a phenomenon called natriuresis of fasting, while in rats during starvation the opposite mechanism, anti-natriuresis, has been observed [60]. Another reason behind the increase in sodium level, as well as the decrease in potassium level, may be altered signaling of the renin-angiotensin-aldosterone pathway. Studies are inconclusive on whether the basal aldosterone level is elevated or unchanged by food restriction in rats [61–63], however, it has been confirmed that dietary restriction sensitizes the distal colon of the rat to aldosterone [62]. Furthermore, there is evidence that the increase in aldosterone concentration after physical activity is greater in patients with AN than in healthy controls [64].

Currently available therapy implemented in the treatment of anorexia is characterized by a high rate of relapse up to 35–41% of patients [65]. In addition to identifying the pathophysiological core of this disease entity, one way to improve treatment outcomes may be to supplement conventional therapy with symptom-relieving interventions. Impaired gut motility, which is highly dependent on the proper functioning of the ENS, may exacerbate the typical spectrum of anorexia symptoms. For this reason, the identification of neuromorphological ENS abnormalities seems crucial in the creation of tailor-made therapeutic management. The extensive network of ENS multipolar neurons (sensory neurons, interneurons, motor neurons) can be classified based on their morphology, biochemical profile or biophysical properties [66]. Immunohistochemical techniques allow to group enteric neurons into two large populations (yet not exclusively), namely cholinergic and nitrergic neurons, immunoreactive for ChAT or NOS, respectively. Acetylcholine (ACh), synthesized by choline acetyltransferase, as a part of the cholinergic system, is a leading neurotransmitter of excitatory pathways in the ENS mediated by nicotinic (rapid) and muscarinic (slow) receptors. Nitrergic enteric neurons, which largely gather within the myenteric plexus, are chiefly responsible for descending inhibitory reflexes. In a healthy state, coordination between these enteric components ensures the neurotransmission necessary for effective intestinal peristalsis. Jejunal cross-sections from ABA rats were characterized by decreased both NOS- and ChAT-immunoreactivity. Yet, double-staining with a PGP 9.5 neuronal marker and c-fos, a marker of neuronal activity, suggests that other neuronal populations of the ENS were also altered and it was not a population of TH-immunopositive neurons. Noteworthy is the fact that activation of muscarinic receptors within glial cells is likely essential for normal gastrointestinal motility [67]. What is more, enteric glial cells can express c-fos in response to inflammation [68]. Thus,

activation of enteric glial cells cannot be ruled out. Our results are preliminary and should be further addressed in all parts of the intestine, with the use of markers for enteric glial cells as well. However, given the unique functions of the small intestine, especially in relation to pain perception, motility, and secretion, its ENS has developed individualized characteristics [69] and thus, sections from the small intestine were chosen as a starting point for the assessment of the influence of ABA on the ENS.

To date, reports on the function and structure of the ENS in AN patients are very limited. The issue is often collaterally addressed in reports focused on functional gastrointestinal diseases (also known as disorders of gut-brain interaction). Mainly because, despite their unknown etiology, AN and DGBI share many common features in terms of symptoms and association with psychological factors. Despite the lack of clear consensus, studies show increasing evidence of ENS pathology in DGBI including lymphocyte infiltration, neuronal degeneration and autoantibody production [70]. However, there is still a lot of uncertainty about the causal relationship between DGBI and eating disorders [18]. Moreover, in AN, a factor that appears to be potentially important in the development of gastrointestinal symptoms is poor diet resulting in severe electrolyte imbalance or use of specific medications like tricyclic antidepressants or laxatives.

Another factor with a recognized impact on the ENS function and structure is the gut microbiota [71]. Besides its influence on postnatal development, experimental depletion of microbiota leads to the degeneration of enteric neurons in adults. It has been recently shown that bacteria stimulate colonic neurogenesis in the ENS through toll-like receptors which appears to be of great importance in balancing neuronal apoptosis [72]. Dysbiosis, widely studied in the AN population, has been repeatedly shown to be characterized by reduced alpha-diversity but reports on the role of individual taxa are as yet inconclusive. As shown in studies on germ-free animals, also proper intrinsic and extrinsic neuronal excitability depends on healthy microbiota composition. Given that ABA also leads to disturbances in the composition of the microbiota, this may be a factor affecting, even despite its short duration, the functional and morphological aspects of ENS.

It has been suggested that AN pathophysiology is associated with a low-grade inflammatory state characterized by elevated concentrations of cytokines and autoimmunization that may affect gut-brain communication. As reported in a large population-based cohort study, the AN population is also at higher risk of developing inflammatory bowel disease (IBD), which is associated with neuronal damage [73]. On the other hand, experiments have shown that increased density of enteric neurons promotes local inflammation in the intestinal wall [74]. Our results indicate “hypoinnervation” in the ABA gut, which would rather determine impaired motility [75]. So far, a number of GI manifestations have been described among AN patients [17, 76], yet no direct links exist in relation to the ENS. Similarly, GI alterations have been

observed in the ABA model [77], yet again histological descriptions of the ENS abnormalities remain scarce.

ABA provides a phenomenon (i.e. excessive running, reduced food intake, and subsequent weight loss) that can be effectively used to model AN. To date, it is thought to be the best-fitting animal model analogous to anorexia nervosa, with clear behavioral and physiological similarities [78]. However, a throughout pain assessment of the model is still missing.

The Von Frey test is considered a gold standard for determining the mechanical pain threshold in rodents. It is rapid, simple and does not require animal restraint [79]. The electronic variation of the test provides a possibility to apply the continuous scale of stimulation, which objectifies the result. Our results suggest a decrease in the mechanical pain threshold in anorexic rats. Numerous mechanisms might be responsible for this phenomenon, such as central sensitization defined as hyperresponsiveness of the central nervous system to normal or subthreshold nociceptive stimuli [80]. Other possible mechanisms include alterations in pain modulation by descending pathways [81], peripheral sensitization, characterized by hypersensitivity of nociceptors due to changes in ion channels activation or levels of different chemicals like serotonin, histamine, bradykinin, prostaglandins but also calcitonin gene-related peptide (CGRP), substance P and nerve growth factor (NGF) [82–84]; and activation of silent nociceptors, which cannot be excited by physiological noxious stimuli but become sensitized to it in presence of inflammation [85, 86].

There is limited evidence on the mechanical pain threshold alterations in humans except for some single reports suggesting its elevation in patients with AN. It has been implied that a strong belief in self-control in these patients could result in the desire to maintain control over pain [28]. This factor is not present in rodents, which supports the possible psychological nature of the proposed elevated pain threshold in patients. However, evidence on this is elusive and further investigation in this field is needed. The major disadvantage of the Von Frey test is its non-specificity, which is caused by the fact that the flexor response in the rodent can also be triggered by non-nociceptive stimulation [87]. It is elicited by low-threshold mechanoreceptors in the paw [79]. For this reason, the test is used to assess the reaction to non-nociceptive stimuli in human studies [29].

The hot plate test is one of the most frequently conducted experiments to study the nociceptive response to fixed heat stimuli. It is rapid, inexpensive and allows repetitive testing on the same animals in a short duration without causing injury to the tissue [79]. Our results were consistent with research conducted so far on humans using radiant heat stimuli of constant intensity [22, 25, 28]. Anorexic rats showed a hypoalgesic profile compared to their prestarvation measurements. There have been many hypotheses on why the thermal pain threshold in AN is elevated. One idea was that an increase in opioid activity might be a possible mechanism. Nevertheless,

naloxone administration did not normalize pain thresholds in anorectic patients [88]. A different concept assumed that the state of starvation is responsible for such changes, but another study, which included subjecting healthy individuals to 21 days of high-calorie deficit, confounded it [89]. Several studies have indicated a connection between this phenomenon and decreased peripheral skin temperature [90, 91]. However, some more recent studies state that it has less influence than we used to think [23, 25]. Other researchers point to hormonal disturbances as a possible trigger of these changes. One study indicated that thermal pain threshold correlates negatively with the level of DHEA and positively with the cortisol/DHEA(S) ratio [22], while a different study revealed that levels of fT3 correlate negatively with it [23]. Moreover, the same piece of research showed an association of reduced thermal pain sensitivity with increased parasympathetic activity [23].

Noteworthy, most of the confounders known to us would decrease the latency time with repeated testing. First, the hot plate technique promotes learning behavioral responses, which decreases reaction times during subsequent exposures to heat stimuli. Sometimes this phenomenon can cause a reaction identical to a nocifensive one even on an unheated plate [79]. Furthermore, this test is sensitive to stress-induced analgesia, which can lead to extended latency time in naive animals, especially when they are poorly habituated [87].

Both tests used in the experiment are based on behavioral methods, which are characterized by subjectivity in the evaluation conducted by an operator. Stereotypical rat behavior can sometimes be confused with the pain response [79]. Finally, the reaction of the animals occurs on a spectrum of intensity, whether we note them as present or not [92]. These are aspects that significantly hinder the unambiguous interpretation of the results obtained by these methods and make the issue of pain sensation so puzzling.

Conclusion

The extent of abnormalities in the structure and function of the nervous system in AN extends beyond its central part and affects peripheral neurons that form the ENS. Exposition to ABA is associated with a reduction in the number and activity of enteric neurons. This is a potential cause of gastrointestinal dysfunction, which is inevitably included in the clinical picture of AN and can exacerbate the course of the disease. AN symptomatology in the field of neurohormonal signaling also includes the issue of altered pain perception. The ABA model promotes disequilibrium in the processes responsible for responding to mechanical and thermal stimuli. The evaluation of the former showed a decrease and the latter an increase in pain threshold. Thus, given the high degree to which peripheral complications of AN are mirrored by the animal model, these findings provide a framework for further research on humans.

Author contributions

Conceptualization, K.S. and P.S.; methodology, P.S., K.S., V.A., M.K.-Ł., M.J. and B.K.-C.; software, K.S, M.J. and P.S.; validation, K.S., M.K.-Ł.; formal analysis, P.S., K.S., V.A. K.C., M.G., and W.S.; investigation, P.S., V.A., and K.S.; resources, V.A., M.K.-Ł. and B.K.-C.; data curation, K.S.; writing—original draft preparation, K.S., P.S., K.C., M.G., and W.S.; writing—review and editing, K.S., M.K.-Ł., K.C., M.G., and W.S.; visualization, P.S., and W.S.; supervision, K.G.; project administration, K.S.; funding acquisition, K.G. All authors have read and agreed to the published version of the manuscript.

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The study was conducted in accordance with universally accepted ethical guidelines for animal research after approval and under the supervision of the local Animal Welfare Committee of Jagiellonian University (331/2019).

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare no conflict of interest.

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