

MORPHOMETRIC CHANGES OF THE MYENTERIC
PLEXUS IN THE COLON OF THE MICE-D-GALACTOSE
AGEING MODEL

Nikolay Genov^{1✉}, Nikola Tomov², Nikolay Dimitrov¹, Todor Kirov³,
Lubomir Petrov^{4,5}, Elina Tsvetanova⁴, Almira Georgieva⁴,
Albena Alexandrova^{4,5}, Nikolai Lazarov^{3,4}, Dimitrinka Atanasova^{1,4✉}

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Abstract

Ageing-related diseases are paramount for understanding the socio-economic impact of the postmodern society. Neuronal degeneration is a known phenomenon in ageing. The D-galactose model of accelerated ageing is widely used in age-associated neuronal cell death because of the oxidative stress it induces, which correlates to reduced cognitive performance and robustly detectable neuronal shrinkage in the CNS. This study aims at demonstrating the morphological changes of neurons of the myenteric plexus in mice treated with D-galactose. D-galactose was given orally with drinking water, resulting in an average dose of 500 mg/kg daily for six weeks. In the D-galactose accelerated ageing model, a significant size reduction of the neuronal perikarya of the myenteric plexus was observed all along the large intestine. The average soma area at the caecum in the control group was 305 μm^2 , which was reduced by almost 20% in the ageing model. In contrast, at the level of the proximal colon in the D-galactose ageing model, the average area of the neuronal soma was 280 μm^2 , a reduction of 30%. The most significant reduction has been observed at the

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level of the distal colon, where the neuronal soma was reduced by 40%. There is a significant reduction of the area of the neuronal bodies in the myenteric plexus among different parts of the mice's large intestine in the accelerated ageing model. It can be inferred that D-galactose-induced morphological changes in the myenteric plexus may be related to the increased gastrointestinal motility dysfunction with ageing.

Key words: myenteric plexus, D-galactose, ageing model, colon, mice

Introduction. The enteric nervous system (ENS) is a complex web of ganglia forming a well-built polysynaptic circuit. Many neuronal cells ensure the independent innervation of the gastrointestinal tract [1]. ENS is composed of almost half a billion enteric neurons, nearly the equivalent of the neurons in the spinal cord [2]. This complex meshwork of enteric ganglia between the longitudinal and circular layer is developing the myenteric plexus [3]. The gut wall muscles, the epithelial tissues, blood vessels and endocrine cells related to the gastrointestinal tract are innervated by this plexus [2,4].

The ageing of the gastrointestinal tract has been studied by many authors and their studies have demonstrated various changes related to its function and morphology. Prior studies have pointed to a significant decrease in myenteric neurons at the level of the esophagus and small and large intestines [5,6]. Some of them have reported that the loss of the neurons in older individuals could be compensated by a corresponding enlargement of the neuronal perikarya and their nuclei [5]. Other research has shown a decrease in the overall number of neurons per square cm. The duodenum presented the most significant amount of neuronal concentration loss, with a reduction of almost 40% [7]. Other studies conducted on guinea pigs have shown a decrease between 40 to 60% in the number of neurons of the myenteric plexus in the small intestines of the ageing models compared with young rodents [8]. In addition to the decreased concentration of neurons, ganglia with hollow spaces were reported to be more common in elderly patients. Cavity-rich ganglia were demonstrated to be increased in size, but the number of neurons that they contained was found to be significantly less. The studies have shown that the appearance of cavities could not be related to the general increase of the area of the intestines with age, as tests conducted on young patients with dilated sigmoid showed no significant increase in the number of ganglia with cavities [8].

Two methods, i.e. normal ageing or age-accelerating models could, be used to study the age-related changes in the gut [9]. The use of long-term injections of D-galactose is a well-known ageing model, with little to no side effects and a high survival rate for experimental animals [10,11]. Moreover, many authors consider D-galactose-induced accelerated ageing as a preferable ageing model as it mimics ageing-typical increases in oxidative stress and cognitive decline [12].

Material and methods. The studies were performed on male ICR mice. The animals were divided into a control group and a D-galactose group. D-galactose was given per os with drinking water, resulting in an average dose of

500 mg/kg daily for six weeks. The housing of the animals has been conducted under an artificial 12-h light/dark cycle and at a temperature of 22 °C. Water and food pellets have been given ad libitum. The experiments with animals were carried out in complete agreement with the Directive 2010/63/EU on protecting animals used for scientific purposes. To conduct the morphometric analyses, all animals were anaesthetized with 87 mg ketamine/kg body weight and 13 mg xylazine/kg after simultaneous intraperitoneal injection.

For the microscopic analyses, we made whole-mount preparations of the mice colon. Flat, whole-mount preparations were fixed into 4% PFA in 0.1 M PB and quickly before the microdissections were transferred into cold PBS. The large intestine was divided into the following segments: caecum, proximal colon, distal colon, and rectum. Each of these areas was cut into separate pieces using micro dissecting scissors with a length of roughly 2 cm. The separation of the myenteric plexus from the mucosa and the circular muscle layer was performed by dissecting under a desk magnifying glass with 300% magnification, removing the mucosa of the intestine and mounting the left serosa, muscle layers and the plexus on gelatin-coated slides, air-drying over an hour and consequent microdissection under stereoscopic microscope (Carl Zeiss 47 50 57 Stereo Microscope w/ Zeiss 46 40 03 W10X/25 Ocular Eyepieces) with microdissection tools. The microdissection aimed to remove the muscle fibres of the circular layer and leave the myenteric plexus attached over the longitudinal layer.

For the histological staining, we used a Nissl methodology. A research microscope Leica DM1000 equipped with a digital camera Leica DFC 290 was used to examine the slides and create images. All the images were processed with Adobe Photoshop 24.0 software.

The image analyses and the measurement of the surface area of the neurons were analysed with the software ImageJ (National Institutes of Health, Bethesda, MD, USA). Statistical analysis was performed using GraphPad Prism8[®] software (San Diego, CA, USA). We applied Dunn's multiple comparisons test to compare the results and considered $p < 0.05$ as significant.

Results. We first measured the sectional area of neuronal perikarya in the myenteric plexus located in the colon region. The tissue sections used for measurements were acquired from the level of the proximal blind pouch of the ascending colon called the cecum (CAE), proximal colon (PC), and distal colon (DC). After all dissections were completed, the samples were micro-dissected and prepared as whole-mount slides on chrome-gelatinized glasses.

The chosen intestinal segments were stained using the Nissl methodology (Fig. 1). This method allowed staining of myenteric neuronal perikarya simultaneously demonstrating fewer morphological features of the surrounding longitudinal muscle layer. The cresyl violet staining, in combination with the whole-mount preparation, allowed a more detailed and precise measuring of the neuronal soma. The non-parametric Kruskal–Wallis test for perikarya surface area with Dunn's

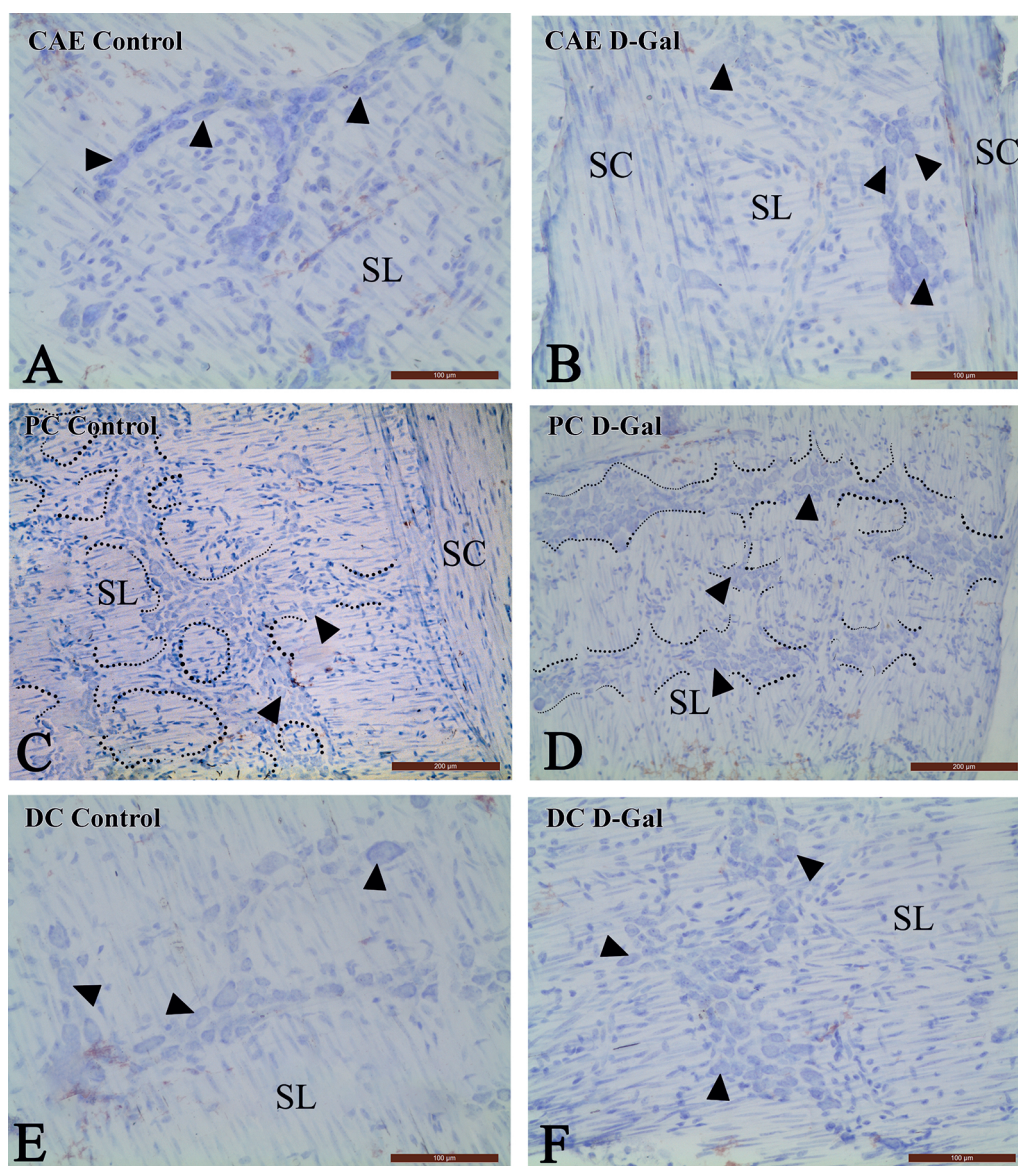


Fig. 1. Photomicrographs of Nissl-stained whole-mount preparations at the level of the caecum (CAE), proximal colon (PC) and distal colon (DC) from both the control group (A, C, E) and the D-galactose (D-Gal)-treated one (B, D, F). Note the ganglionated network of the myenteric plexus (dotted line) and perikarya of myenteric neurons (arrowheads), SL – *stratum longitudinale*, SC – *stratum circulare*. Scale bar = 100 µm (A, B, E, F), 200 µm (C, D)

multiple analysis was used to compare the medians (Med) of the control groups in three regions of the large intestine [cecum of the control group (CAE Control), proximal colon of the control group (PC Control) and distal colon from the control group (DC Control)] and their corresponding colon sections in mice treated with

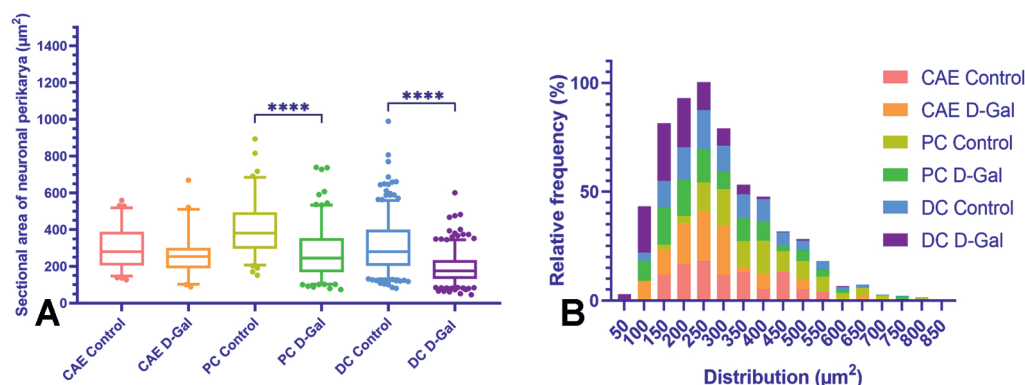


Fig. 2. (A) Box plot diagrams showing statistical comparison of the sectional area of neuronal perikarya in whole-mount preparations at the level of the caecum (CAE), proximal colon (PC) and distal colon (DC) from both the control group (CAE Control, PC Control, DC Control) and the D-galactose (D-Gal)-treated one (CAE D-Gal, PC D-Gal, DC D-Gal) from the ICR mice myenteric plexus. The data are presented as box plots, where the line within the box represents the median and the boxes represent the second and third quartiles (25–75%). Individual points are the corresponding values. The data compared using the Dunn's multiple comparisons test, where **** $p < 0.0001$. (B) Stacked bar charts showing the relative frequency in percentage of a distribution of values for the whole-mount area of neuronal perikarya

D-Galactose to induce an accelerated ageing model (CAE D-Gal, PC D-Gal and DC D-Gal).

The measurements of the neuronal soma from the myenteric plexus at the different levels of the large intestine of the control group and the D-galactose ageing model (D-Gal) showed significant differences in all of the three examined regions proven by the Kruskal–Wallis test $H(2) = 237.5$, $p < 0.0001$ (Fig. 2A). A statistically significant difference in the median of the neuronal cell bodies was presented between the PC Control ($402 \mu\text{m}^2$) and PC D-Gal ($277 \mu\text{m}^2$) and between DC Control ($310 \mu\text{m}^2$) and DC D-Gal ($190 \mu\text{m}^2$).

The frequency distribution test showed that 50% of the surface area of the myenteric plexus neurons in the caecum of the control animal (CAE Control) and the D-Gal ageing model group (CAE D-Gal) were in the field of $250 \mu\text{m}^2$ to $400 \mu\text{m}^2$ (Fig. 2B). At the level of the proximal colon, 57% of the neurons of the control group (PC Control) had an area ranging from $250 \mu\text{m}^2$ to $400 \mu\text{m}^2$. In contrast, in the ageing model (PC D-Gal), almost 60% of the neuronal soma ranged between $100 \mu\text{m}^2$ and $250 \mu\text{m}^2$. The measuring of myenteric perikarya of the distal colon showed that in the control animals (DC Control), half of the examined neuronal perikarya was in the field of $250 \mu\text{m}^2$ to $400 \mu\text{m}^2$. In the D-galactose ageing model, more than 70% of the examined neurons of the myenteric plexus were distributed from $50 \mu\text{m}^2$ to $200 \mu\text{m}^2$. The total number (n) of the examined nerve cell bodies was 1024.

Discussion. In this study, we have compared the neuronal perikarya at the three levels of the large intestine in control and experimental mice. The tissue samples were created using a whole-mount technique. This technique, although challenging to execute due to the constant rupturing of the myenteric plexus, as already reported by other authors [4, 13], allowed much more accurate measurement of the mean area of the neuronal soma. The most common perikaryal size reported in other studies has to be $200\text{ }\mu\text{m}^2$ to $400\text{ }\mu\text{m}^2$ [14] that is also confirmed in our study. Indeed, in our control groups, 65% of the neuronal perikarya in the myenteric plexus at the three regions of the colon ranged between $200\text{ }\mu\text{m}^2$ and $400\text{ }\mu\text{m}^2$.

The ageing gastrointestinal tract is characterized by various changes including decreased motility, reduction of the concentration of neurons per square cm, and increase of the average area of hollow ganglia [5, 7, 8]. We used two methods to study the age-induced changes in the myenteric plexus, natural ageing and accelerated ageing. We found that the first method is rather difficult, time-consuming and more expensive, whereas the accelerated models deliver better results and are faster and cheaper [9]. In addition, the D-galactose ageing model induces cognitive deficit, mitochondrial dysfunction, neurodegeneration and an increase in reactive oxygen species [9]. Elevated levels of oxidative damage in a tissue specimen have been reported by many authors as the leading cause of neurodegeneration [15].

We found that the average surface area of the neuronal soma at the level of the colon of the D-galactose treated mice shows a statistically significant decrease. The biggest difference observed in this study is at the level of the proximal and distal colon in which almost 70% of the neuronal perikarya are distributed between $100\text{ }\mu\text{m}^2$ to $200\text{ }\mu\text{m}^2$. This statistically significant decrease corresponds to already reported age-related changes in the myenteric plexus.

In conclusion, D-galactose-induced changes in the morphology of the myenteric plexus may be related to the increased gastrointestinal motility dysfunction in the elderly. Moreover, it seems that the influence of D-galactose causes a significant reduction in the surface area of the neuronal perikarya of the myenteric plexus at the level of the proximal and distal colon. Therefore, the use of D-galactose to generate a model of accelerated ageing could be fast and reliable method for studying the ageing myenteric plexus.

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¹*Department of Anatomy, Faculty of Medicine, Trakia University,
11 Armejska St, 6000 Stara Zagora, Bulgaria
e-mails: nikolay.genov@trakia-uni.bg, nikolay.dimitrov@trakia-uni.bg,
dimitrinka.atanasova-dimitrova@trakia-uni.bg*

²*Institute of Anatomy, University of Bern, Baltzerstrasse 2, 3012 Bern, Switzerland*
e-mail: nikola.tomov@unibe.ch

³*Department of Anatomy and Histology, Faculty of Medicine, Medical University of Sofia,*
1 St. G. Sofiiski St, 1431 Sofia, Bulgaria
e-mails: nlazarov@medfac.mu-sofia.bg, tkirov@medfac.mu-sofia.bg

⁴*Institute of Neurobiology, Bulgarian Academy of Sciences,*
Akad. G. Bonchev St, Bl. 23, 1113 Sofia, Bulgaria
e-mails: d.atanasova@inb.bas.bg, nlazarov@bio.bas.bg, e.tsvetanova@bio.bas.bg,
l.petrov@inb.bas.bg, a.georgieva@inb.bas.bg, a.alexandrova@inb.bas.bg

⁵*National Sports Academy "Vassil Levski",*
21 Akad. Stefan Mladenov St, 1700 Sofia, Bulgaria
e-mails: dr.lubomir.petrov@gmail.com, a_alexandrova_bas@yahoo.com