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Original article

# Tripeptidyl peptidase I activity in porcine lumbar spinal ganglia – a histochemical study

A.P. Vodenicharov<sup>1</sup>, M. Dimitrova<sup>2</sup>, N.S. Tsandev<sup>1</sup>, I.S. Stefanov<sup>3</sup>

<sup>1</sup> Department of Veterinary Anatomy, Histology and Embryology, Faculty of Veterinary Medicine Trakia University of Stara Zagora, Bulgaria,

<sup>2</sup> Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Science, Sofia, Bulgaria,

<sup>3</sup> Department of Anatomy, Faculty of Medicine, Trakia University of Stara Zagora, Student Town 6000, Bulgaria

## Abstract

Distribution of tripeptidyl peptidase I (TPPI) activity in the structures of porcine lumbar spinal ganglia (LSG) was studied by enzyme histochemistry on cryostat sections from all the ganglia using the substrate glycyl-L-prolyl-L-methionyl-5-chloro-1-anthraquinonyl hydrazide (GPM-CAH) and 4-nitrobenzaldehyde (NBA) as visualization factor. Light microscopic observations showed TPPI activity in almost all the LSG structures. The enzyme reaction in different cell types was compared semi-quantitatively. Strong reaction was observed in the small neurons, satellite ganglia cells and some nerve fibers. Weak reactivity was found in the large sensory somatic neurons, whereas moderate reaction for TPPI was determined in the middle sensory somatic neurons and some nerve fibers. Statistical analysis by one-way ANOVA showed no significance of difference (when  $p < 0.05$ ) for the number of TPPI positive neurons per  $\text{mm}^2$ . The original data obtained by the enzyme histochemistry method give us a reason to presume that TPPI actively participates in the functions of all the neuronal structures in porcine LSG. According to our results, it could be suggested that TPPI activity is important for the functions of autonomic and somatic sensory neurons.

**Key words:** enzyme histochemistry, pig, spinal ganglia, tripeptidyl peptidase I

## Introduction

Tripeptidyl peptidase I (TPPI, EC 3.4.14.9) is a lysosomal serine peptidase widely expressed in the central nervous system (CNS) and other organs of mammals and humans. It cleaves off tripeptides from oligo- and polypeptides, participates in the hydrolysis of collagen and

regulates the activities of a number of neuropeptides and peptide hormones (Kida et al. 2001, Koike et al. 2002, Dimitrova et al. 2009, Atanassova and Lazarov 2015).

TPPI activity is crucial for the neuronal functions. Its genetically determined deficiency causes the classical form of late-infantile neuronal ceroid lipofuscinosis (LINCL), a severe neurological disorder leading

to a shortened life expectancy (see e.g. Steinfeld et al. 2002). Data about the enzyme distribution in the central and peripheral nervous system of different mammalian species would be useful both for elucidation of the enzyme functions and for the application of animal models of LINCL and other neurological diseases (Dimitrova et al. 2017b). The expression of TPPI in laboratory animals and in human autopsy material has been studied by immunohistochemical and biochemical methods (Kida et al. 2001, Koike et al. 2002). Histochemical methods for the visualization of the enzyme activity were developed in our laboratory (Dikov et al. 2000, Ivanov et al. 2009). It has been shown that positive staining for TPPI is distinct in many cell types in the CNS, including not only neurons but also vascular endothelial cells, glial cells, ependymal cells, epithelial cells of choroid plexuses, and meningeal cells. The localization pattern of TPPI in the CNS of rats and mice were essentially similar to those in humans (Kida et al. 2001, Kurachi et al. 2001).

Information on the distribution of TPPI in pig organs and tissues is very limited and includes only a small amount of data on *Sus scrofa* (wild boar) (McDonald et al. 1985), whereas in the domestic pig (*Sus scrofa domesticus*) the enzyme has hardly been studied. A case of LINCL in a Vietnamese pot-bellied pig has been described, in which the symptoms are quite different from those in human disease, but the brain damage caused by the enzyme deficiency is no less serious (Cesta et al. 2006). Studies on the importance of TPPI for the functioning of both central and peripheral nervous system are based on animal models (Bond et al. 2013). With the publication of the pig genome, it became clear that the domestic pig could be successfully used as a model system for the study of various human diseases (Lunney 2007).

In our research, we focused on dorsal root ganglion (DRG) neurons. DRG neurons are the first neurons of the sensory pathway. They are activated by a variety of sensory stimuli that are then transmitted to the CNS. In vertebrates, all somatosensory pathways (with the exception of those coming from the head) begin with the activation of DRG neurons. These neurons are responsible for thermoreception (sensing temperature), nociception (feeling pain), mechanoreception (sensing pressure), and proprioception (sensing body spatial position) (Nascimento et al. 2018). Both somatic and autonomic sensory neurons also send collaterals to pre-vertebral sympathetic ganglia (Matthews and Cuello 1982, Aldskogius et al. 1986). The soma of that primary afferent neurons is surrounded by sheaths of cells known as satellite glial cells (SGCs; Pannese 1981, 2010). This unique arrangement of SGCs allows them to exert a highly regulated control over the neuronal

microenvironment. SGCs may represent a reservoir of multipotent cells that could replenish damaged neurons within the ganglia.

Recently, data have been accumulated on the expression of a number of biologically active peptides and enzymes in porcine lumbosacral DRG neuronal subpopulation: calcitonin gene-related peptide (CGRP), substance P (SP), neuronal nitric oxide synthase (nNOS), nicotinamide adenine dinucleotide phosphate diaphorase, neurofilament 200kDa (NF200), transient receptor potential vanilloid 1 (TRPV1) and isolectin B4 (IB4), pituitary adenylate cyclase-activating polypeptide, neuropeptide Y (NPY), somatostatin (SOM), and vasoactive intestinal polypeptide (VIP) which indicate that the lumbar and sacral pathways play different roles in sensory transmission, especially in the urinary bladder (Russo et al. 2013, Pidsudko 2014, Stefanov et al. 2017, Kozłowska et al. 2018a). This intensified interest is due to the understanding that perturbing of signaling pathways between the SGC and the neuronal soma as well as between adjacent SGCs, can lead to the development of novel therapeutics for dealing with neuropathic pain (George et al. 2018).

The aim of the present study was to determine TPPI distribution in the structures of porcine lumbar spinal ganglia (LSG). The results can help to elucidate the enzyme importance for different sensitive sensory functions of those ganglia and may be useful in view of the increasing significance of domestic pig in biomedical research.

## Materials and Methods

### Lumbar ganglia

Thirty six entire spinal lumbar ganglia (LSG) were obtained from 6 (3 males and 3 females) 6 month-old, 90 – 100 kg b. w. pigs slaughtered in a licensed abattoir for a meat consumption in accordance with the European Union's and Bulgarian legislations.

### TPPI histochemistry

The entire right and left LSG were removed immediately after slaughtering the animals and were fixed in 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.0 for 20 hours at 4°C. After that, they were washed in modified Holt's solution (20% sucrose and 1% gum Arabic in distilled water) for 48 hours at 4°C, frozen in liquid nitrogen and cryostat sections (10 µm) were prepared using cryotom Reichert-Jung 2000M (FRG). The sections were mounted on glass slides, covered with 0.5% collodion solution in amyl acetate (Sigma-Aldrich, Taufkirchen, Germany) and incubated in acetate buffer (pH 4.5) containing 0.5 mM of the substrate

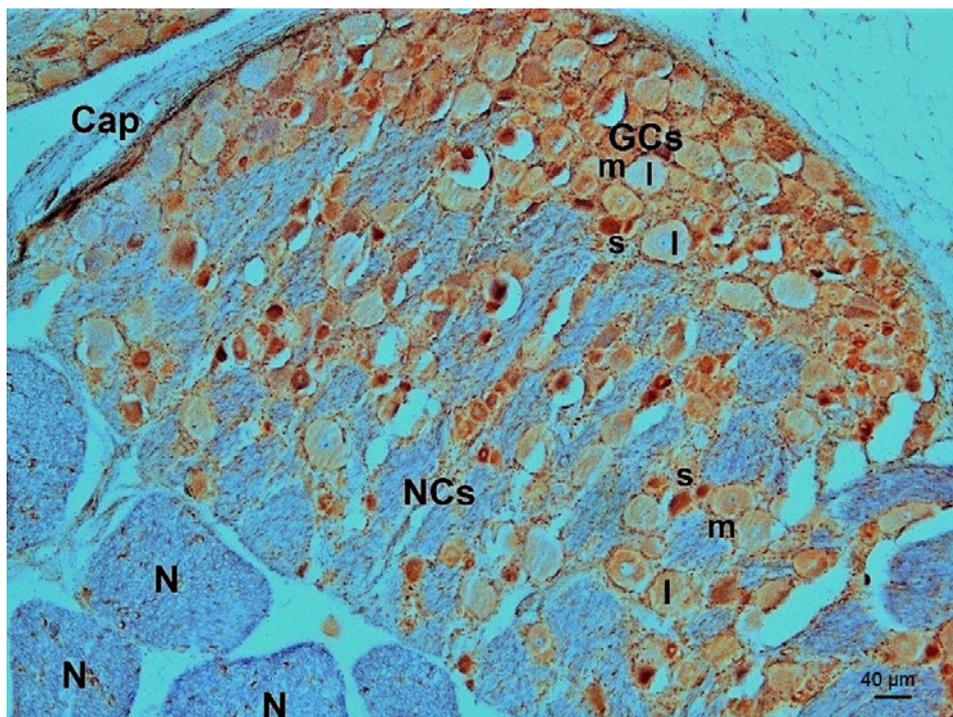


Fig. 1. Left Vth lumbar ganglion. Cap – capsula; GCs - zone of concentration of the neurons; NCs – columns of nerve fibers and neurons and N – nerves. Different degrees of tripeptidyl peptidase I activity in the small (s), middle (m) and large (l) neurons. Bar = 40 µm.

GPM-CAH and 0.1 mg/ml 4-nitrobenzaldehyde (NBA) for 70 minutes at 37°C. Finally, the sections were fixed in buffered 4% formaldehyde for 15 minutes at room temperature, slightly counterstained with hematoxylin according to the standard procedure and embedded in glycerol/jelly (1:1) (Dikov et al. 2000). Control sections were prepared in the same manner, but the incubation medium was supplied with 1.0 µM TPPI specific inhibitor AAP-CMK. Detailed description of the histochemical method as well as the application of TPPI inhibitor can be found in Dimitrova et al. (2017a). All the sections were studied under the microscopes Leica – DM 1000 and DM 5000B.

### Reagents

The substrate – glycy-L-prolyl-L-methionyl-5-chloro-1-anthraquinonyl hydrazide (GPM-CAH) was synthesized exactly as described previously (Dikov et al. 2000). The TPPI specific inhibitor Ala-Ala-Phe-chloromethylketon hydrochloride (AAP-CMK) was purchased from Bachem (Switzerland). All the other chemicals were from Sigma-Aldrich (Germany).

### Statistical analysis

The number of TPPI-positive neurons per mm<sup>2</sup> was evaluated using a light microscope (Leica DM1000), digital camera (Leica DFC 290) and software (LAS V4.10.0 2016). The data were processed by GraphPad

Prism 6 for Windows (GraphPad Software, Inc., USA) using one-way analyses of variance (one-way ANOVA). The results after using Tukey-Kramer's post-hoc test are presented as mean ± SD. *p*-values < 0.05 were considered statistically significant. The percent of positive neurons and the degree of positive reaction in satellite cells and vascular endothelium, were also estimated.

The activity of TPPI in different types of cells was estimated semiquantitatively by visually assessing the color (from light brownish to dark brown) of the enzyme reaction product and the number of TPPI-positive granules, as follows: +++ strong (many dark-brown granules in the cytoplasm), ++ moderate (substantial quantity of light brown granules) and + weak reaction (light brownish slightly granulated product).

Neurons (with a visible nucleus) were classified according to their size in the pig (Bossowska et al. 2009), as follows: small (diameter up to 30 µm), medium-sized (diameter from 31 to 50 µm) and large neurons (diameter > 51 µm).

The study has been approved by Commission of human affair to animals at the Faculty of Veterinary Medicine, Trakia University, Stara Zagora (Bulgaria).

### Results

Light microscopic observations showed a concentration (as a compact body cell mass) of the three types of positively reacted spinal sensory neurons under the

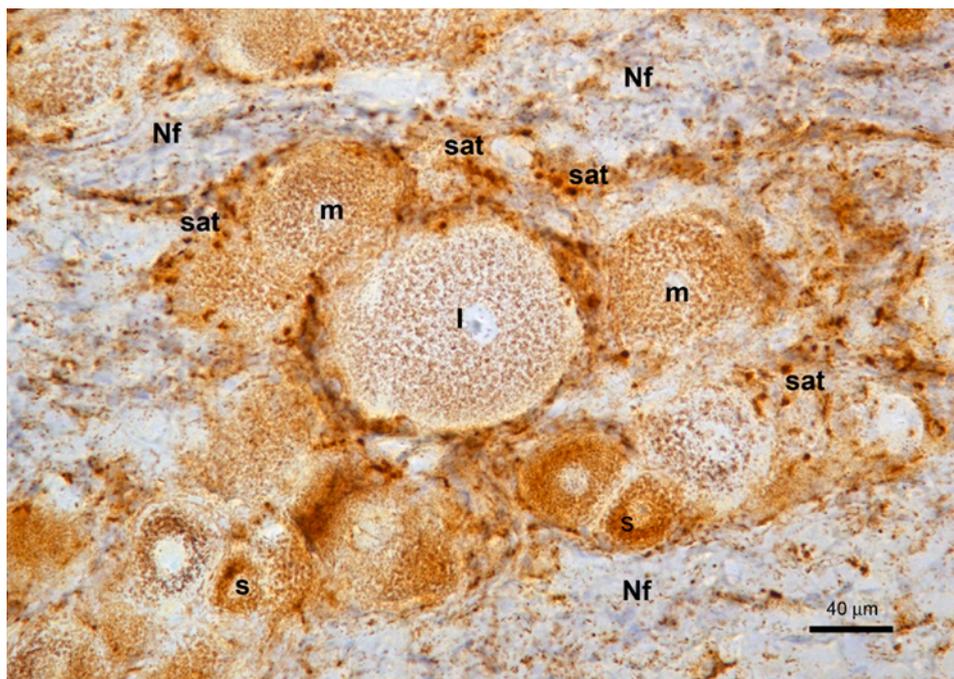


Fig. 2. TPPI activity in large (l), middle (m) and small (s) sensitive ganglia neurons, satellite cells (sat) and nerve fibers (Nf). Bar = 40  $\mu$ m

Table 1. Number of TPP positive neurons/mm<sup>2</sup> in spinal ganglia in male and spinal ganglia in female pigs.

Size of neurons	Number of TPP neurons in Spinal ganglia of male pigs	Number of TPP neurons Spinal ganglia of female pigs
Large	1 $\pm$ 0.0	1.33 $\pm$ 0.58
Medium	11.33 $\pm$ 1.53 ***	9.67 $\pm$ 0.58 ***
Small	23.67 $\pm$ 3.51 ****	24.00 $\pm$ 2.00 ****

\*\*\*\*  $p < 0.0001$ ; Statistical significant difference between the number of small and medium neurons

\*\*\*  $p < 0.001$  Statistical significant difference between the number of medium and large neurons

ganglionic capsule. Four times larger “mixed” zone was visible, consisting of column-like arrangement of nerve fibers and sensory neurons located between them. It should be noted that all the three types of neurons – small, middle and large were found in the “mixed” zone. The nerve fibers were seen to contain rarely distributed TPPI positive granules (Fig. 1). TPPI was differently distributed in the LSG structures (Figs. 1 and 2). The bodies of small sensory neurons (corpus neuroni, perikaryon) showed a strong enzyme histochemical reaction. The middle sensory neurons showed moderate degree of the expression, while the perikarya of the largest neurons exhibited comparatively weak reaction (Fig. 1). TPPI activity in the nerve fibers was estimated as moderate to strong (Fig. 2).

The reaction product within the body sensory neurons was demonstrated as fine brown to dark-brown granules. The granules were almost equal in size and were regularly distributed between the nucleus and cytolemma, filling up the entire cytoplasm. TPPI reaction in satellite cells surrounding all types of neurons

was strong (Figs. 1 and 2). Endothelial cells of ganglia vessels had a moderate to strong enzyme reaction.

Additionally, no differences were observed between ganglia of male and female animals.

The measurements showed that the number of TPPI positive small neurons was the highest, followed by the number of medium and large ones in both spinal ganglia (Table 1). There was no statistical significance between the parameters of both spinal ganglia.

*Control sections:* The inhibitor used in the control sections is specific for the enzyme and suppresses fully its activity. No residual enzyme activity was observed on the sections and no specific background was formed.

## Discussion

The results of our study demonstrate for the first time the histochemical localization of TPPI within porcine lumbar spinal ganglia at light microscopic level. Almost all the ganglia structures showed TPPI activity,

which varied from strong to moderate and weak. It can be assumed that the enzyme is involved in the intracellular degradation of neurotransmitters and/or other reactive proteins. The strong reactivity in satellite cells, surrounding all the three types of sensory neurons deserves more attention in view of the recent knowledge about their structure and functions. It is known that the distance between satellite cells and neuronal surfaces is about 20 nm (Panesse 1981), and therefore the extracellular space around the neurons is very small. The neurons send numerous fine processes (microvilli), some of which fit into invaginations of SGCs (Panesse 2002). The role of these structures is little understood yet, but they increase the neuronal surface area considerably (by 30–40%), and may allow an extensive exchange of chemicals between the two cell types. This unique arrangement allows them to exert a highly regulated control over the neuronal microenvironment. The activation of SGCs might in turn influence neighboring neurons. Thus, SGCs are likely to participate in signal processing and transmission in sensory ganglia. Damage to the axons of sensory ganglia is known to contribute to neuropathic pain. Such damage also affects SGCs, and it can be proposed that these cells have a role in pathological changes in the ganglia (Hanani 2005, George et al. 2018). The DRG are a novel target for neuromodulation, and DRG stimulation is proving to be a viable option in the treatment of chronic intractable neuropathic pain (Esposito et al. 2019). It would be useful to follow up the possible changes in TPPI activity throughout such pathological alterations.

According to Kida et al. (2001) distribution of TPP I in human tissues under normal and pathological conditions suggests that it is involved in general protein turnover and that its expression may be controlled by various regulatory mechanisms, which highlights the importance of this enzyme for normal functioning of human cells and organs.

In humans, TPP I constitutes an integral part of the lysosomal proteolytic apparatus, which includes numerous hydrolytic enzymes, mostly cysteine proteases but also serine and aspartic proteases. The combination of endo- and exopeptidase activities of these enzymes allows for efficient digestion of the diverse proteins transported to the lysosomes, releasing free amino acids and dipeptides that are transported back to the cytoplasm and reused according to the metabolic needs of the cell (Gołabek 2006). On the other hand, the enzyme also participates in a number of pathological processes including neurodegeneration (Dimitrova et al. 2017b). Our findings together with the well-known similarities between enzymatic systems of humans and domestic swine (Schook et al. 2005, Kuzmuk and

Schook 2011) give us a reason to suppose that TPPI in the pig fulfils the similar/or same functions as in humans. According to the present results from our study it could be presumed that the method initially elaborated for rodent TPP (Dikov et al. 2000, Ivanov et al. 2009) is useful also for TPPI analyses in the domestic pig.

In conclusion, the original data about distribution and activity of TPP in porcine LSG, obtained in this study, convincingly demonstrate that the enzyme is involved in the functions of all the neuronal types and satellite cells. Its high activity in small neurons and ganglionic satellite cells gives a reason to hypothesize that these two cell populations play a key role in sensory functions and in the maintenance of local microenvironment. In the latter probably and the vascular endothelial cells are also involved.

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