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Distribution of cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and galanin (GAL) in the pterygopalatine ganglion of the domestic duck (*Anas platyrhynchos f. domestica*)

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Abstract

Introduction. Cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and galanin (GAL) act as neurotransmitters and neuromodulators in both the central and peripheral nervous systems. Their presence has been found in different taxonomic groups, in particular in mammals. However, only few investigators have studied these neuropeptides in the class *Aves* (birds). The aim of the present study was to describe the distribution of CART, NPY and GAL in the pterygopalatine ganglion (PPG) of the domestic duck (*Anas platyrhynchos f. domestica*).

Material and methods. The experiment was conducted on 16 one-year-old domestic ducks of the Pekin breed of both sexes (8 males and 8 females). Frozen sections of the PPG were subjected to immunofluorescence staining using primary mouse monoclonal antibodies directed against CART and GAL and rabbit polyclonal antibody directed against NPY. Secondary antibodies were conjugated with Cy3 and FITC fluorochromes.

Results. CART, NPY, and GAL were present in the PPG of the domestic duck. The highest immunoreactivity (IR) in the ganglionic cells was found for CART in the majority (83–85%) of neurons of both superior (SPPG) and inferior (IPPG) PPG. CART-IR was also found in small aggregations of neurons on the medial surface of the Harderian gland, and on the course of the palatine branch of the facial nerve. CART-IR was also observed in the nerve fibers of these neurons' aggregations; however, it was low in comparison to the immunoreactivity of the perikarya. Immunoreactivity of NPY was found in ganglionic neurons, but above all in numerous fibers of the SPPG and IPPG and within aggregations on the surface of the Harderian gland. NPY-IR cells were distributed irregularly over the cross-sections of the tested aggregations, and constituted from 36% to 43% of the SPPG and from 37% to 40% of the IPPG of all cross-sectioned neurons. GAL-immunoreactive perikarya, distributed irregularly across the sections, were observed in the SPPG, where they constituted 61–65%, and in the IPPG, where they made up 50–57% of all neurons. All immunoreactive neurons were characterized by immunopositive neuroplasm and immunonegative cell nuclei.

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Conclusions. The presence of CART, NPY, and GAL in the PPG of the domestic duck suggests that these peptides may contribute to the secretory innervation of the glands of the mucosa of the palate and nasal cavity, the Harderian gland, and the lacrimal gland. *(Folia Histochem Cytobiol. 2016, Vol. 54, No. 1, 25–31)*

Key words: domestic duck; pterygopalatine ganglion; immunoreactivity; CART; NPY; GAL

Introduction

According to the classical approach, the pterygo-palatine ganglion (PPG) in birds is a parasympathetic structure; however, sympathetic and sensory components can be also distinguished. In birds, this ganglion is topographically and functionally related to the Harderian gland and to the palatine branch of the facial nerve. The Harderian gland is very well developed in *Aves* and is located in the nasal part of the orbit [1, 2]. It performs two important functions: produces oily substance that wets the nictitating membrane and plays a role in the local immune system of the oculonasal region, as evidenced by the presence of lymphoid tissue and immunoglobulins (IgA, IgG and IgM) [3–5]. The PPG primarily innervates in a secretory fashion Harderian gland, the lacrimal gland, and the glands of the mucosa of the palate and nasal cavity [1, 2]. In the previous study [2] it was shown that in domestic duck, the PPG is made up of many neuron aggregations of different shapes and sizes. Among the numerous small aggregations of ganglionic cells that occur in the palatine branch of the facial nerve and the medial surface of the Harderian gland, two large aggregations are distinguished: the superior ganglion (SPPG) and the inferior ganglion (IPPG), named by their location in relation to the gland. The SPPG, which is typically star-shaped and sometimes triangular or elongated, occurs in the upper part of the Harderian gland, in the intranasal end of the palatine branch at the crossing with the optic nerve. The IPPG is typically elongated and is located on the medial surface of the gland below the superior ganglion (Figure 1).

Acetylcholine is the main chemical transmitter in the parasympathetic autonomic nervous system. Cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and galanin (GAL) belong to the group of neuropeptides and are present in both the central and peripheral nervous systems. CART inhibits hunger (anorexigenic factor), plays a role in secretion of neuroendocrine hormones, and influences the effects of addictive substances [6–8]. It elevates blood pressure and heart rate and has an impact on the functions of the gastrointestinal and reproductive systems (regulation of gonadotropin release) [9–11]. CART also plays a role in the response to stressors and in anxiety behaviors [12].
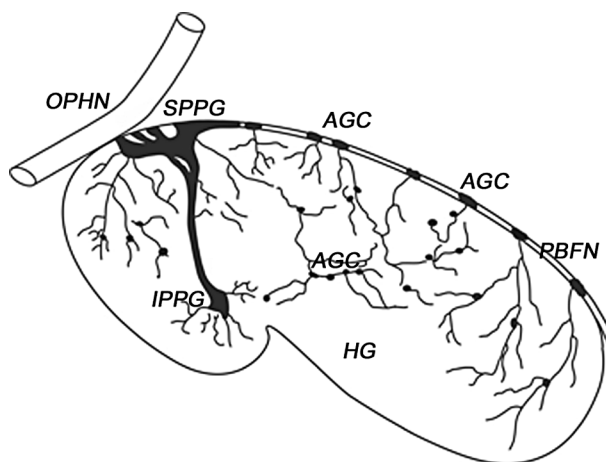
Figure 1. Topography and morphology of the domestic duck pterygopalatine ganglion. Abbreviations: SPPG — superior pterygopalatine ganglion; IPPG — inferior pterygopalatine ganglion; HG — Harderian gland; PBFN — palatine branch of the facial nerve; OPHN — ophthalmic nerve; AGC — aggregations of ganglionic cells

NPY in the central nervous system is associated with different processes, for example, it enhances hunger (orexigenic factor), affects memory processes, and anxiety behaviors [13–15]. Its peripheral impact involves among others intestinal passage, inhibition of smooth muscle contraction in the reproductive and respiratory systems, endocrine function of the epithelium of the gastrointestinal and the excretory systems, contraction of the smooth muscle of blood vessels, and may play a role in blood pressure regulation and heart failure [13, 16, 17]. Clinical studies in animal models and in patients have shown reduced levels of NPY in the plasma and cerebrospinal fluid in depressive disorders [18, 19]. These studies, as well as those of Malva et al. [20], demonstrate a neuroprotective role of NPY under conditions of stress, depression, anxiety, and convulsion, and in other diseases of the nervous system.

Galanin influences the frequency of ingestion, and reduces energy expenditure, which favors obesity [21]. It participates in the regulation of the activity of the cardiovascular system, causing increased blood pressure and tachycardia [22]. Furthermore, it has a stimulating impact on the secretion of certain pituitary

hormones, such as growth hormone and prolactin, as well as on the secretion of neurotransmitter — serotonin [23, 24]. It has also been found that it modulates pain impulses [25].

In this paper we present the first description of the presence of CART, NPY and GAL in PPG of the domestic duck, and suggest their possible role in the autonomic innervations of the avian head.

Material and methods

The study was conducted on 16 one-year-old domestic ducks of the Pekin breed of both sexes (8 males and 8 females). The heads of the animals were collected immediately after economic slaughter. Two common carotid arteries were cannulated for perfusion, and then ligated. The arteries were rinsed with 80 mL of 0.1 M phosphate buffer (pH 7.4). For perfusion 80 mL of ice-cold 4% buffered paraformaldehyde solution (pH 7.4) was used. The heads of the birds were then cut in a medial plane, exposing the region of the Harderian gland. The dissected gland, together with the pterygopalatine ganglion, was fixed in paraformaldehyde for 1 h, and rinsed with 0.1 M phosphate buffer for 3 consecutive days. It was then stored in 19% sucrose and, when it dropped to the bottom, it was transferred to 30% sucrose solution. The next step involved freezing the gland and cutting it into sections of 8 μm thickness using a HM cryostat (Microm, Waldorf, Germany). The frozen sections were subjected to a routine technique of single immunofluorescence staining. The sections were incubated for 16 h at room temperature with primary mouse monoclonal antibodies directed against CART (1:2,000, MAB163, R&D Systems, Minneapolis, MN, USA) and GAL (1:2,000, MAB5854, R&D Systems), and rabbit polyclonal antibody directed against NPY (1:2,000, AHP 2189, AbD Serotec, Kidlington, UK). The sections were subsequently incubated for 1 h with suitable secondary antibodies conjugated with Cy3 (1:6,000, 711-165-150, 711--165-152, Jackson ImmunoLabs, West Grove, Pennsylvania, USA) and FITC (1:600, 715-095-150, Jackson ImmunoLabs) fluorochromes. The sections were encapsulated in buffered glycerol and analyzed under an Olympus BX51 fluorescence microscope equipped with a CC12 camera connected to a PC (Olympus, Tokyo, Japan). The photographs were taken using AnalySIS software (Soft Imaging System, Münster, Germany). In order to test specificity of antibody and staining technique, standard controls were applied, omitting the primary antibodies.

Cross-sections of various parts of the SPPG and IPPG from each animal were used for statistical analysis. As the ganglionic cells varied in diameter from 9 to 53 μm [2], every seventh section was used to avoid double counting of the same neurons. For each individual, the number of immunoreactive perikarya was calculated as a proportion of all ganglionic cells in the whole cross-section area

(132348.34–225412.78 μm^2; mean = 179819.60 μm^2; SD = 36481.10 μm^2). To test the null hypothesis that there are no differences between the analyzed proportions of the immunoreactive cells against the alternative that not all the analyzed proportions are equal, the chi-square test for differences among more than two proportions was applied [26].

Results

The immunofluorescence study allowed demonstrating the presence of CART, NPY, and GAL immunoreactivities (IR) in PPG of the domestic duck. The analyzed ganglionic structure is differentiated in terms of the cell number per cross-section. In the SPPG, there are 57–84 cells (mean = 70.30; SD = 10.30), while the IPPG has 69–85 cells (mean = 77.96; SD = 7.02). In small aggregations forming the plexoganglionic complex in the medial surface of the Harderian gland and the palatine branch of the facial nerve, there are from 3 to 25 neurocytes (mean = 9.25; SD = 4.89).

The highest immunoreactivity of the ganglionic neurons was found for CART. CART-IR was observed in perikarya over the entire area of the SPPG cross-sections (Figure 2A), its highest expression was found in the caudal and central part of the ganglion. The number of CART-IR cells ranges from 49 to 72 (mean = 60.30; SD = 9.50), which constitutes from 83.08% to 88.24% of all neurocytes in the cross-sections. In the IPPG, CART-positive neurons are distributed irregularly over the entire surface of the sections and are numerous: from 60 to 71 (mean = 65.20; SD = 6.07), accounting for 81.13–86.36% of the cells on the sections (Figure 2B). There were no differences between the analyzed proportions of CART-IR cells in SPPG and IPPG as determined by χ^2 test (SPPG: χ^2 = 7.4478; p = 0.9437; IPPG: χ^2 = 8.9925; p = 0.8779). Additionally, CART-IR nerve fibers were seen in both aggregations of the ganglion; however, the immunoreactivity was low compared with the immunoreactivity of the perikarya. The CART-IR neurocytes are also numerous in the small aggregations belonging to the PPG.

NPY-IR was found in ganglionic perikarya, but mainly in numerous fibers of the superior (Figure 2C) and inferior (Figure 2D) ganglion and in the small cell aggregations on the surface of the Harderian gland. These fibers have a characteristic varicose form. NPY-IR cells are distributed irregularly over the cross-sections of the aggregations. In the SPPG, their number ranges from 21 to 36 (mean = 27.60; SD = 5.09), accounting for 35.84–43.41% of all cells in the cross-sections. In the IPPG, their number ranges
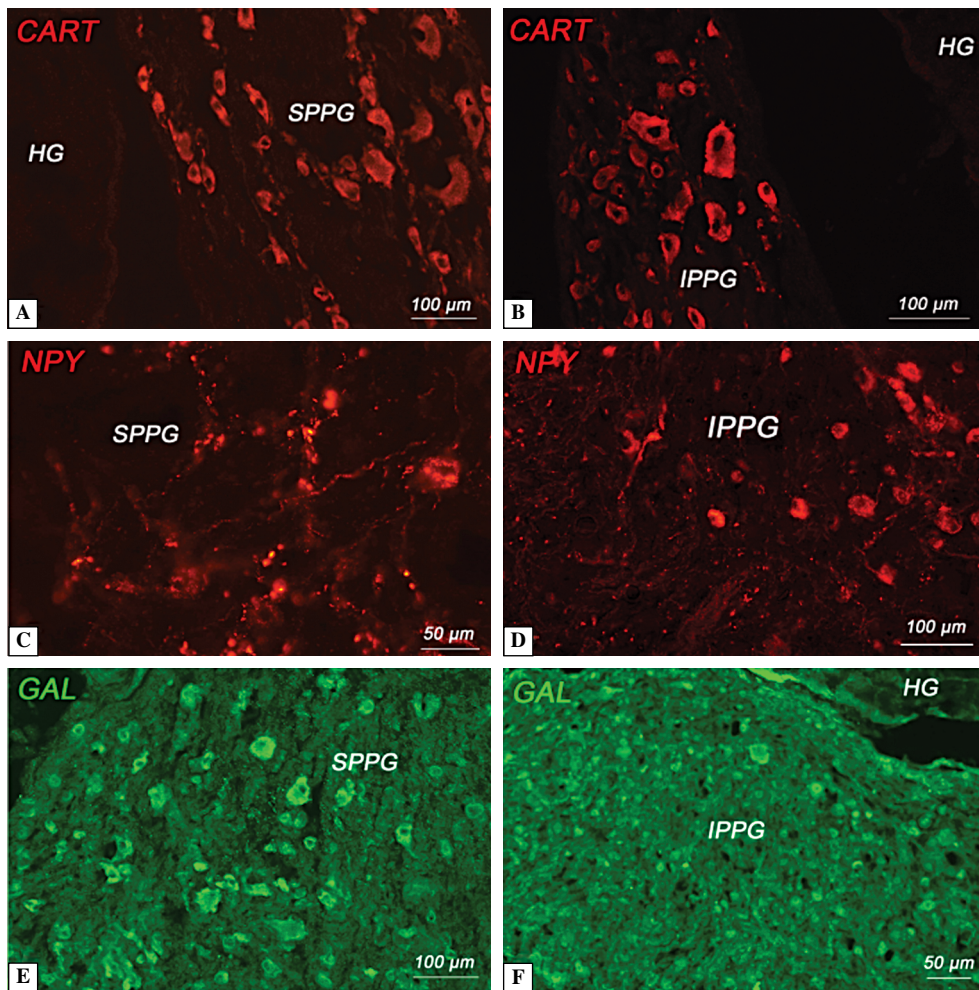
Figure 2. Immunoreactivity of neuropeptides in superior (SPPG) and inferior (IPPG) pterygopalatine ganglion of the domestic duck. **A, B.** High cocaine- and amphetamine-regulated transcript (CART)-immunoreactivity (IR) in ganglionic neurons (mainly in perikarya); **C, D.** Neuropeptide Y (NPY)-IR in numerous varicose fibers and in few ganglionic perikarya; **E, F.** Irregularly distributed galanin (GAL)-IR ganglionic neurons on the cross-sections through the ganglion. Sections were stained by simple immunofluorescence technique as described in Material and methods. Abbreviations: HG — Harderian gland

from 26 to 33 (mean = 29.80; SD = 3.65) accounting for 37.14–39.84% of the ganglionic cells. There were no differences between the analyzed proportions of NPY-IR cells in SPPG and IPPG as determined by χ^2 test (SPPG: χ^2 = 8.1837; p = 0.9162; IPPG: χ^2 = 1.9817; p = 0.9999).

GAL-IR perikarya are present in SPPG (Figure 2E) and IPPG (Figure 2F) pterygopalatine ganglion, as well as in the small aggregations constituting a plexoganglionic complex on the medial surface of the Harderian gland. They are irregularly distributed in the cross-sections, more numerous in the SPPG, ranging from 36 to 53 cells (mean = 44.60; SD = 6.76) accounting for 60.71–64.81% of ganglionic neurons than in the IPPG, where the number of

GAL-IR cells is in the range 38–47 (mean = 41.40; SD = 4.66) accounting for 50.40–57.21% of all the cells in the subsequent cross-sections. There were no differences between the analyzed proportions of GAL-IR cells in SPPG and IPPG as determined by χ^2 test (SPPG: χ^2 = 3.6133; p = 0.9988; IPPG: χ^2 = 8.8333; p = 0.8861).

All the immunoreactive neurons containing the studied neuropeptides were characterized by immunopositive neuroplasm and an immunonegative nucleus. No differences in the immunoreactivity of CART, NPY, or GAL depending on the diameter of ganglion cells in the structure were observed. Moreover, no differences in the immunoreactivity between males and females were found.

Discussion

The neuropeptides under investigation are present in different structures of the nervous system. CART was found primarily in the brain, but is also present in the plexuses of the stomach muscular layer of mammals, *e.g.* in pig and wild boar (where it coexists with other biologically active neuropeptides, such as GAL, SP, and NPY) [27], sheep [28], various parts of rat's gastrointestinal [10], intramural ganglia of the pig urinary bladder in colocalization with pituitary adenylate cyclase-activating polypeptide (PACAP) [29] and in cardiac ganglia of a number of animals, including guinea pig and rat [30–32]. In the enteric nervous system CART is regarded as a strong neuroprotective factor. In rat [33] CART was found also in parasympathetic cephalic ganglia, pterygopalatine (5.25% of neurons) and otic (4.23%) ganglia as well as in the sensory trigeminal ganglion (1.26%). It was demonstrated that in the PPG, CART coexisted with NPY in 20.07%, with vasoactive intestinal peptide (VIP) in 21.22%, and with enkephalin (ENK) in 11.69% of cells. In the otic ganglion, it coexisted with NPY in 26.24%, with VIP in 33.57%, and with ENK in 2.49% of cells [33]. As demonstrated by the present study, the CART immunoreactivity of the PPG neurons in duck is very high, being over 80%, which is completely different from the corresponding mammalian ganglion. This is probably related to the fact that in birds PPG innervates the Harderian gland which is much more developed in than in mammals.

Apart from the presence in the brain, NPY is present in the ganglia of the sympathetic trunk in rat [34], as well as in the cardiac ganglia of rat and guinea pig [31, 35]. In rat PPG, the highest immunoreactivity was reported for VIP (99.0% of all cells), then for NPY (54.1%), and then for ENK (10.5%) [36]. These substances colocalized together in rat ganglion neurocytes in the following way: VIP/NPY (47.4%), VIP/NPY/ENK (6.6%), and VIP/ENK (3.9%) [36]. In rat it was found that NPY was predominant in the neurons from which the postganglionic fibers extend to the Harderian gland [36]. In pig PPG NPY was found in 25% of all neuronal perikarya, mainly in those of large diameter, where it colocalized with VIP, however, NPY-IR fibers in the PPG were scarce [37]. The present study demonstrates that in domestic duck about 40% of perikarya are NPY-positive and that there numerous fibers in the PPG, different from the observations in rat and pig PPG [36, 37].

Galanin presence was demonstrated in the plexuses of the stomach muscular layer of pig and wild boar [27] and in the PPG of pig, chinchilla and cat [37–39]. GAL-IR has been observed in chinchilla in a few perikarya (2–3 per section) and in individual intraganglionic nerve fibers of the PPG [38]. In pig PPG a small number (approximately 6%) of GAL-IR neurons and single nerve fibers was found with all GAL-positive neurons containing also VIP [37]. Grimes et al. [39] investigated the autonomic innervations of cat's eye and found galanin in as many as 80% of cells of the PPG, closer to our findings in domestic duck, with galanin present in more than half of the PPG perikarya.

Galanin and PACAP were also demonstrated in the PPG of chicken [40]. GAL-IR was observed in numerous ganglionic cells, especially those of a larger size. The PACAP27-IR neurons were less numerous than the GAL-IR neurons, and the PACAP38-immunoreactive cells were the least numerous. These substances were also present in nerve fibers innervating the Harderian gland.

Walcott et al. [41] studied the presence of the vasoactive intestinal polypeptide and substance P (SP) in nerve fibers innervating the Harderian gland and in the adjacent PPG in chicken. They observed a variable degree of VIP-IR in nearly all large nerve cell bodies of the PPG and in the preganglionic fibers extending with the palatine branch of the facial nerve. The SP-immunoreactivity was detected in preganglionic fibers and in numerous varicose ganglionic fibers situated around immunonegative perikarya. VIP and SP were also present in nerve fibers passing through the gland. This study indicates that in birds these neuropeptides may have an impact on the function of Harderian gland.

The presence of CART and NPY has not previously been investigated in the PPG of birds. In the present study on the domestic duck, very high CART-immunoreactivity was observed in the perikarya of both the SPPG and IPPG as well as in small aggregations situated on the surface of the Harderian gland and along the palatine branch of the facial nerve. NPY-IR was detected in a very high number of nerve fibers in these aggregations but only fewer perikarya showed NPY-immunoreactivity neuropeptide. Our study confirms the high immunoreactivity of galanin in ganglionic cells, which Hiramatsu et al. [40] investigated in the PPG of chicken. Thus, the demonstration in our study of the presence of CART, NPY, and GAL in the investigated structures of the domestic duct, suggests a role in the secretory innervations of the Harderian gland, lacrimal gland, and the glands of the mucosa of the palate and nasal cavity.

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