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Galanin-like Immunoreactive Neural Elements in Domestic Ruminant Pancreas

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ABSTRACT. The distribution and ontogeny of the galanin-like immunoreactive (Gal-IR) neural structures in the pancreas of cattle, sheep and goat were investigated immunohistochemically. The present study confirmed the previous findings on the immunolocalization of galanin both in the neural elements and endocrine cells of cattle, and reported for the first time its exclusive localization in the neural elements of sheep and goat. The frequency of Gal-IR nerve fibers and nerve cell bodies was high in cattle and low in sheep and goat. Their first detection was at the first fetal trimester in cattle and third trimester in sheep and goat. In cattle, a marked increase in the frequency of Gal-IR nerve fibers was observed from the third trimester to early neonatal stage followed by a decrease after three months postnatal. In contrast to the non-preferential distribution pattern in sheep, the Gal-IR nerve fibers in cattle and goat pancreas were predominantly associated with the acini, excretory ducts and blood vessels, but rarely detected in the pancreatic islets. The Gal-IR nerve cell bodies were observed as isolated bodies in the intra- and interlobular connective tissues and as a group within the intrapancreatic ganglia. At the vicinity of the nerve cell bodies, Gal-IR nerve fibers were observed. The present findings may suggest that: (1) galanin regulates pancreatic function as neurotransmitter/neuromodulator in ruminants; (2) galanin plays a more important role in large than in small ruminants; and (3) particularly in cattle, it exerts its most dramatic effect during perinatal development.
KEY WORDS: galanin, immunohistochemistry, pancreas, ruminant.

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Galanin, a 29-amino acid peptide, was first isolated from the pig upper intestine [22]. One of the extensively studied biological effect of galanin is its ability to regulate insulin secretion in pig [16], dog [5, 9, 15], rat [8, 12, 18], mouse [11] and human [1, 3]. This physiological action of galanin coupled with its effect on pancreatic exocrine secretion [18, 23] and release of other pancreatic hormones such as pancreatic polypeptide [3, 20], glucagon [3, 5, 9, 11, 12, 16] and somatostatin [3, 5, 9, 16] have substantiated its influential role in the pancreas.

Immunohistochemical studies have demonstrated galanin in the neural elements of the pancreas of several mammalian species such as pig [14, 16], dog [5, 14, 23], cat [6], rat and mouse [13], baboon [23] and human [1, 14, 19]. Recently, we have reported striking findings on the presence of galanin-like immunoreactivity not only in the neural elements but also in the endocrine cells of the large islets of cattle, a large ruminant species [2, 17]. Large islet is one of the two distinct types of pancreatic islets in cattle [4]. Compared to the small islets, the islets of Langerhans, which are embedded in the exocrine tissue, and present in the fetus, young and adult, the large islets are enmeshed in the interlobular connective tissue, and prominent in the fetus and neonate but negligible in the adult [4]. Such type of islet, which has also been reported in sheep [23] and goat [unpublished observation], may be speculated as a characteristic feature of ruminants. Whether the peculiarity in the distribution of galanin-like immunoreactive (Gal-IR) structures in the cattle pancreas is also true to all ruminants or not remains to be clarified.

The present study was, therefore, conducted to investigate and compare the distribution and ontogeny of the Gal-IR structures in the pancreas of sheep and goat, both small ruminant species, with those in cattle.

MATERIALS AND METHODS

Collection and preparation of tissue samples: Pancreata were collected from Holstein cattle, *Bos taurus* (8 fetuses, 16 calves and 3 cows), sheep, *Ovis aries* (6 fetuses of Scottish Greyface ewes crossed with Border Leicester rams, 6 suffolk lambs and 4 adult suffolk rams) and Philippine native goats, *Capra hircus* (1 fetus, 4 kids and 3 does) (Table 1). The fetal pancreata were obtained from the fetuses of sacrificed animals, while the pancreatic tissues from young and adult animals were sampled after the animals were anaesthetized with xylazine and sacrificed by exsanguination from the left common carotid artery. The fetal ages of cattle [7] and goat [21] were estimated on the basis of the crown-rump length measurement, while those of sheep were based on actual days after mating. These ages were classified according to trimesters as follows: (1) for

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Table 1. Animals used in the present study

Age	Cattle	Sheep	Goat
Prenatal			
1.6 months		1	
2.1months	1		
2.3 months		2	
3.2 months	1		
3.6 months		1	
4.1 months	1		
4.3 months		2	
4.9 months			1
5.3 months	2		
7.3 months	1		
8.0 months	1		
8.4 months	1		
Postnatal			
1 day	1		1
3 days		3	
4 days			1
5 days	2		
7 days	2		1
10 days	1		1
13 days	1		
21 days	2	2	
1 month	1		
1.5 months		1	
2 months	1		
3 months	1		
4 months	1		
6 months	1		
11 months	1		
1 year	1		
1.5 years	1		
2 years			1
3 years	1		2
4 years		2	
5 years		2	
8 years	1		

cattle: first trimester (up to 3 months of fetal age), second trimester (more than 3 months to 6 months of fetal age), and third trimester (more than 6 months of fetal age to birth); and (2) for sheep and goat: first trimester (up to 1.6 months of fetal age), second trimester (more than 1.6 months to 3.2 months of fetal age), and third trimester (more than 3.2 months of fetal age to birth). The gestation periods were estimated at 9–10 months for cattle and about 5 months for sheep and goat. The animals were classified as the adult at age > 1 year in cattle and > 6 months in sheep and goat.

Tissue blocks from the body of the pancreas were collected and then fixed in 10% formalin in 0.1 M sodium phosphate-buffered saline (PBS), pH 7.4. To remove the excess fixative, the blocks were washed for several hours in water continuously displaced from the container at a very low flow rate. They were immersed and allowed to settle in 10% and then 30% sucrose solution in PBS. After embedding them in Tissue-Tek® O.C.T. compound (Miles, Elkhart, U.S.A.), they were frozen in liquid nitrogen and sectioned at 10 and 20 μm for cattle and 10 μm thickness for sheep and goat by the use of a cryostat (1720 Digital Kryostat, Leica, Wetzlar, Germany).

Immunohistochemistry: The cryostat sections were mounted on glass slides coated with poly-L-lysine and processed for immunohistochemistry employing avidin-biotin-peroxidase complex (ABC) method [10]. The incubation periods were 30 min for 10 μm sections and 2 hr for 20 μm sections with normal goat serum (1:50 dilution), overnight with rabbit anti-pig galanin (1:3,000 dilution, Cat. No. IHC-7153, Lot Nos. 950503–3 and 950503–2, Peninsula Laboratories, Inc., Belmont, CA, U.S.A.; or 1:3,000 dilution, Cat. No. AB1985, Lot. No. 20040629, Chemicon International, Inc., Temecula, CA, U.S.A.), 30 min with biotinylated anti-rabbit IgG in goat (1:200 dilution, BA-1000, Vector Laboratories, Inc., Burlingame, CA, U.S.A.), and 30 min with ABC reagent (1:2 dilution, PK-6100, Vector Laboratories, Inc.). The immunoreactive site was revealed with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2. The sections were then dehydrated through graded series of ethanol, cleared in xylene, mounted and examined under conventional light microscope.

Negative controls were carried out by substituting the primary antiserum with PBS and pre-absorption of galanin antiserum with excess amount of its corresponding antigen (pig galanin, Cat. No. 7153, Lot. No. 036749, Peninsula Laboratories, Inc., San Carlos, CA, U.S.A.), i.e., 5, 10, 15, 20 μg antigen per m*l* of diluted antiserum (1:3,000). In these cases, no immunoreactivity was observed. Furthermore, Peninsula Laboratories, Inc. has claimed negative cross reactivity of its galanin antibody to porcine secretin and neuropeptide Y, human peptide histidine methionine-27, galanin message-associated peptide 1–41 and 44–59, and vasoactive intestinal peptide, while Chemicon International, Inc. has declared no cross reactivity to CCK octapeptide, CCK-38 and galanin 10–29. Nevertheless, the immunoreactive structures observed in the present study were referred to as Gal-IR since possible cross reaction with other still unknown peptides can not be excluded.

Semi-quantitative evaluation of Gal-IR nerve fibers: The frequency of occurrence of Gal-IR nerve fibers was subjectively rated as follows: not detected (-); rare (+/-); a few (+); moderate (++); numerous (+++); and most numerous (++++).

Quantitation of Gal-IR nerve cell bodies in the intrapancreatic ganglia: The frequency of occurrence of Gal-IR nerve cell bodies was determined from the ratio of the number of Gal-IR nerve cell bodies to the total number of nerve cell bodies (both Gal-IR and immunonegative nerve cell bodies) per ganglion.

RESULTS

The present study demonstrated galanin-like immunoreactivity in the neural elements of the pancreas of cattle, sheep and goat. It was also detected in the endocrine cells of the large islets of cattle but not in those of sheep and goat. In the ruminant species studied, no immunoreactivity was detected in the endocrine cells of the islets of Langerhans. The details of Gal-IR endocrine cells in the large islets of the cattle pancreas have already been described in our previous report [2]. The present study, therefore, focused on the Gal-IR neural elements in the ruminant pancreas.

Gal-IR nerve fibers: The Gal-IR nerve fibers were either thin or thick, and showed varicose or non-varicose appearance. In the sections, they were observed as dotted or short fragments, or wavy running course.

As shown in Table 2, the frequency of occurrence of Gal-IR nerve fibers differed among the ruminants, i.e., high in cattle and low in sheep and goat. Their topographical distribution profiles, on the other hand, were basically similar in cattle and goat. They were distributed predominantly in association with the pancreatic acini (Fig. 1a) and excretory ducts (Fig. 1b) of the exocrine pancreas, and blood vessels (Fig. 1c). In the blood vessels, they were frequently detected in the tunica adventitia of the arteries and arterioles, and occasionally around the veins. Perivascular Gal-IR nerve fibers associated with the capillaries were also observed. In the nerve bundles running in the intra- and interlobular connective tissues, Gal-IR nerve fibers were detected (Fig. 1d). In the endocrine pancreas, they were rarely observed around and within the islets of Langerhans

Table 2. Frequency of occurrence of galanin-like immunoreactive nerve fibers in the cattle, sheep and goat pancreas at various stages of development

	Exocrine pancreas		Endocrine pancreas		
Developmental stage	Acinus	Duct	Islet of Langerhans	Large islet	Blood vessel
Cattle					
Fetal (Trimester)					
First	+/–	–	–	–	–
Second	++	++	+/–	+/–	++
Third	+++	++	+/–	+/–	++
Young					
up to 1 week old	++++	++	+/–	+	+++
2 weeks old	++++	++	+/–	+	+++
3 weeks old	++++	+++	+/–	+	+++
1 month old	++++	+++	+/–	+	+++
2 months old	++++	+++	+/–	+	+++
3 months old	++++	++	+/–	+	+++
4 months old	++	++	+/–	+	+++
6 months old	++	++	+/–	+	+++
11 months old	++	++	+/–	+/–	+++
1 year old	++	+	+/–	+/–	++
Adult					
1.5 years old	+	+	+/–	+/–	++
3 years old	+	+	+/–	+/–	++
8 years old	+	+	+/–	+/–	+
Sheep					
Fetal (Trimester)					
First	–	–	–	–	–
Second	–	–	–	–	–
Third	+/–	+/–	+/–	+/–	+/–
Young					
3 days old	+	+	+	+	+
3 weeks old	+/–	+/–	+/–	+/–	+/–
6 weeks old	+/–	+/–	+/–	+/–	+/–
Adult					
4 years old	+/–	+/–	+/–	+/–	+/–
5 years old	+/–	+/–	+/–	+/–	+/–
Goat					
Third fetal trimester	+	+/–	+/–	+/–	+
Young					
up to 4 days old	++	+/–	+/–	+/–	++
7–10 days old	+	+/–	+/–	+/–	++
Adult					
2 years old	+/–	+/–	+/–	+/–	++
3 years old	+/–	+/–	+/–	+/–	++

–, not detected; +/–, rare; +, a few; ++, moderate; +++, numerous; ++++, most numerous.

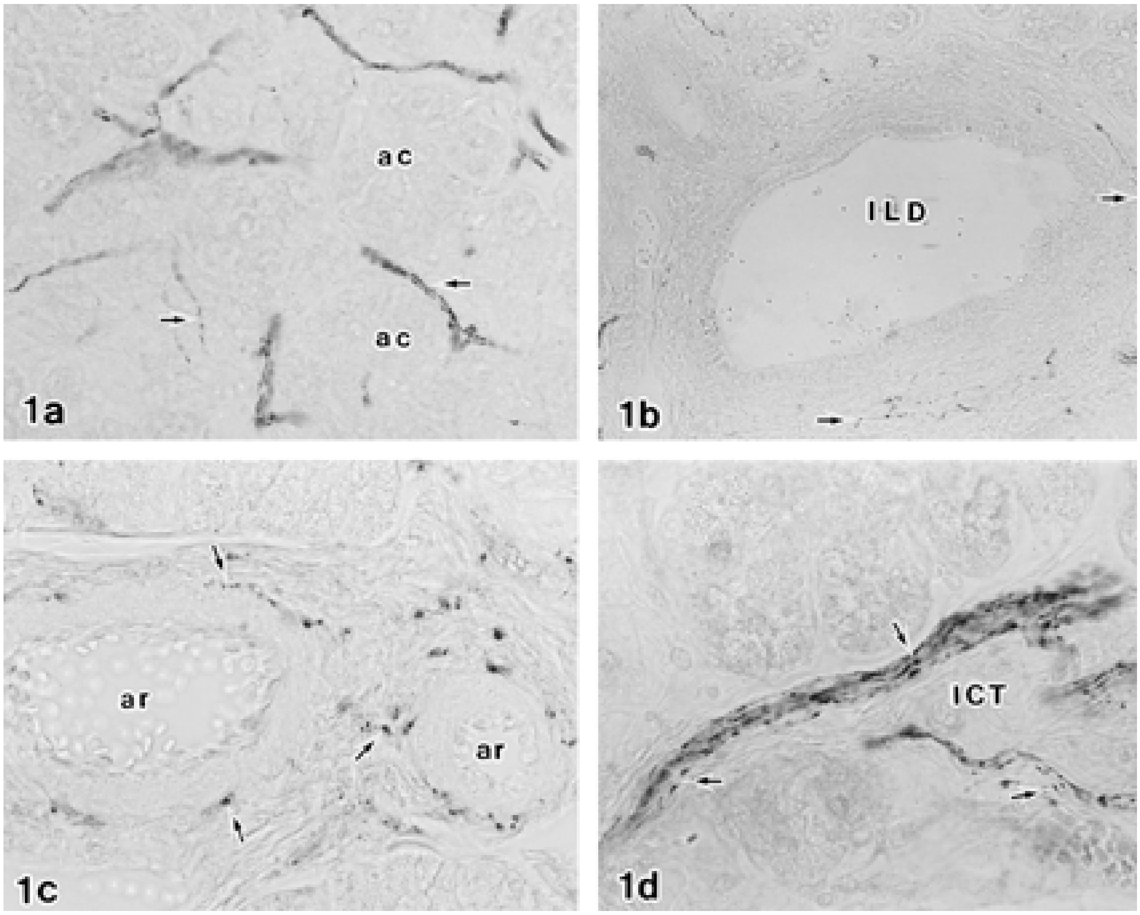

Fig. 1. Photomicrographs showing galanin-like immunoreactive (Gal-IR) nerve fibers (arrows) in association with the acini (ac) of 5-day-old calf (a), interlobular duct (ILD) of 1-day-old kid (b), arterioles (ar) of 1-day-old calf (c), and in the nerve bundles running in the interlobular connective tissue (ICT) of 1-day-old kid (d) pancreas. a: × 625; b: × 170; c: × 417; d: × 500.

(Fig. 2a) and large islets (Fig. 2b, d). In sheep, however, such preferential topographical distribution was not noted probably due to their extremely low frequency of occurrence. Nonetheless, they were observed, although very rare, in the acini, excretory ducts, blood vessels, islets of Langerhans and large islets (Fig. 2c). They were also found in the nerve bundles.

As to the ontogeny of the Gal-IR nerve fibers in the cattle pancreas, they were first detected as early as the first fetal trimester (2.1 months fetal age) but only in association with the pancreatic acini (Fig. 3). Their association with the excretory ducts, blood vessels, islets of Langerhans and large islets was observed at the second fetal trimester (4.1 months fetal age). As shown in Table 2, their frequency of occurrence increased progressively as the fetal age advanced. Shortly before birth, a considerable increase in frequency was observed until a peak was reached at the first neonatal week. This high frequency of Gal-IR nerve fibers was sustained until three months of age, wherein they began to decrease appreciably thereafter. In the adult, moderate to a few were observed.

In the sheep pancreas, the Gal-IR nerve fibers were first detected at the third fetal trimester (3.6 months fetal age). At this stage, they were found in association with the pancreatic acini, excretory ducts, blood vessels, islets of Langerhans and large islets. In goat, the first appearance of Gal-IR nerve fibers could not be defined due to limited fetal samples, though they were already observed in the pancreas of 4.9 months old fetus, the earliest stage examined. Contrary to the pronounced change in the frequency of occurrence of Gal-IR nerve fibers among the various stages of development in cattle, the extent of variation was found minimal and moderate in sheep and goat, respectively.

Gal-IR nerve cell bodies: The Gal-IR nerve cell bodies were round to oval in shape (Fig. 4). The galanin-like immunoreactivity in the nerve cell bodies showed granular appearance in the cytoplasm and was not localized in the nucleus. These Gal-IR nerve cell bodies together with immunonegative ones were observed in the intrapancreatic ganglia, which varied in size and frequency of occurrence

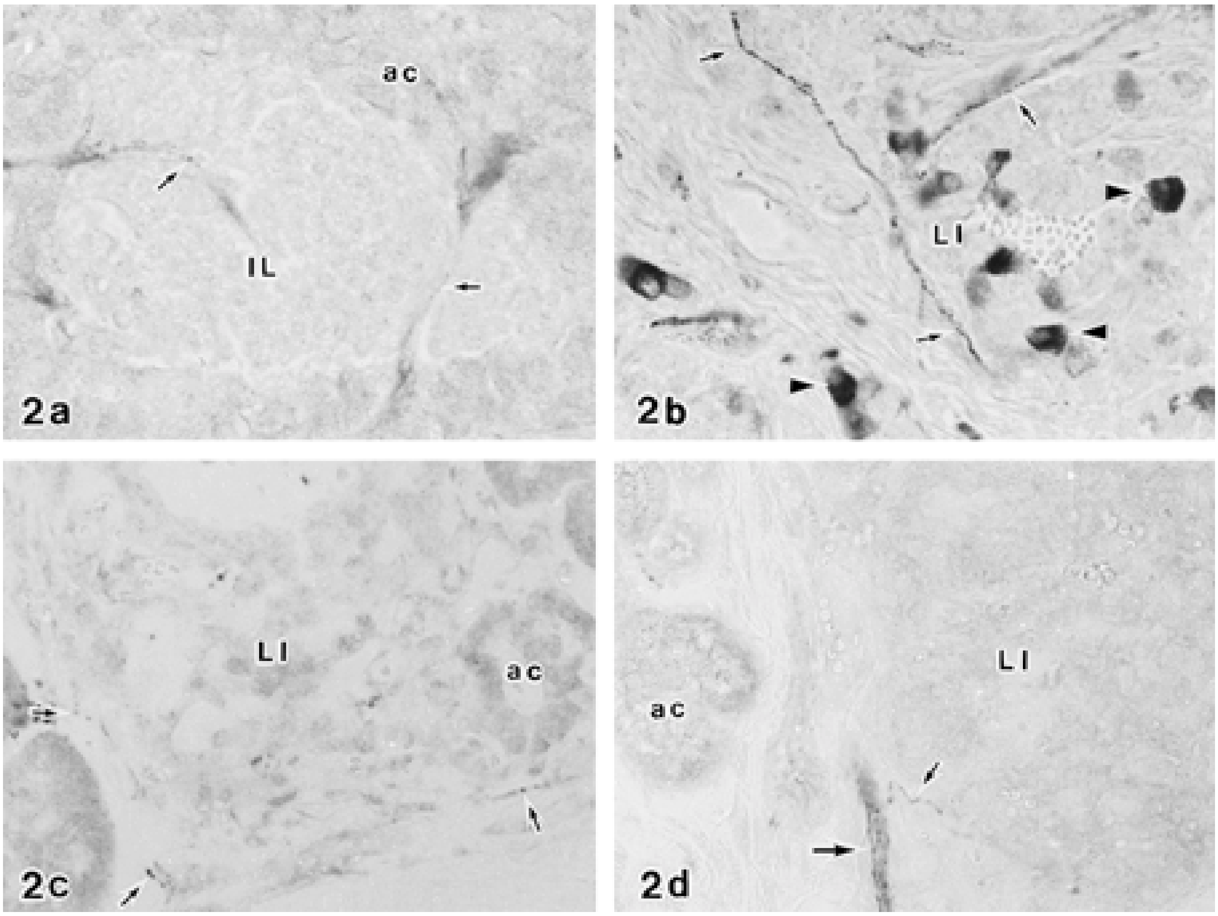

Fig. 2. Photomicrographs showing Gal-IR nerve fibers in the islet of Langerhans of 5-day-old calf (a), and large islet of 1-day-old calf (b), 3-day-old lamb (c) and 1-day-old kid (d) pancreas. Small arrows, Gal-IR nerve fibers; arrowheads, Gal-IR endocrine cells; large arrow, nerve bundle with Gal-IR nerve fibers; ac, acini; IL, islet of Langerhans; LI, large islet. a: × 396; b: × 156; c: × 354; d: × 354.

among the species studied, i.e., larger in size and higher in frequency in cattle than in sheep and goat.

Within the intrapancreatic ganglia, a considerable number of Gal-IR nerve cell bodies were detected. In the fetus, young and adult cattle, 89.1 ± 7.0 (number of intrapancreatic ganglia examined, n=10), 73.5 ± 24.6 (n=37) and 89.0% (n=2), respectively, of the total population of the nerve cell bodies in the intrapancreatic ganglia were immunoreactive for galanin. The Gal-IR nerve cell bodies in the pancreas of cattle were first detected as isolated bodies in the interlobular connective tissue at the first fetal trimester (2.1 months fetal age)(Fig. 3) and in the intralobular connective tissues at the first neonatal week (1 day old). The Gal-IR nerve cell bodies within the intrapancreatic ganglia was first observed at the third trimester (7.3 months fetal age). Among the various stages of development of cattle, their frequency of occurrence in the intrapancreatic ganglia was basically comparable.

In sheep and goat, the number of intrapancreatic ganglia was too small to deduce definite data on the frequency of occurrence of Gal-IR nerve cell bodies. Nonetheless, to the observed few, the proportion of Gal-IR nerve cell bodies in the intrapancreatic ganglia appeared to be relatively high (Fig. 4b, c). At the vicinity of both the Gal-IR and immunonegative nerve cell bodies of the intrapancreatic ganglia, Gal-IR nerve fibers were observed. In sheep, the Gal-IR nerve cell bodies were first observed in the intrapancreatic ganglia at the third fetal trimester (4.3 months fetal age). In goat, they were already observed at the third trimester (4.9 months fetal age). Isolated Gal-IR nerve cell bodies were also detected in the intralobular connective tissue of young goat. Since the number of intrapancreatic ganglia observed in sheep and goat was too small, definite data on their frequency of occurrence among the different stages of development could not be deduced.

DISCUSSION

The present study showed the exclusive localization of galanin-like immunoreactivity in the neural elements of sheep and goat pancreas. It also confirmed previous findings on its presence not only in the neural elements but also

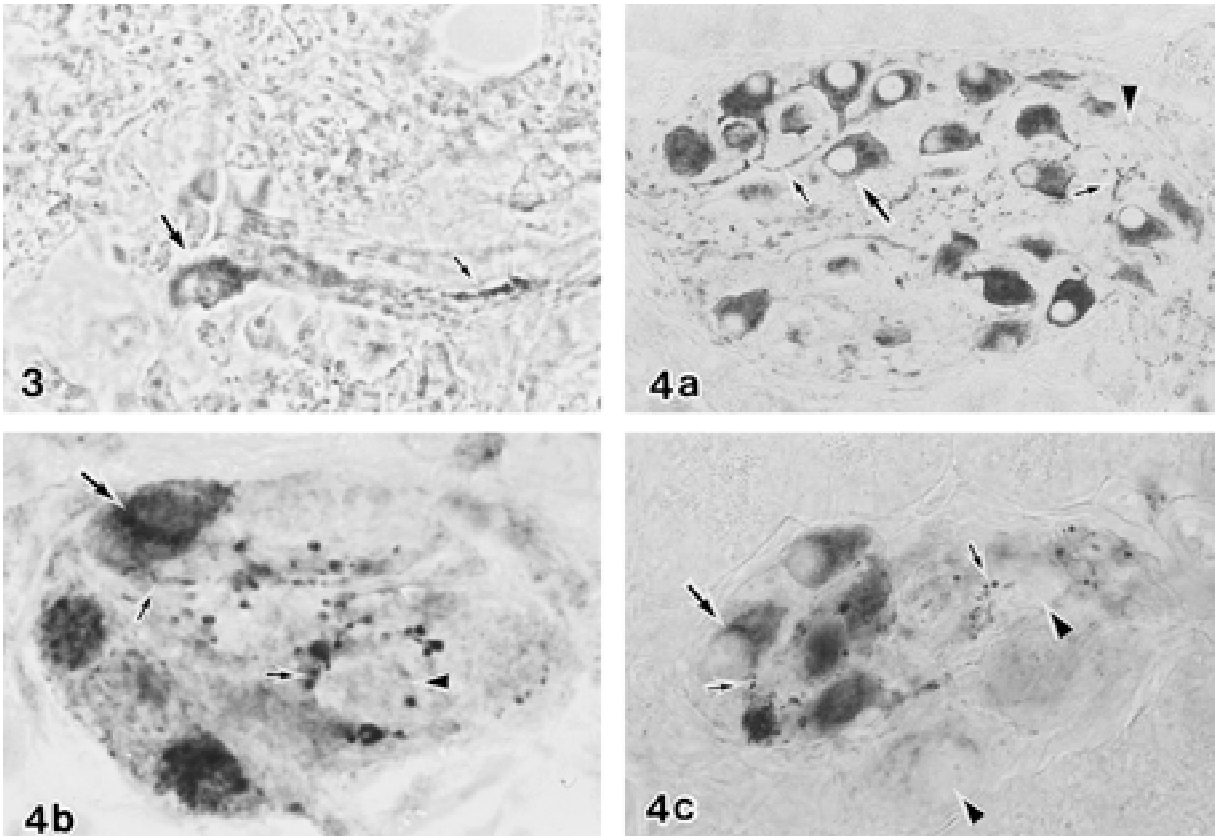

Fig. 3. First detection of Gal-IR nerve fiber (small arrow) and nerve cell body (large arrow) in the pancreas of 2.1-month-old fetus of cattle. × 896.

Fig. 4. Photomicrographs showing Gal-IR nerve cell bodies in the intrapancreatic ganglia of 5-day-old calf (a), 3-day-old lamb (b) and 1-day-old kid (c) pancreas. Gal-IR nerve fibers (small arrows) were observed in the area surrounding both the Gal-IR (large arrows) and immunonegative (arrowheads) nerve cell bodies. a: × 314; b: × 229; c: × 563.

in the large islet endocrine cells of cattle [2, 17]. The Gal-IR endocrine cells in the large islets may be a peculiar morphological feature of the large but not the small ruminant species.

The localization of galanin-like immunoreactivity in the neural elements of the pancreas of cattle, sheep and goat is consistent with those in other mammalian species such as pig [14, 16], dog [5, 14, 23], cat [6], rat and mouse [13], baboon [23] and human [1, 14, 19]. However, topographical distribution pattern and frequency of occurrence of the Gal-IR neural elements in the pancreas varied not only among the ruminants but also with those of the other species.

Contrary to the non-preferential topographical distribution pattern of Gal-IR nerve fibers in the sheep pancreas, dense innervation in the exocrine pancreas and minimal innervation in the pancreatic islets by Gal-IR nerve fibers were observed in cattle. In goat, a relatively higher frequency was observed in the exocrine pancreas than in the pancreatic islets. Similar distribution pattern as in cattle has also been reported in human [14, 19], pig [14, 16] and baboon [23]. In the dog, however, the opposite has been observed, i.e., dense innervation by the Gal-IR nerve fibers in the endocrine pancreas and minimal in the exocrine pancreas [5, 14, 23]. Moreover, the Gal-IR nerve fibers in mouse and rat have shown minimal frequency of distribution both in the exocrine and endocrine pancreas [13]. The present findings on the appreciable innervation by Gal-IR nerve fibers in the acini, excretory ducts and blood vessels of the exocrine pancreas may suggest that galanin is a key peptide influencing the ruminant pancreatic exocrine function such as regulation of the exocrine secretion, modulation of the excretory duct activity and control of vasomotor functions.

Difference in the frequency of occurrence and developmental profile of pancreatic Gal-IR nerve fibers was also noted among the ruminant species studied. The frequency of occurrence of Gal-IR nerve fibers was relatively high in cattle and low in sheep and goat. Moreover, irregularity was observed in their developmental profiles, i.e., first detection at the first fetal trimester in cattle and at the third trimester in sheep, and in their postnatal diminishing phase, i.e., three months in cattle and first week in sheep and goat. The higher frequency of occurrence as well as longer duration of appreciable density of Gal-IR nerve fibers in cattle than in sheep and goat pancreas may suggest that demand of galanin is higher in the large than in the small ruminant species.

As to the distribution of Gal-IR nerve cell bodies in the pancreas, the present findings were in sharp contrast with the reported absence of Gal-IR nerve cell bodies in the intrapancreatic ganglia of pig [16] and their very rare occurrence in the dog [23]. In cattle, a considerable proportion of Gal-IR nerve cell bodies were found in a substantial number of intrapancreatic ganglia. In sheep and goat, on the other hand, the proportion of Gal-IR nerve cell bodies relative to the total number of the nerve cell bodies in the observed few intrapancreatic ganglia appeared to be relatively high. Also, in the ruminant species studied, isolated Gal-IR nerve cell bodies were detected in the intra- and interlobular connective tissues. Other than the extrinsic origin of the nerve fibers in the pancreas, the present findings may suggest possible intrinsic source of the Gal-IR nerve fibers in the cattle, sheep and goat pancreas.

It may be worth noting the time frame at which the frequency of Gal-IR nerve fibers increased to maximum in cattle, i.e., during late prenatal to early postnatal stage. Interestingly, a sharp increment to maximum in the volume density of Gal-IR endocrine cells in the large islets of cattle was also observed precisely at this period [2]. It may be suggested that galanin, either as a neural element or endocrine cell constituent, exerts its most influential role during the perinatal development of cattle wherein substantial metabolic alteration occurs as the animal system adapts from intra- to extra-uterine life.

In conclusion, the present findings provide immunohistochemical evidences to support the following hypotheses: (1) galanin in the neural element may act as neurotransmitter and/or neuromodulator regulating various functions including ganglion transmission, exocrine secretion, pancreatic hormone release and blood circulation in ruminant pancreas; (2) galanin may play a more important role in large than in small ruminants; and (3) galanin may exert its most dramatic effect in the pancreas during perinatal development of cattle.

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