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THE LIGHT MICROSCOPIC IMMUNODETECTION OF GALANIN (GAL) INTRINSIC INNERVATION IN THE SPLEEN OF THE GRASS-SNAKE, NATRIX N. NATRIX

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Abstract: The spleens of two specimens of grass-snakes were investigated for the immunohistochemical detection of the galanin (GAL) by using the PAP technique. The study has revealed an extensive network of galanin immunomarked nerve fibbers and many autonomic ganglions. It represents the first demonstration of the occurrence and topographic distribution of these neural structures inside the spleens of ophidians, where their functional significance has been explained in connection with the adaptative migration of the pancreatic islets in this organ. The above findings supporting the good phylogenetic conservation of this neuropeptide are discussed in connection with the results previously reported in men, mammals, and other poikilothermic vertebrates.

Keywords: galanin, GAL, snakes, spleen, immunohistochemistry

INTRODUCTION

Galanin is a 29-amino acid residue neuropeptide isolated from porcine intestine by Tatemoto et al., 1983. As one of the most recently discovered neuropeptides, galanin (GAL) shows a lot of interesting physiological and behavioral actions, which may be relevant to disease state and clinical therapeutics. Its name derived from the first and last amino acids sequence of the porcine galanin. Preprogalanin is a 123 amino acid precursor, which is processed into galanin and message-associated peptide (Sillard et al., 1992). The first 15-N terminal amino acids are highly conserved during phylogeny. Only the human species of GAL contains C-terminal amidation and shows also a second molecular form. In addition, peptides immunoreactive with antiserum raised against porcine galanin have been identified in the chicken (Jozsa and Mess, 1993), in reptiles, including the turtle (Jiminez et al., 1994) and alligator (Wong and Conlon, 1994), in amphibians, including frogs (Lazar et al., 1991) and mudpuppies (McKeon et al., 1990), in several teleost fishes (Batten et al., 1990), and in an insect, the blowfly (Lundquist et al., 1991).

As regard the quantitative distribution of GAL in the mammalian central nervous system this has been well documented especially in rat (Melander et al., 1986a; Skofitsch and Jacobowitz, 1986). Galanin immunoreactivity was detected in high concentration in the hippocampus, amygdala, nucleus accumbens and in other brain formations and in moderate concentrations in the cerebral cortex and cerebellum (Skofitsch and Jacobowitz, 1986). Coexistence of galanin immunoreactivity within the same cell bodies as neurotransmitters included coexistence with norepinephrine, with serotonin, with acetylcholine, with vasopressin, with glutamic acid decarboxylase and with tyrosine hydroxylase (Melander et al., 1986b). Galanin-containing pathways in the brain and spinal cord of several species have been also extensively described (Merchenthaler et al., 1993). Finally, galanin immunoreactivity is present in many peripheral organs including the pituitary, pancreas, genital tract, gastrointestinal tract, urinary bladder, respiratory tract, adrenal gland and in autonomic ganglia and nerve fibbers innervating, the heart, liver, kidney and spleen (Crawley, 1995).

MATERIALS AND METHODS

Animals

Two specimens of grass-snakes, captured in springtime (April-May) from the surroundings of Bucharest, were kept unfed in terraria for 3 days.

Tissue preparation

The animals were killed under chloroform anesthesia and the spleens were removed. Fragments of the organ were immersed in Bouin’s fluid for 36h, dehydrated in increasing concentrations of ethanol, cleared with toluene and paraffin-embedded. Serial sections of 6μm-thickness, prepared on a sledge microtome,

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were mounted apart on poly-L-lisine (Sigma, USA) – coated slides.

Primary antibody

The primary antiserum - polyclonal rabbit anti-goat galanin (GAL) - was purchased from Biotrend Chem. GmbH, Köln, Germany.

Immunohistochemical protocol

The deparaffinized and rehydrated sections were treated according to the peroxidase anti-peroxidase (PAP) technique (Sternberger, 1974) modified as follows:

-the second and third incubation steps in the original protocol were replaced with donkey anti-rabbit IgG peroxidase linked the whole antibody produced by Amersham Pharmacia Biotech, UK.

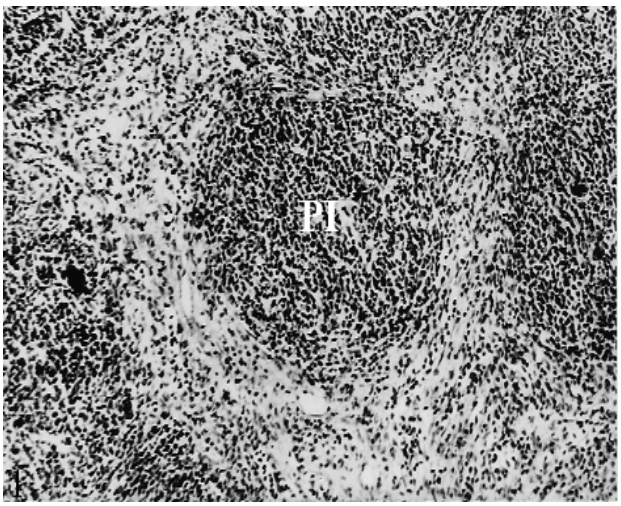
The 1:400 concentration of the primary antiserum, which maximally stains the immunoreactive structures without any other unspecific reaction, was chosen. The sections were finally dehydrated in ethanol, cleared with xylene, mounted in Entellan (E.Merck, Germany) and examined in a Zeiss (Oberkochen, Germany) Photomicroscope II. The adjacent sections to those already immunolabelled were stained with hemalaun-eozine or hemalaun-trichrome.

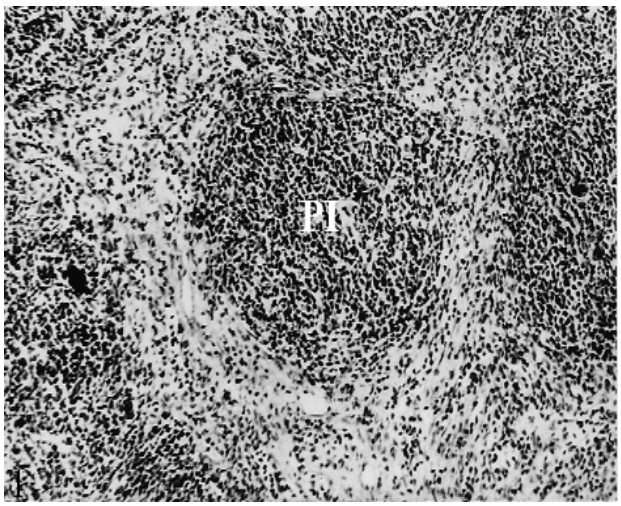
Specificity controls

The immunoreaction specificity was tested by replacing the primary antibody and donkey anti-rabbit IgG peroxidase linked antiserum with phosphate saline (PBS) or with Tris-saline (TBS) buffers. The specificity of the primary antibody was tested by preadsorbtion (24h at 4o C) with the corresponding antigen (pituitary human GAL) produced by Peninsula Labs., Germany.

RESULTS AND DISCUSSIONS

The first demonstration of the occurrence and of the topographic distribution of intrinsic ganglions and of the nerve fibbers immunopositive for GAL in the spleen of the grass-snake doesn't seem to represent an isolated phenomenon, at least among ophidians, where the very elongated forms of their body implied an adaptative migration of the pancreatic islets in this organ (Figs. 1,2). Regarding the above, early studies on the physiological actions of this neuropeptide have showed that the intravenous administration of galanin to conscious dogs produced hyperglycemia and inhibition of insulin release (McDonald et al., 1985). The ability of GAL to inhibit glucose-stimulated insulin release has been reported in rat (Schnuerer et al., 1987) and pig (Lindskog et al., 1990) However, GAL may not be effective at inhibiting glucose-stimulated insulin secretion in humans (McDonald et al., 1994).





Figs.1,2. Pancreatic islets (PI) stained with hemalaun-eozin (Fig.1) and with hemalaun-trichrome (Fig.2) in the spleen of the grass-snakes; both figures x173

Neuronal activation causes the release of GAL from sympathetic nerve terminals innervating the pancreas (Dunning et al., 1990). Electrical stimulation of the autonomic pancreatic nerves in dogs produced a co-release of galanin and norepinephrine, while noradrenergic drugs simultaneously regulated GAL and NA, suggesting their co-localizations in sympathetic nerves (Scheurink et al., 1992). Therefore in this case is highly possible that the releasing of galanin from the sympathetic terminals spread almost throughout the spleen, to be responsible for insulin release from the pancreatic islets (Figs. 3-6). As a matter of fact, as Dunning et al. have advanced (Dunning et al., 1990), GAL could be considered a sympathetic neurotransmitter for the endocrine pancreas, in our opinion irrespective of its localization (in the pancreatic exocrine tissue or in spleen).

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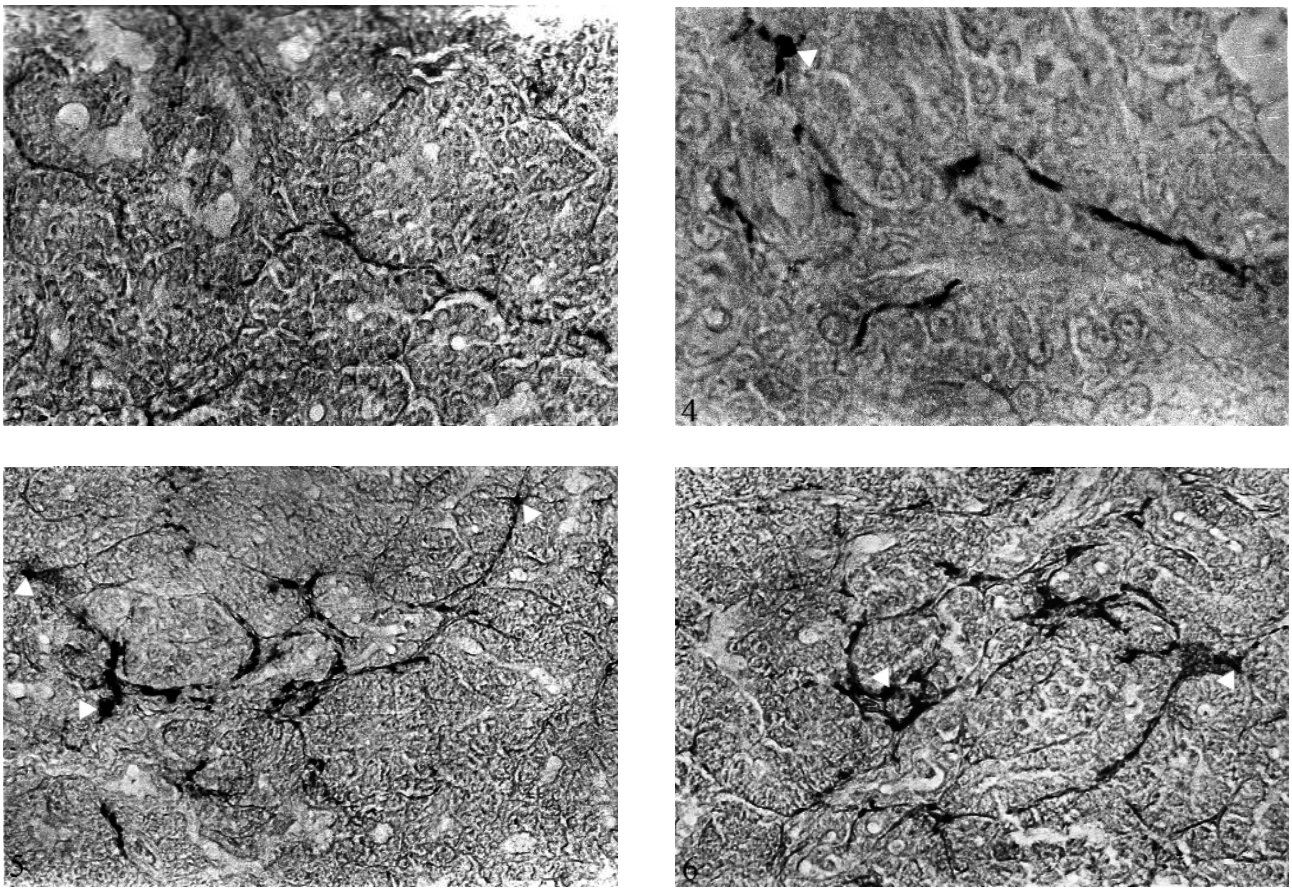

Figs.3-6. Galanin immunoreactive nerve fibbers distributed in the spleens of the grass-snakes. To note also the presence of autonomic ganglia (arrowheads) in the figures 4-6. Figs. 3,5,6 x82; Fig.4 x280

Reduced levels of galanin were seen in the pancreas of the ob/ob mouse (Dunning and Ahrén, 1992), a spontaneous mutant mouse model of obesity for which the genetic locus has been identified (Zang et al., 1994). It is interesting also to speculate that GAL may be involved in some forms of human obesity, either at the level of pancreatic insulin secretion or hypothalamic regulation of feeding behaviors (Crawley, 1995).

Among others quoted effects of GAL - administration are those related to food consumption. Thus, central administration of the neuropeptide induces feeding in satiated rats (Schick et al., 1993) and ground squirrels (Boswell et al., 1993). The anatomical sites at which galanin induces food consumption includes the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala (Corwin et al., 1993). GAL may be acting by releasing norepinephrine or acting on the mesolimbic reward pathway as it increased the levels of dopamine (Rada et al., 1994). The role of endogenous galanin on the regulation of food consumption remains, however, controversial. No study has been published until now on GAL in human obesity.

As regard the endocrine, sexual and reproductive functions galanin expression appears to be regulated by gonadal steroids. A lot of data showed that this neuropeptide is an important element in the hypothalamic-pituitary circuitry of neuroendocrinological regulation of sexual and reproductive behaviors (see Crawley, 1995). In addition, the visualization of galanin-like immunoreactivity in the pituitary adenomas (Hsu et al., 1991) raised the possibility that GAL is involved in the etiology of pituitary tumors.

Galanin is also dramatically overexpressed in neurons after mechanical or pharmacological lesions of their axons (Cortés et al., 1990; Hökfelt et al., 1987; Hökfelt et al., 1994). The neuropeptide induction by axotomy appears to be mediated by leukemia inhibitory factor (LIF), a cytokine which influences neuronal differentiation and the expression of several neuropeptides (Rao et al., 1993). Finally, it is interesting to speculate that GAL is a relatively "plastic" neuropeptide, which is induced in response to neuronal injury and may serve to limit neuronal damage by inhibiting the release of excitatory amino acids.

Wisenfeld-Hallin and coworkers have elucidated the actions of galanin on spinal reflex responses to painful stimuli (Hökfelt et al., 1994; Wisenfeld-Hallin et al., 1992a; Wisenfeld-Hallin et al., 1992b). These actions of GAL on spinal nociceptive reflexes may depend on interactions with other neuropeptides. All the data suggest that endogenous galanin serves an antinociceptive function in the circuitry mediating sensory transmission in the spinal cord, and that these neuropeptide agonists may be clinically useful in potentiating the analgesic effects of morphine.

Degeneration of cholinergic neurons of the basal forebrain is characteristic of Alzheimer's neuropathology (Coyle et al., 1983), in which lesions in the cholinergic pathways in rats and monkeys produce deficits in learning and memory (Chan-Palay, 1988). Galanin hyperinnervation of cholinergic neurons of the basal forebrain was also seen in postmortem analysis of victims of Alzheimer's disease and Parkinson's disease when accompanied by dementia (Chan-Palay, 1988). Investigations into the functional role of galanin in cholinergic pathways relevant to memory and learning suggest that this neuropeptide has an inhibitory effect which produces performance deficits on learning and memory tasks when administrated intraventricularly to rats. The inhibitory effects on working memory are more severe when cholinergic transmission is reduced by scopolamine (Robinson and Crawley, 1993). Therefore, these behavioral findings with exogenously administered GAL raised the possibility that this endogenous neuropeptide hyperinnervating the neuronal cell bodies in Alzheimer's disease may act to reduce acetylcholine release in the few remaining cholinergic neurons and to exacerbate the memory deficits (Hökfelt et al., 1987a,b; 1994).

Finally, based on the fact that the major source of circulating galanin is the gut (Harling and Holst, 1992), a series of investigations have studied its effects on gastrointestinal smooth musculature (Crawley, 1995)

CONCLUSIONS

Exogenously administered galanin induces several major biological actions such as inhibitory effects on memory and learning, inhibition of acetylcholine and glutamate release, stimulation of feeding and of pituitary hormones release, inhibition of insulin release and inhibition of spinal nociceptive reflexes. Expression of galanin is regulated by gonadal steroids and the neuropeptide is dramatically overexpressed after neuronal injury and in Alzheimer's disease. Clinical investigations of galanin levels, its receptor and their gene sequences in human disorders related to these functions may provide a steady argument for a novel neuropeptide-based therapeutics for Alzheimer's disease, stroke, neuropathic pain, obesity, and endocrine disorders. Coming back to the ophidians, in which the pancreatic islets are localized inside the spleen, the revealing of autonomic ganglia and nerve fibbers immunolabelled for galanin may be related with the inhibition of insulin release.

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