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Laboratory of Cell Biology and Histology, Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium

Development of Galanin-Containing Nerve Fibres in Rat Tibia

M. Gajda[1]*, J. A. Litwin[1], O. Zagólski[2], G. J. Lis[1], T. Cichocki[1], J.-P. Timmermans[3] and D. Adriaensen[3]

Addresses of authors: [1]Department of Histology, Jagiellonian University Medical College, Kopernika 7, 31-034, Kraków, Poland; [2]Diagnostic and Therapeutic Medical Centre 'Medicina', Barska 12, 30-307 Kraków, Poland; [3]Laboratory of Cell Biology and Histology, Department of Biomedical Sciences, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium; *Corresponding author: Tel.: 0048 12 422 7027; fax: 0048 12 422 7027; e-mail: mmgajda@cyf-kr.edu.pl

With 8 figures

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Summary

Galanin exerts tonic inhibition of nociceptive input to the central nervous system. Recently, this peptide was demonstrated in several neuronal and non-neuronal structures in bones and joints. In this study, the time of appearance and topographic localization of galanin-containing nerve fibres in bone were studied in rats from gestational day 16 (GD16) to postnatal day 21 (PD21). The tibia was chosen as a model of developing long bone and indirect immunofluorescence combined with confocal laser scanning microscopy was used to identify galanin-immunoreactive (GAL-IR) nerve fibres. The earliest, sparse GAL-IR fibres were observed on GD21 in the perichondrium of both epiphyses and in the periosteum of the diaphysis. From PD1 onwards, GAL-IR fibres were also seen in the bone marrow cavity and in the region of the intercondylar eminence of the knee joint. Intramedullary GAL-IR fibres in proximal and distal metaphyses appeared around PD1. Some of them accompanied blood vessels, although free fibres were also seen. GAL-IR fibres located in the cartilage canals of both epiphyses were observed from PD7, in the secondary ossification centres from PD10 and in the bone marrow of both epiphyses from PD14. The time course and localization of galanin-containing nerve fibres resemble the development of substance P- and CGRP-expressing nerve fibres, thus suggesting their sensory origin.

Introduction

Mature long bones have been shown to be densely innervated (Mach et al., 2002). Using immunohistochemistry, neuronal tracing and denervation studies, two main components of the skeletal innervation have been identified: (1) more numerous sensory fibres, originating from dorsal root ganglia and expressing substance P (SP) and CGRP (Bjurholm et al., 1988a), and (2) less numerous autonomic fibres of both adrenergic and cholinergic nature, originating from paravertebral ganglia of the sympathetic chain and mostly accompanying blood vessels (Bjurholm et al., 1988b). The highest density of innervation was observed in the periosteum, although nerve fibres were also located in the bone marrow and in vascular canals (Mach et al., 2002).

Galanin, 29 amino acid (30 in humans) amidated peptide, has been reported to perform numerous physiological functions (Vrontakis, 2002). It acts via three so far characterized G-protein coupled receptors: GAL1, gal2 and gal3 (Branchek et al., 2000). There is substantial evidence indicating that galanin is involved in processing of sensory information (Xu et al., 1996; Ackermann et al., 2003; Yoon et al., 2003).

In normal conditions, very small proportion of dorsal root and trigeminal ganglion neurons express galanin mRNA and the protein product detected by in situ hybridization and immunohistochemistry, respectively (Skofitsch and Jacobowitz, 1985; Xu et al., 1996; Yoon et al., 2003; Brumovsky et al., 2006). Most of galanin-containing cells in sensory ganglia belong to a subpopulation of small-sized neurons coexpressing also CGRP and known to process nociceptive and thermal impulses (Ju et al., 1987). In electrophysiological and behavioural studies, galanin was demonstrated to exert tonic inhibition of nociceptive input to the central nervous system (Wiesenfeld-Hallin et al., 1989, 1990). In gene-targeted mice overexpressing galanin, decrease in sensibility to pain was reported (Grass et al., 2003; Hygge-Blakeman et al., 2004).

Significant increase in galanin immunoreactivity was observed in dorsal root ganglia and associated roots after experimental nerve injury (Zhang et al., 1996; Hu and McLachlan, 2001). Galanin expression was upregulated in central and peripheral nerves following axotomy and such effect is believed to influence neural regeneration (Zhang et al., 1996, 1998; Hu and McLachlan, 2001; Ackermann et al., 2003; McDonald et al., 2003). However, the role of galanin in pain processing is complex: inhibitory, excitatory or even biphasic actions have been described for this peptide, partly dependent on the type of the activated receptor (Liu et al., 2001; Flatters et al., 2003; Brumovsky et al., 2006). Pro-nociceptive effect of galanin was observed in capsaicin-induced inflammatory pain (Jimenez-Andrade et al., 2004).

The presence of galanin was demonstrated in bones by radioimmunoassay and immunoelectron microscopy (Qinyang et al., 2005). The mean concentration of this peptide was the highest in bone marrow, followed by periosteum and cortical bone. The immunolabelling for galanin was low in osteoblasts, osteoclasts and osteocytes, but much higher (even 10 times) in myelinated and unmyelinated nerve fibres. During bone fracture repair, 32-fold higher concentration of galanin was found in bone one week after injury and a 2.4-fold increase in galanin concentration in peripheral blood was observed one week later (McDonald et al., 2003). Such effects suggest an involvement of galanin in fracture healing, a process known to reflect the mechanisms of bone development (Vortkamp et al., 1998).

The aim of the present study was to describe the time of appearance and spatial distribution of nerve fibres expressing galanin in the developing long bone. Tibia of rat hindlimb was used as a model and indirect immunofluorescence combined with confocal laser scanning microscopy was applied to identify galanin-containing nerve fibres.

Materials and Methods

Animals

Adult Wistar rats of both sexes and timed pregnant females were obtained from Charles River Laboratories (Brussels, Belgium). The animals were housed separately in acrylic cages with wood shavings in air-conditioned room (22 ± 3°C, 12 h dark/light cycle). They had unlimited access to water and standard rodent pellets. National and international principles of laboratory animal welfare (conforming to NIH publication nr 86-23, revised 1985) were followed and the experiments were approved by the local ethics committee of the University of Antwerp.

Pregnant rats were sacrificed by an overdose of sodium pentobarbital (Nembutal, Sanofi, Belgium) administered intraperitoneally. Foetuses were obtained at gestational days (GD) 16 (n = 8 from two different mothers), GD17 (n = 8 from two different mothers), GD19 (n = 8 from two different mothers), and GD21 (n = 8 from two different mothers). The lower limbs of foetuses were dissected out for further processing.

Offspring from different litters were euthanized at postnatal day (PD) 1 (day of birth; n = 4), PD2 (n = 4), PD3 (n = 4), PD4 (n = 4), PD7 (n = 4), PD10 (n = 3), PD14 (n = 3), PD21 (n = 3) and PD28 (n = 4) using an overdose of Nembutal. Hindlimbs were dissected and the skin was removed to allow better penetration of the fixative.

Deeply anaesthetized (as described above) animals older than PD7 were first transcardially perfused with cold Krebs-Ringer solution followed by 4% phosphate-buffered (0.1 M, pH = 7.4) paraformaldehyde. Then, limbs were post-fixed as described below.

Tissue preparation

Dissected hindlimbs were fixed overnight by immersion in paraformaldehyde solution at 4°C, followed by rinsing in phosphate-buffered saline (PBS, 0.01 M, pH = 7.4). Hindlimbs from animals older than GD21 were decalcified in 10% EDTA in 0.1 M Tris-HCl buffer (pH = 7) at 4°C for 5–14 days. The solution was refreshed every 2–3 days. The hindlimbs were then rinsed in PBS and immersed overnight in 25% sucrose in PBS with 0.01% sodium azide at 4°C. Tissue blocks were mounted in TissueTek OCT compound (Sakura, Tokyo, Japan) on cryostat holders and snap-frozen. Fifteen micrometre-thick cryosections were cut in the sagittal plane, thaw-mounted on poly-L-lysine-coated slides and air-dried.

Immunohistochemistry

A pre-incubation step with 10% normal goat serum in PBS containing 0.01% sodium azide, 0.05% thimerosal, 0.1% bovine serum albumin and 0.5% Triton X-100 was applied for 40 min to reduce non-specific binding and to increase penetration of the antibodies. An indirect immunofluorescence procedure was applied: the sections were incubated overnight at room temperature in humid chambers with primary rabbit anti-galanin antibody (Biogenesis, Poole, UK, #4600-5004; diluted 1:200). After rinsing in PBS, sections were incubated for 2 h at room temperature with a Cy3-conjugated goat anti-rabbit serum (Jackson IR, West Grove, PA, USA, 111-165-144; diluted 1:500). After final rinse, the sections were mounted in Vectashield medium (Vector, Burlingame, CA, USA, H-1000) to minimize photobleaching. In the controls, the primary or secondary antibodies were omitted and replaced by non-immune serum.

Fluorescence microscopy

Sections were preliminarily examined under an Olympus BX50 (Olympus, Tokyo, Japan) epifluorescence microscope equipped with filter set U-MNG for visualization of Cy3 (red) fluorescence. For precise demonstration of the examined antigen, the images were also registered with a Zeiss LSM 410 (Zeiss, Jena, Germany) confocal laser scanning microscope. A helium-neon laser (λ = 543 nm) and appropriate dichroic mirror and emission filter (FT560, LP570) were used for excitation of the fluorochrome and acquisition of its emission. Stacks of acquired optical sections were stored as graphic files and further processed with 3D-reconstruction software (Imaris 3.0, Bitplane AG, Zürich, Switzerland) working on an Indigo2 station (Silicon Graphics, Mountain View, CA, USA). Final images were obtained as a result of 'extended focus/maximal intensity projection' transformation and presented as TIFF files at a resolution of 512 × 512 pixels.

Results

In foetuses from GD16 to GD20, there was no immunoreactivity for galanin in the close vicinity of the tibial rudiment. The earliest, sparse galanin-immunoreactive (GAL-IR) nerve fibres appeared on GD21 in the perichondrium of both epiphyses (Fig. 1) and in the periosteum of the shaft (Fig. 2). Perichondrial/periosteal fibres were located in both layers: thick fibres and nerve bundles were present in the superficial fibrous layer, while thin fibres and nerve terminals were seen in the deeper cellular lining (Fig. 3). A majority of these fibres showed parallel orientation to the long axis of the bone rudiment. From PD1 onwards, GAL-IR fibres were also present in the bone marrow cavity of the shaft (Fig. 4) and in the region of inter-condylar eminence of the knee joint (Fig. 5). The density of GAL-IR fibres located in the bone marrow was lower than that of perichondrial/periosteal fibres. Bone marrow fibres mostly accompanied blood vessels (Fig. 4), although 'free' fibres, running between bone marrow cells were also observed (Figs 6 and 8).

Intramedullary GAL-IR fibres in proximal and distal metaphyses appeared around PD1 (Fig. 6). In both epiphyses, they were observed in the cartilage canals from PD7, in the secondary ossification centres from PD10 (Fig. 7) and in the bone marrow from PD14 (Fig. 8). The topographic distribution of GAL-IR fibres did not change in animals older than PD14, although their density seemed to decrease.

GAL-IR fibres located in all the mentioned tibial regions showed a characteristic, fine varicose appearance (see Fig. 2 as an example).

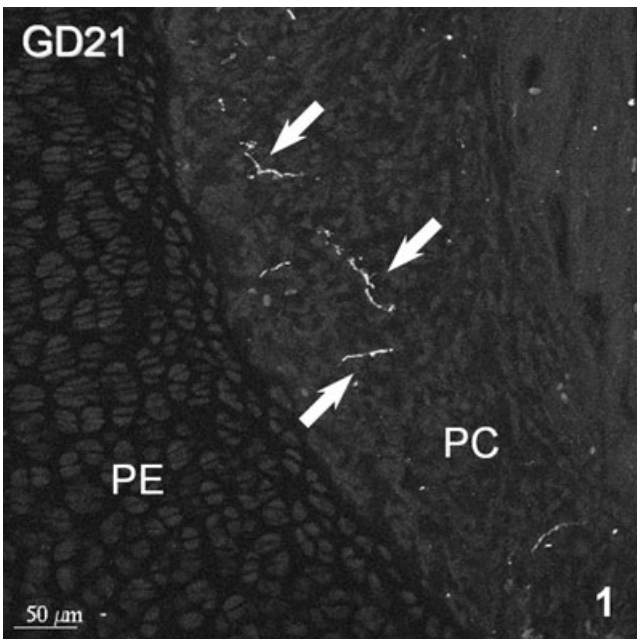

Fig. 1. GD21. Galanin-immunoreactive (GAL-IR) fibres *(arrows)* in the perichondrium of the proximal epiphysis. PE, proximal epiphysis; PC, perichondrium.

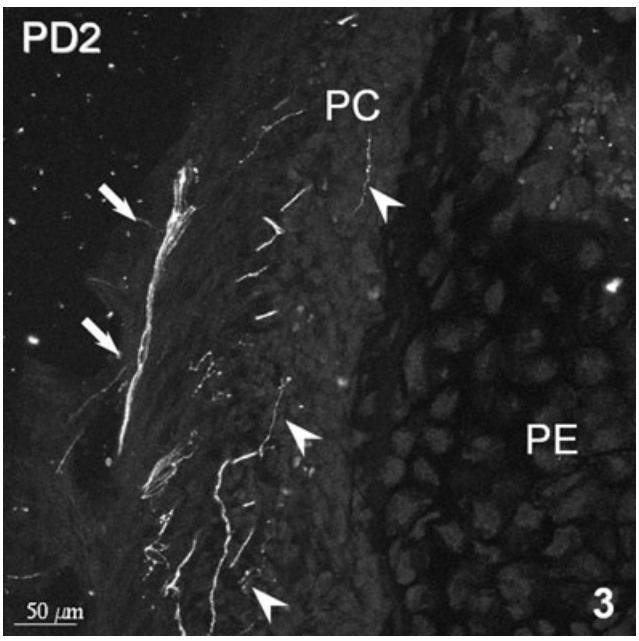

Fig. 3. PD2. GAL-IR fibres in the perichondrium of the proximal epiphysis: thick fibres and nerve bundles are present in the outer fibrous layer *(arrows)*, thinner fibres and terminals in the deep cellular lining *(arrowheads)*. PE, proximal epiphysis; PC, perichondrium.

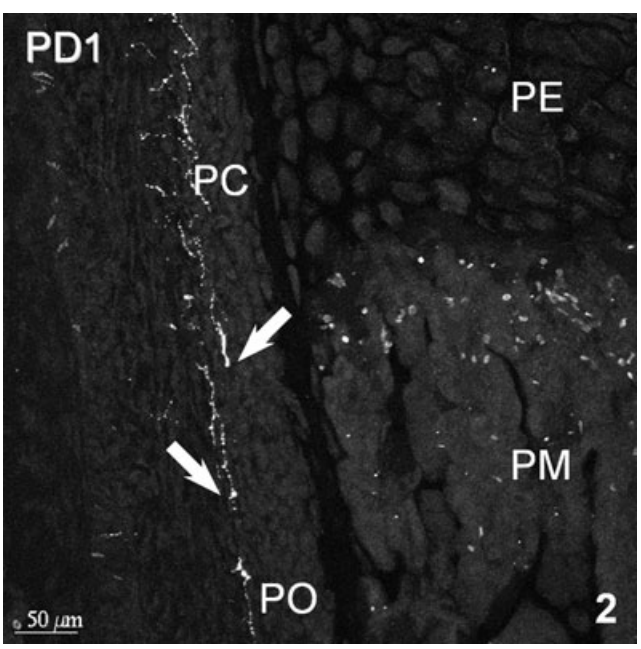

Fig. 2. PD1. GAL-IR fibres *(arrows)* in the perichondrium/periosteum of the metaphyseal region. PE, proximal epiphysis; PC, perichondrium; PO, periosteum; PM, proximal metaphysis.

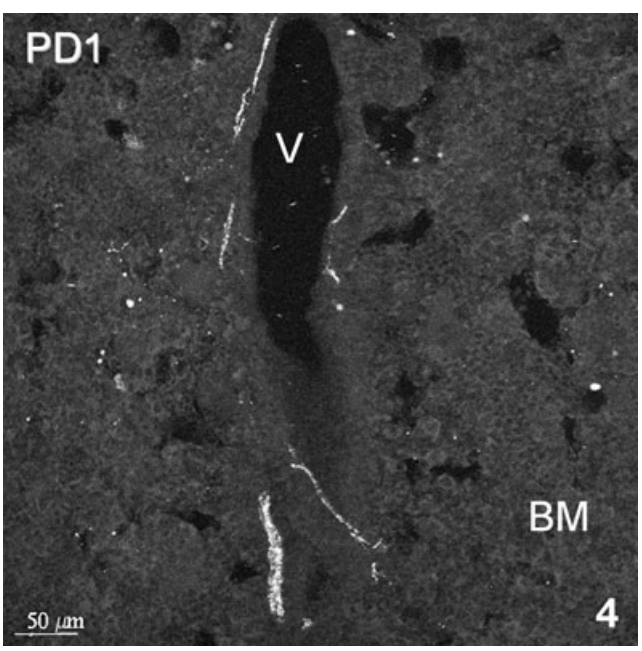

Fig. 4. PD1. Blood vessel-associated GAL-IR fibres in the bone marrow of the diaphysis. V, blood vessel; BM, bone marrow.

Discussion

This study presents for the first time – to the best of our knowledge – the spatiotemporal pattern of galanin-containing innervation in the developing bone. There are only few reports in the literature dealing with ontogeny of peripheral nerves in long bones (Calvo and Forteza-Vila, 1969; Sisask et al., 1995, 1996; Hara-Irie et al., 1996; Gajda et al., 2000, 2005) but none concerns galanin as a component of developing osseous innervation.

In our study, we found first galanin-expressing nerve fibres related to the bone rudiment on GD21. Similarly, first CGRP- and SP-IR sensory nerves in rat tibial rudiment could be discerned on GD21 in the perichondrium of both epiphyses and in periosteum of the shaft (Gajda et al., 2005). The earliest CGRP-IR intramedullary fibres were seen occasionally on

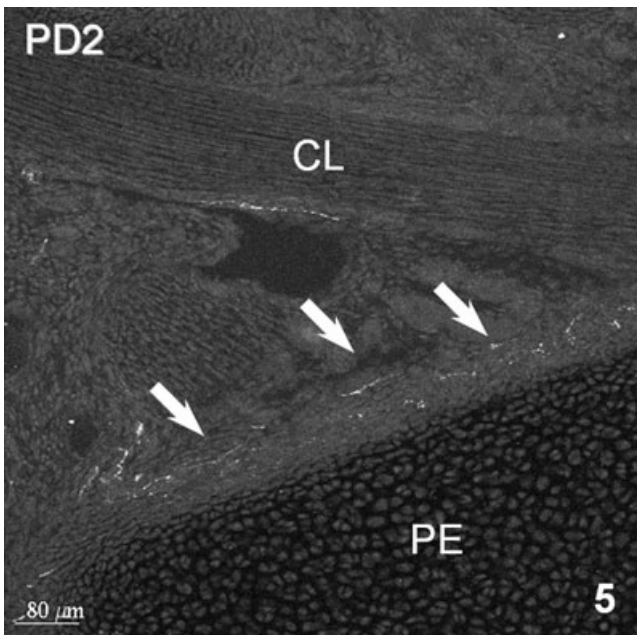

Fig. 5. PD2. GAL-IR fibres in the intercondylar eminence of tibia *(arrows)*. PE, proximal epiphysis; CL, cruciate ligament.

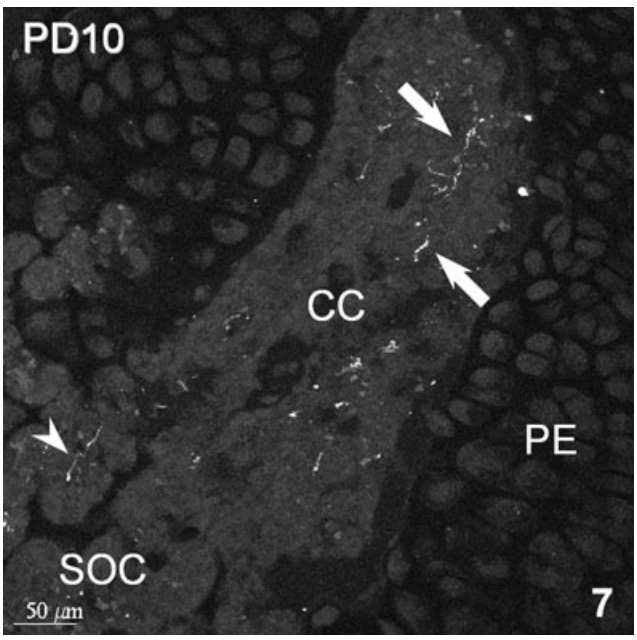

Fig. 7. PD10. GAL-IR fibres in the cartilage canal (*arrows*) of the distal epiphysis and in the secondary ossification centre (*arrowhead*). CC, cartilage canal; SOC, secondary ossification centre.

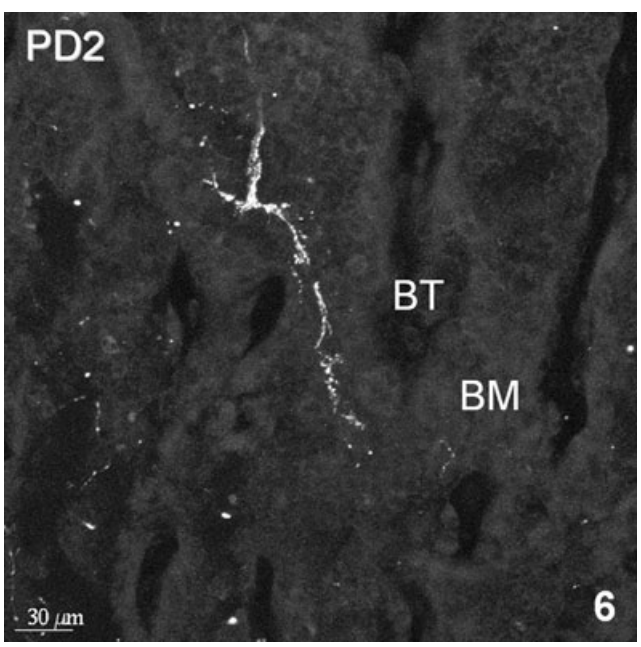

Fig. 6. PD2. GAL-IR fibres in the bone marrow of the distal metaphysis. BM, bone marrow; BT, bone trabecule.

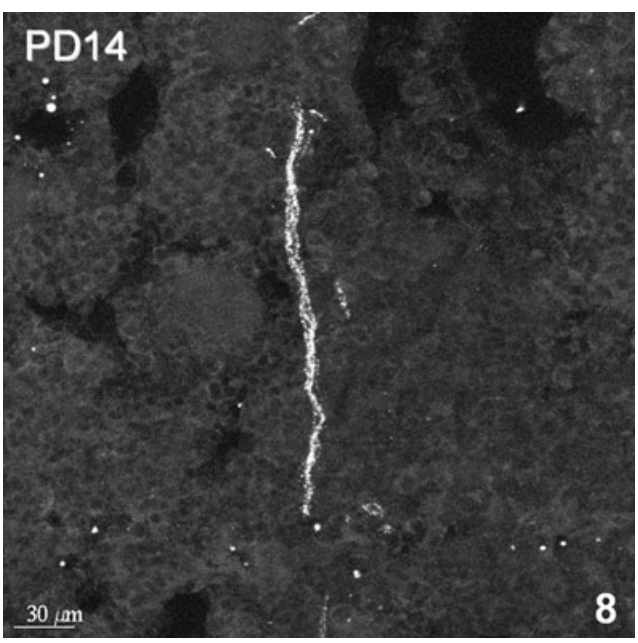

Fig. 8. PD14. GAL-IR fibres in the bone marrow of the proximal epiphysis.

GD21, whilst first galaninergic fibres in bone marrow cavity appeared on PD1, one day later. Peptidergic nerve fibres in epiphyseal cartilage canals, secondary ossification centres and in bone marrow of the epiphyses appear at the time as these structures develop during endochondral bone formation. In the mentioned locations, temporal occurrence pattern of galanin-containing fibres exactly matches that of CGRP- and SP-IR fibres. These observations strengthen the notion that the GAL-IR nerve fibres observed during development of the tibia are sensory in nature. Galanin was shown to coexist in the same neurons in dorsal root and trigeminal ganglia (Yoon et al., 2003) as well as in peripheral nerves (Brumovsky et al., 2006).

On the other hand, autonomic neurons can also express galanin, although this is a rare phenomenon. In immunocytochemical studies of rat stellate ganglia during ontogenesis, less than 1% of neurons displayed galanin labelling (Masliukov and Timmermans, 2004). In gene-targeted mice

over-expressing galanin, numerous GAL-IR neurons were seen in the superior cervical ganglion; however, in wild-type animals, they were only occasionally present. Similarly, in the hindpaw skin of naive mice only a few galaninergic nerve fibres were seen and they were not related to sweat glands, structures known to have extensive sympathetic innervation (Brumovsky et al., 2006).

The temporal and spatial pattern of the appearance of galanin-containing fibres and adrenergic or cholinergic autonomic fibres during long bone development shows numerous discrepancies. Generally, first autonomic nerve fibres appear later that the galanin-expressing ones. NPY-IR (adrenergic) nerve fibres are seen within bone marrow cavity from PD4. VIP-IR (cholinergic) fibres exhibit a similar temporal pattern and are also not observed in the bone marrow before PD4. Furthermore, autonomic nerve fibres extend into the metaphyses on PD6-8 and finally into epiphyses on PD10 (Sisask et al., 1996). GAL-IR fibres can be detected much earlier in these locations. It suggests that autonomic origin of galanin-containing fibres in the developing bone is unlikely.

Several observations suggest an involvement of galanin in the developmental processes. In rat and human dorsal root ganglia and spinal motoneurons, galanin is expressed at higher levels pre-natally than post-natally (Marti et al., 1987). This pattern of expression resembles that of other factors involved in the development and growth of axons, e.g. growth-associated protein 43 (GAP-43/B-50) (Oestreicher et al., 1997; Gajda et al., 2000). In adult mice carrying 'loss-in-function' mutation in galanin gene, a 13% loss of cells in dorsal root ganglia associated with increased apoptosis was reported (Holmes et al., 2000). Hence, it cannot be excluded that galanin plays a particularly important role during development of neural structures and that its upregulation observed after axotomy (Villar et al., 1989) or following bone fracture (McDonald et al., 2003) mainly represents activation of the ontogenetic program.

Early expression of galanin, its receptors and GAL-message associated peptide in various developing organs of the rat further support an important, albeit still unclear role of galanin during the prenatal period (Xu et al., 1996). In the same study, strong signal for galanin mRNA was observed as early as on GD19 in some regions of bone formation.

These findings put galanin in a different perspective and support attempts to search for its trophic function which potentially could be even more significant than neurotransmitter-like activity.

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