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Expression of galanin and a galanin receptor in several sensory systems and bone anlage of rat embryos

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ABSTRACT **Using *in situ* hybridization and immunohistochemistry the expression of, respectively, prepro-galanin (prepro-GAL) mRNA and GAL receptor-1 mRNA, as well as GAL-like and GAL message-associated peptide-like immunoreactivities, were studied in rats from embryonic day 14 (E14) to postnatal day 1. GAL expression was observed already at E14 in trigeminal and dorsal root ganglion neurons and at E15 in the sensory epithelia in developing ear, eye, and nose, as well as at E19 during bone formation. Also, GAL receptor-1 mRNA was expressed in the sensory ganglia of embryos but appeared later than the ligand. These findings suggest that GAL and/or GAL message-associated peptide may have a developmental role in several sensory systems and during bone formation.**

The 29-amino acid peptide galanin (GAL), originally isolated from porcine intestine (1), has a widespread distribution in the central nervous system (2–4) and is also present in primary sensory neurons (5, 6). GAL has been suggested to be involved in numerous neuronal and endocrine functions, with the main emphasis on a role as an intercellular messenger molecule/transmitter/modulator/hormone (7). The GAL precursor contains a second tentatively bioactive peptide, the GAL message-associated peptide (GMAP) (8). Recently, the first GAL receptor (GAL-R1) has been cloned (9–11).

An interesting feature of GAL is its low expression in several adult neuronal systems and its dramatic up-regulation after certain stimuli, such as nerve injury; one example is dorsal root ganglion (DRG) neurons (12, 13). In general terms, it has been postulated that compounds that are up-regulated after nerve injury are of importance for survival and regeneration of neurons, whereas transmitters and transmitter-synthesizing enzymes often are down-regulated (14–16). However, thus far there is little evidence that GAL is of importance for survival and/or regeneration, but neuropeptide tyrosine, another peptide up-regulated after nerve injury (17), has recently been shown to increase neurite outgrowth (18). In addition, GAL has been reported to have trophic actions in the pituitary (19).

Several neuropeptides exhibit interesting patterns of expression in discrete brain regions during development. For example, somatostatin appears very early during ontogeny in the rat brain but is then down-regulated early postnatally in some systems and may, therefore, play a developmental role (20, 21). With regard to GAL, Marti *et al.* (22) have provided evidence that GAL in rat and human DRGs and spinal motoneurons is expressed at higher levels prenatally than postnatally, and Gabriel *et al.* (23) have obtained similar results with radioimmunoassay on whole-brain preparations.

In the present study, we have analyzed rat embryos from embryonic day 14 (E14) to E21 and pups on postnatal day 1 (P1) for expression of GAL, GMAP, and prepro-GAL (ppGAL) mRNA and GAL-R1 mRNA using immunohistochemistry and *in situ* hybridization. The results suggest that GAL/GMAP may play a developmental role not only in several sensory systems but also during bone formation.

MATERIALS AND METHODS

Fetuses at E14, E15, E17, E19, and E21 and P1 pups were obtained from adult, timed-pregnant Sprague Dawley rats (Bantin & Kebo Universal, Stockholm). Fetuses were dissected from dams anesthetized with ether and frozen or postfixed by immersion in formalin. P1 rats were anesthetized and either frozen or perfused with formalin and postfixed. The whole body (E14–E19), head and body (E21), or brain, spinal cord, and DRGs (P1) were cut at 14-μm thickness in a cryostat (Microm, Heidelberg, Germany).

Immunocytochemistry. The sections were processed for indirect immunofluorescence staining or a modified method using biotin amplification (24) and a commercial kit (TSA kit, DuPont) and antiserum to GAL (E. Theodorsson, unpublished observations) or GMAP (25). Sections were first incubated overnight at 4°C with primary antiserum (dilution, 1:400–8000), rinsed, and incubated with anti-rabbit horseradish peroxidase-labeled secondary antibodies, followed by biotinylated tyramine. The reactions were detected with fluorescein-labeled streptavidin (DuPont). The sections were examined under a Nikon Optiphot II microscope or a Bio-Rad MRC-600 laser scanning confocal imaging system. Some sections were restained with propidium iodide. The specificity of the antisera was tested by absorption with synthetic peptides (GAL from Peninsula Laboratories; GMAP was a gift from T. Bartfai) for 24 hr at 4°C.

***In Situ* Hybridization Histochemistry.** Cryostat sections (14 μm) of unfixed embryo tissue were processed for *in situ* hybridization histochemistry as described earlier (26). Briefly, the sections were hybridized with ^{35}S-labeled oligonucleotide probes complementary to nucleotides 152–199 of rat ppGAL (27) or to nucleotides 4–51, 506–553, 784–831, and 975-1022 of the rat GAL-R1 (10, 11), exposed to NTB2 nuclear track emulsion (Kodak) (2–4 weeks), developed with D19 (Kodak), fixed in Kodak 3000 A&B, and analyzed. Some sections were stained with bisbenzimide (Sigma) (28). Control hybridizations were carried out with an excess of nonradioactive probe (100-fold).

RESULTS

Sensory Ganglia and Spinal Cord. Strong expression of ppGAL mRNA was observed from E14 and thereafter (Figs. 1 *A* and *B* and 2 *A* and *C*); GAL/GMAP-LI was first observed at E15 (Fig. 1 *D*, *E*, *H*, and *K*) in both DRGs (Fig. 1 *A, B, D, E, H,* and *K*) and trigeminal ganglia (Fig. 2 *A* and *C*). At the mRNA level, the signal from the trigeminal ganglion was still strong at E19 but was attenuated in DRGs at this stage (Fig. 1*F*). At high power magnification in the confocal microscope,

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Abbreviations: GAL, galanin; GMAP, GAL message-associated peptide; GAL-R1, GAL receptor 1; DRG, dorsal root ganglion; E14–E21, embryonic days 14–21; P1, postnatal day 1; ppGAL, prepro-GAL.

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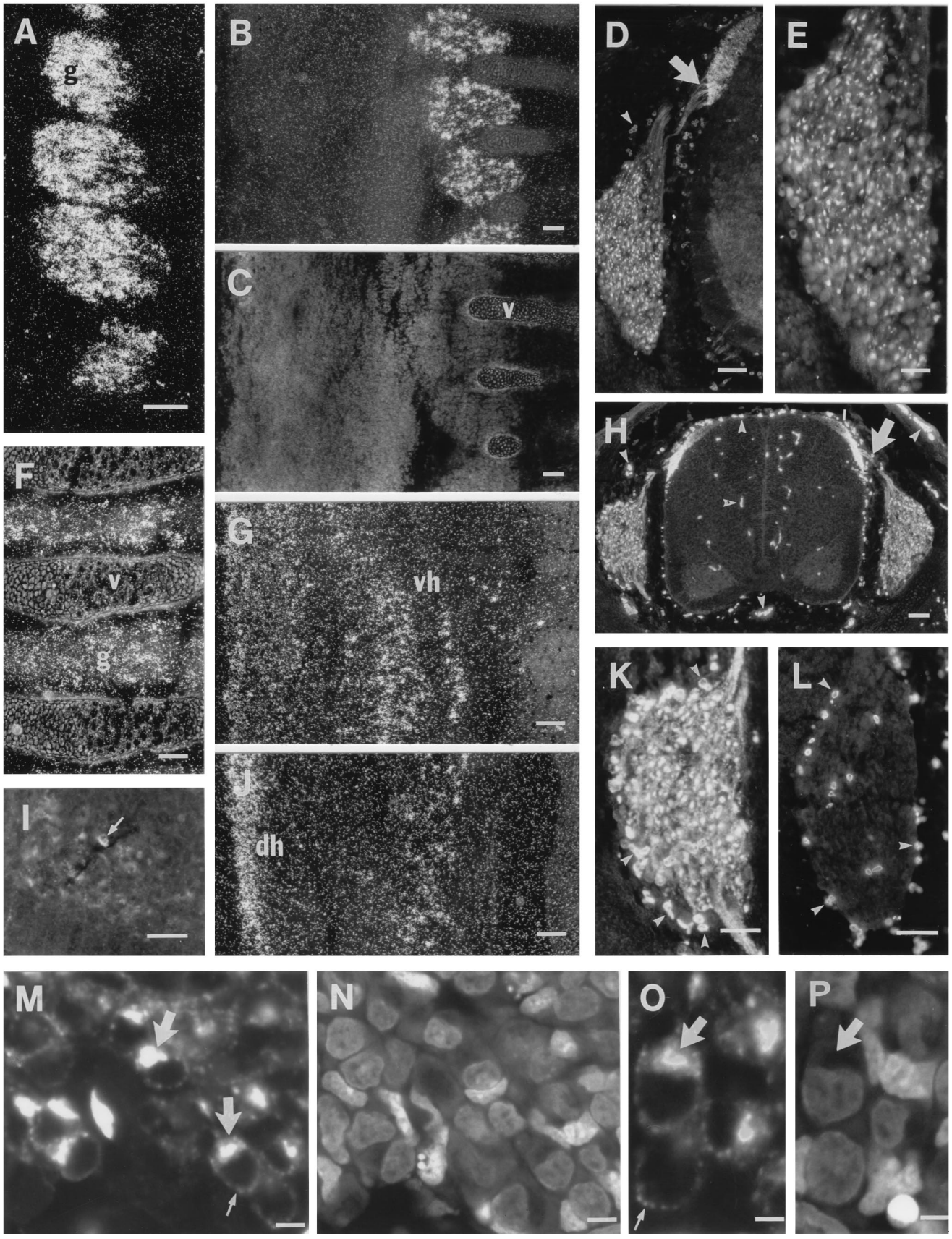

FIG. 1. Darkfield micrographs of DRGs (*A–C* and *F*) and spinal cord (*G* and *J*) after hybridization with probe complementary to ppGAL mRNA and fluorescence micrographs after incubation with GMAP antiserum (*D, E, H, I, K–M,* and *O*). *C* shows the same section as *B* stained with bisbenzimide. *L* shows semiadjacent sections to *K* incubated with GMAP antiserum preabsorbed with an excess of GMAP. *N* and *P* show the same sections as *M* and *O,* respectively, after staining with propidium iodide. Distinct ppGAL-mRNA and GMAP expression is seen in DRGs (g) at E15 (*A, D,* and *E*), E17 (*B, H,* and *K*), and E19 (*F*). Immunoreactive fibers (arrows) run from the DRGs to the dorsal horn (*D, H,* and *K*). GMAP-

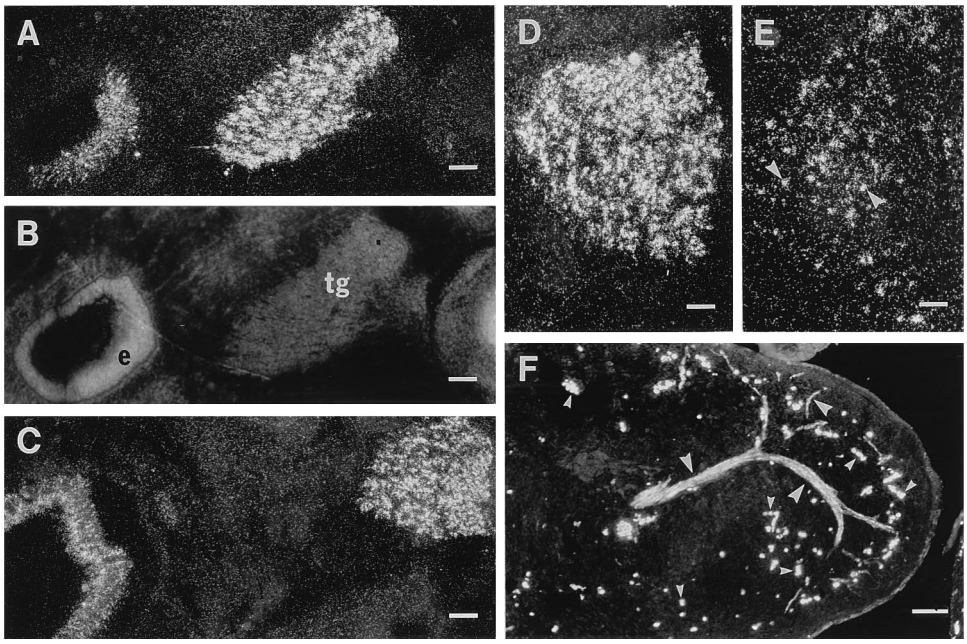

FIG. 2. Darkfield (*A* and *C–E*) and fluorescence micrographs (*F*) of inner ear (*A* and *C*), trigeminal ganglion (*A* and *C–E*), and lip (*F*) showing ppGAL mRNA (*A* and *C–E*) and GMAP-LI (*F*). *B* shows propidium iodide counterstaining of sections in *A*. *D* and *E* are semiadjacent sections. ppGAL bmRNA expression is seen in the trigeminal ganglion (tg) at E15 (*A*) and E17 (*C* and *D*). GAL-R1 mRNA is present in apparently fewer cells (*E*). Also, the sensory epithelium (e) of the inner ear shows GAL mRNA expression (*A* and *C*). GMAP-LI can be seen in a nerve extending branches in the skin, including into the epithelium (big arrowheads) (*F*). Small arrowheads point to nonspecific staining. (*A–C*, bar = 100 μm; *D* and *E*, 50 μm; *F*, bar = 200 μm.)

the immunoreactivity was found in the cytoplasm, especially in the presumable Golgi region, but also showing a dot-like appearance in the thin cytoplasmic rim surrounding part of the nucleus (Fig. 1 *M* and *O*). GAL/GMAP-LIs could be seen in axons running from DRGs to the dorsal horn of the spinal cord, where an intense immunoreactivity was seen in the superficial layers (Fig. 1 *D* and *H*). GAL/GMAP-immunoreactive neurons were seen in the ventral horn (Fig. 1*I*). From the trigeminal ganglia, immunoreactive processes could be seen running toward the medulla oblongata. GAL/GMAP-immunoreactive fibers were seen at E15–E17 in peripheral tissues, with strongly fluorescent fiber bundles and single terminals penetrating into the epithelium (Fig. 2*F*). A stronger-than-background signal for ppGAL mRNA was seen in the skin, just below the epithelium.

GAL-R1 mRNA was observed both in trigeminal ganglia at E17 (Fig. 2*E*) and in DRGs at E21. The numbers of GAL-R1 mRNA-positive neurons appeared lower than those for GAL mRNA-positive ones in both types of sensory ganglia (cf. Fig. 2 *D* and *E*). GAL-R1 mRNA was also observed in the spinal cord, where, at E17, a strong signal was seen in the ventral horn, presumably representing motor neurons (Fig. 1*G*), and at E19 a strong signal was found in the dorsal horn (Fig. 1*J*).

Inner Ear. ppGAL mRNA was observed in the sensory epithelium of the inner ear at E15 (Fig. 2*A*) with an increased intensity at E17, where the highest activity was seen in the central parts of the epithelium (Fig. 2*C*). The signal then decreased in intensity.

Eye. GAL expression in the eye was first detected at E14, and the strongest signal for both mRNA and peptide was seen at E17 (Fig. 3 *A, D,* and *F*). At E15 (Fig. 3*E*) and E19 (Fig. 3 *G* and *J*), the signal was weaker. The highest mRNA levels were observed in the inner layers of the retina. The immunoreactivity was seen in elongated cell bodies with radiating processes (Fig. 3 *D* and *F*). At E17, no certain signal for GAL-R1 mRNA could be seen (Fig. 3*C*).

Nasal Mucosa. Strong ppGAL mRNA was observed in the dorsal aspects of the nasal, presumably the olfactory mucosa at E15 (Fig. 3*H*), remaining high at E21 (Fig. 3*K*). GAL/GMAP-LI was strongly expressed at E17 (Fig. 3*L*).

Bone. GAL mRNA expression was observed at E19 in some regions of bone formation. The signal was strong and seen in large cells, presumably representing osteoclasts (Fig. 4 *A-C*).

Brain and Intestine. GAL and GAL-R1 signals were seen in brain and intestine at E14 and thereafter.

Controls. Absorption of antisera with GAL or GMAP resulted in a complete disappearance of the structures described above. However, with the TSA kit, numerous unspecifically labeled cells were observed (cf. Fig. 1 *K* and *L*), and further examples have been indicated by arrowheads on other micrographs (Fig. 1 *D, H,* and *K,* Fig. 2*F,* and Fig. 3 *B, F, G,* and *L*). The mRNA signals described above were completely blocked by the addition of an excess of cold probe.

DISCUSSION

The present results demonstrate an early expression of GAL/GMAP in five sensory systems, trigeminal ganglia, DRGs, the retina, and the sensory epithelia in the ear and nasal mucosa and of GAL receptor mRNA in the trigeminal and DRGs. This expression cannot be seen in adult rats or is at least markedly attenuated. High levels of ppGAL mRNA and/or GAL- and GMAP-LIs were detected in all systems, with a maximal signal at E15–E17. In fact, ppGAL mRNA was detected as soon as E14 [i.e., when the small dark, isolectin B4-positive DRG neurons are generated (29)]. At least in some of these systems,

positive cell bodies are shown in the ventral horn (*I*). Confocal micrographs show immunoreactivity in mainly the Golgi compartment (arrows) but also as dot-like structures in the thin perinuclear cytoplasm (small arrows) (*M–P*). The GAL-R1 receptor mRNA is at E17 and is found mainly in the ventral horn (vh) (*G*) and at E19 in addition in the dorsal horn (dh) (*J*). Arrowheads in *D, H, K,* and *L* point to nonspecific staining, as revealed in the absorption experiments (*L*). v, vertebra. (*A–D*, *F–H*, and *J*, bar = 100 μm; *I*, *K*, and *L*, bar = 50 μm; *E*, bar = 25 μm; *M* and *N*, bar = 5 μm; *O* and *P*, bar = 2.5 μm.)

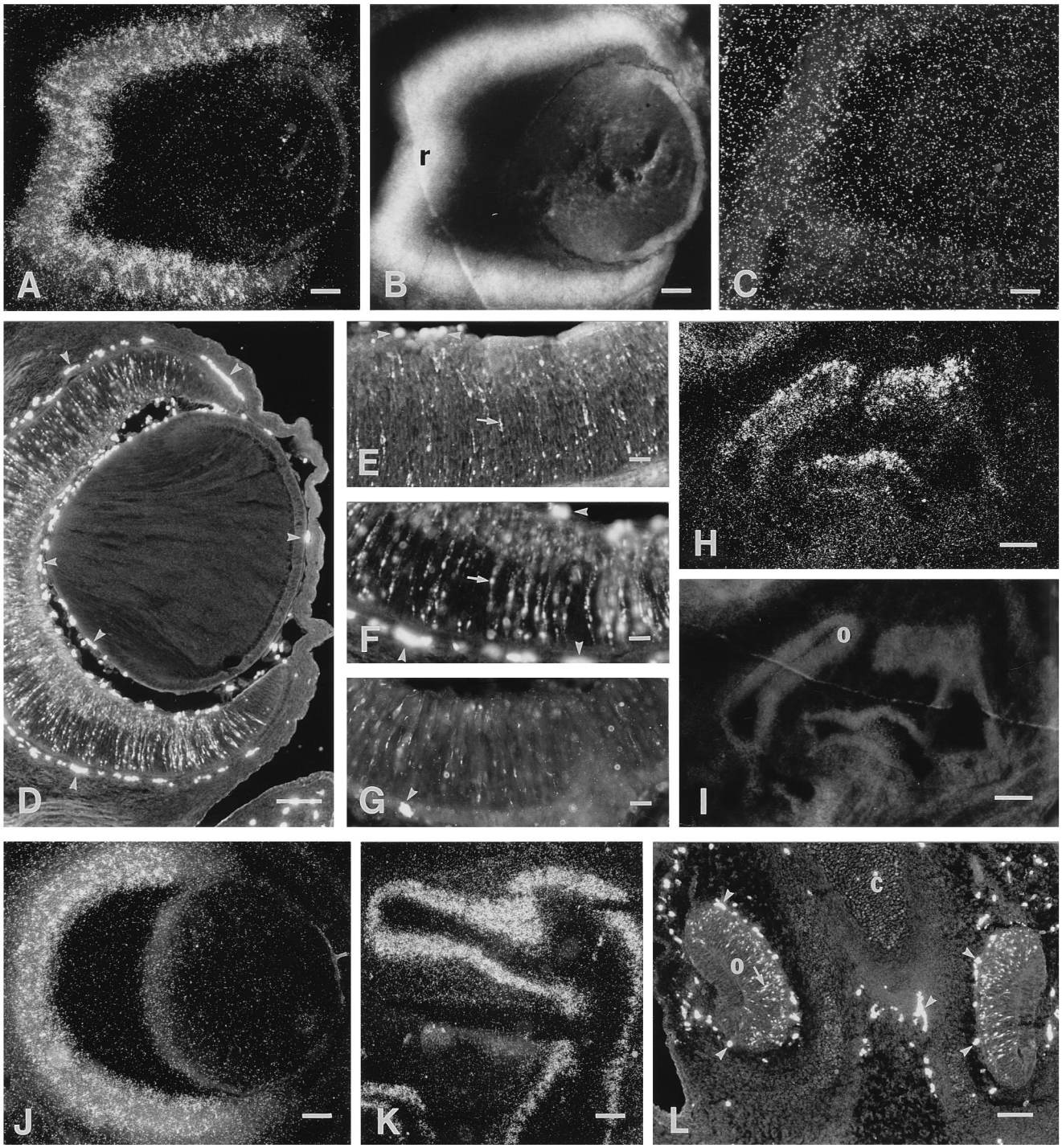

FIG. 3. Darkfield (*A, C, H, J,* and *K*) and fluorescence (*D–G* and *L*) micrographs of the eye (*A–G* and *J*) and nasal mucosa (*H* and *K–L*) showing ppGAL mRNA (*A, H, J,* and *K*) GAL-R1 mRNA (*C*) and GMAP-LI (*D–G* and *L*) expression. *B* and *I* show bisbenzimide counterstaining of the same sections as in *A* and *H,* respectively. GAL expression (arrows) is seen in the retina (r), with the highest level at E17 (*A, D,* and *F*) and lower levels at E15 (*E*) and E19 (*G* and *J*). No distinct GAL-R1 mRNA signal is seen at E17 (*C*). A strong ppGAL mRNA signal is seen in the dorsal aspects of the nasal mucosa at E15 (*H*) and E21 (*K*). GMAP-LI (arrow) is seen in olfactory epithelium (o) (*L*). c, cartilage. Small arrowheads indicate nonspecific staining. (*A–G, J,* and *K*, bar = 100 μm; *H, I,* and *L*, bar = 200 μm.)

it could be shown that the mRNA is translated into peptides. The findings on DRGs confirm and extend earlier immunohistochemical studies by Marti *et al.* (22) and, on in situ hybridization analysis, by Parker *et al*. (11). In the DRGs, GAL/GMAP-positive fibers could be followed into the superficial layers of the dorsal horn and in peripheral branches, suggesting that GAL/GMAP is both centrally and peripherally transported at least as early as E15. In the central nervous system, prenatal expression of GAL/GMAP was observed. These locations were not studied systematically but support the biochemical studies of Gabriel *et al.* (23). We also confirm early expression in the intestine (30). Finally, a prenatal mRNA signal was also detected in the zone of bone formation.

The early expression of GAL, GMAP, and a GAL receptor suggests that one important role of GAL may occur during the prenatal period. This pattern of expression resembles that of other factors involved in development and growth. For example, growth-associated protein (GAP)-43 (also called Protein F1,

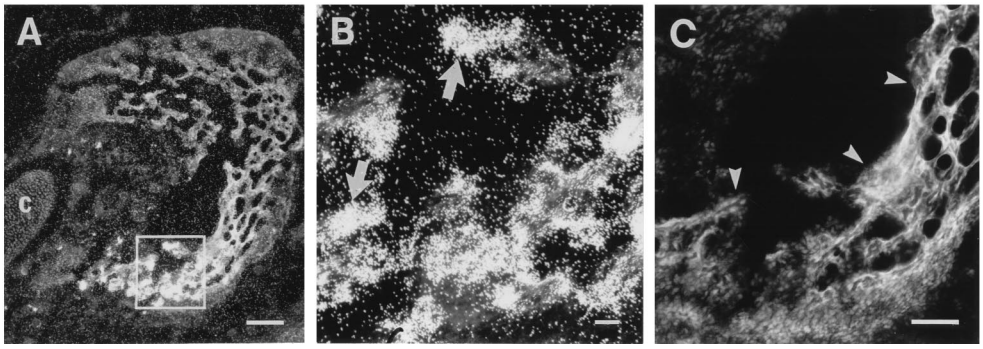

FIG. 4. *In situ* hybridization autoradiographs of bone formation (*A* and *B*). *B* is a high magnification of *A,* as indicated by small box. *C* shows bisbenzimide counterstaining of part of the section shown in *A*. A strong ppGAL mRNA signal (arrows) is seen in cellular elements (arrowheads) in the zone of bone formation. c, Cartilage. (*A*, bar = 200 μm; *B* and *C*, bar = 50 μm.)

B50, GAP-48, or neuromodulin) (31, 32) is closely related to axonal growth during development, with high concentrations in growth cones. GAP-43 is then down-regulated in most systems but can be reactivated after nerve injury, as has been shown for primary sensory neurons (33, 34). In fact, it cannot be excluded that the main role of GAL/GMAP is during development, and that the up-regulation seen after axotomy (12, 13) mainly represents activation of an ontogenetic program.

A developmental role for GAL/GMAP is also suggested by their early and transient appearance in three other sensory systems: the retina, the inner ear, and the nasal, presumably olfactory, mucosa. The exact cellular localization of GAL/GMAP in the retina and ear remains to be established. Taken together, these findings put the neuropeptide GAL into a different perspective and reinforce attempts to search for roles other than that of neurotransmitter-like activity. In fact, several other peptides, e.g., vasoactive intestinal polypeptide (35), have been shown to exert multiple trophic actions.

A completely unexpected finding was the expression of ppGAL mRNA in bone tissue. Thus far, GAL expression has mainly been confined to neurons and certain endocrine systems such as the pituitary gland (7). However, bone and the nervous system share several molecules during development, e.g., osteogenic protein, which is important not only in several peripheral tissues but also in the central nervous system, including the eye (36).

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