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ORIGINAL ARTICLE

# Gangliocytes in Neuroblastic Tumors Express Alarin, a Novel Peptide Derived by Differential Splicing of the Galanin-Like Peptide Gene

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## Abstract

In neuroblastic tumors a relationship of differentiation of the tumor to galanin receptor expression and antiproliferative and apoptotic effects upon activation of galanin receptors in neuroblastoma cells was reported. To elucidate the expression of other components of the galanin peptide family in neuroblastic tumors, RT-PCR analysis of a variety of human neuroblastic tumor tissues was performed. Ganglioneuroma tissues revealed the presence of a splice variant of the galanin-like peptide (*GALP*) mRNA, which results in exclusion of exon 3 and a frame shift after the signal peptide sequence of *GALP*. This generates a peptide of 25 amino acids, which we have termed alarin because of the N-terminal alanine and the C-terminal serine. The novel neuropeptide alarin does not reveal significant homology to other peptides. Immunohistochemistry with antibodies directed against synthetic alarin peptide detected specific cytoplasmic granular staining in ganglia of human ganglioneuroma and ganglioneuroblastoma, as well as differentiated tumor cells of neuroblastoma tissues. Undifferentiated neuroblasts of these tumor tissues did not show alarin-like immunoreactivity and alarin-specific mRNA. Our findings indicate that alarin expression is a feature of ganglionic differentiation in neuroblastic tumor tissues.
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**Index Entries:** Neuroblastic tumor; galanin-like peptide; alarin; gangliocytes; differential splicing; differentiation.

## Introduction

Neuroblastic tumors, such as neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, are neuroendocrine neoplasms that arise from primordial sympathogenic neural crest cells. These tumors reflect a variable mixture of primitive malignant neuroblasts, more differentiated ganglion-like cells (gangliocytes), Schwannian stroma, and endothelial cells. Neuroblastomas are composed primarily of neuroblasts, exhibiting limited or absent ganglionic differentiated cells, and are therefore classified as

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differentiated, poorly differentiated, or undifferentiated neuroblastomas. The benign ganglioneuroma consists only of cells that are well differentiated (Shimada et al., 1999). Neuroblastic tumors have been shown to express a variety of neuropeptides such as somatostatin, neuropeptide Y, chromogranins, gastrin-releasing peptide, vasoactive intestinal peptide, neurotensin, cholecystokinin, gastrin, and galanin (Kogner, 1995; Reubi, 2003; Berger et al., 2005; Gustafson et al., 2005). For some of these peptides a role in neuroblastoma cell growth was demonstrated (Reubi, 2003; Gustafson et al., 2005). In recent studies it was shown that galanin is expressed and secreted by neuroblastic tumors. No correlation of the level of galanin peptide to tumor differentiation was observed. However, galanin receptor expression was correlated to tumor differentiation (Berger et al., 2002; Perel et al., 2002). In addition, activation of the galanin receptor GalR2 exogenously expressed in neuroblastoma cells by galanin leads to inhibition of cell proliferation and apoptosis (Berger et al., 2004; Lang et al., 2005).

Galanin-like peptide (GALP) was originally discovered as an endogenous ligand of the galanin receptor GalR2 in the porcine hypothalamus (Ohtaki et al., 1999). GALP (9-21) is identical to galanin (1-13), and the sequence homology among species is high. In the rat brain, *GALP* mRNA and GALP protein have been found in cell bodies residing exclusively in the hypothalamic arcuate nucleus and median eminence, and this distribution is conserved across all species studied to date (Kerr et al., 2000; Larm and Gundlach, 2000; Gundlach et al., 2001; Shen et al., 2001; Takatsu et al., 2001). GALP-immunoreactive fibers also project to various regions of the forebrain. In the macaque monkey, Cunningham et al. (2004) have shown recently that GALP neurons play a role in integrating metabolic signals that are related to circuits controlling gonadotropin-releasing hormone release.

Ligand-binding assays using the human neuroblastoma cell line SH-SY5Y, transfected with the respective galanin receptors, revealed that human GALP displayed the highest affinity for the galanin receptor subtype GalR3, followed by GalR2 and GalR1 (Lang et al., 2005). Similar to galanin, GALP is also able to induce cell death in GalR2-expressing neuroblastoma cells.

To elucidate if galanin-related peptides could be important factors in neuroblastic tumor biology, the expression of *GALP* mRNA and the existence of possible splice variants in ganglioneuroma and neuroblastoma tissues was analyzed.

## Materials and Methods

### *Patient Material*

Primary tumor samples were obtained from children with neuroblastomas ($n$ = 15), ganglioneuroblastomas ($n$ = 5), and ganglioneuromas ($n$ = 5). Patient selection was based solely on tissue availability (from 1992 to 2004). The tissues were embedded in paraffin or snap frozen in liquid nitrogen within 30 min of surgery and stored at –80°C. Clinical diagnosis and staging were performed according to the International Neuroblastoma Staging System (Brodeur et al., 1988).

### *RT-PCR Analysis*

Total RNA (1 μg) was reverse-transcribed with 200 U Superscript II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD). cDNA (100 ng) was used for PCR amplification with Thermo Start polymerase (ABgene, Surrey, UK) in the presence of 10 pmol of each primer. The primers used were as follows: GALP, acc. no. NM_033106 (66–84, 376–357) and GAPDH, acc. no. NM_002046 (391–414, 644–621). For GAPDH RT-PCR an initial denaturation step at 95°C for 5 min was performed, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 2 min. GALP RT-PCRs were performed with a denaturation step at 95°C, followed by 50 cycles each consisting of 10 s at 95°C, a primer annealing step at 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on an agarose gel.

### *Generation of Polyclonal Alarin Antibodies*

A rabbit polyclonal antiserum was custom made using the synthetic human alarin peptide 6-24-Cys (SSTFPKWVTKTERGRQPLRC) (NeoMPS, Strasbourg, France). Briefly, alarin 6-24 was coupled via a C-terminal cysteine residue to the carrier protein keyhole limpet hemocyanin. Immunization was carried out on day 0, 14, 28, and 56.

For affinity purification of the antiserum the immunogenic peptide human alarin 6-24-Cys (4 mg) was coupled to a HiTrap *N*-hydroxy-succimide (NHS)-activated HP column (1 mL) according to the manufacturer's instructions (Amersham Biosciences; Buckinghamshire, UK). The polyclonal anti-alarin antiserum (4 mL) of the final bleeding was diluted 1:1 with phosphate-buffered saline (PBS), filtered through a 0.45-μm filter, and loaded onto the column. The column was washed with 50 m*M* Tris-HCl (pH 8),

0.1% Triton X-100, and 500 m*M* NaCl, followed by 50 m*M* Tris-HCl (pH 9), 0.1% Triton X-100, 500 m*M* NaCl and, finally, 50 m*M* sodium phosphate (pH 6.3), 0.1% Triton X-100, and 500 m*M* NaCl. The antibody was eluted with 50 m*M* glycine (pH 2.5), 0.1% Triton X-100, and 0.15 *M* NaCl, and the eluate was immediately neutralized with 20 m*M* Tris-HC (pH 9).

### *Immunohistochemistry*

Cryosections (10 μm) of the tumor tissues were air-dried, fixed in 4% formaldehyde for 10 min, and washed with 1× PBS. Immunostaining was performed according to the protocol of Level 2 USA Ultra Streptavidin Detection System (Signet Laboratories, Dedham, MA), with modifications. The endogenous peroxidase was quenched in 3% $H_2O_2$ for 5 min. Sections were incubated with blocking reagent for 30 min at room temperature, followed by an overnight incubation at 4°C with affinity-purified alarin antibody, diluted 1:200 in PBS. After three washes with PBS and incubation with linking reagent for 1 h, sections were treated with labeling reagent for 20 min and washed with PBS. Alarin-like immunoreactivity (alarin-LI) was visualized using 3,3′-diaminobenzidine as a chromogenic substrate. Sections were counterstained with Mayer's Hemalum solution (Merck KGaA, Darmstadt, Germany) and mounted with Kaiser's glycerol gelatin. The specificity of immunostaining was tested by preabsorbtion of the affinity-purified alarin antiserum with 3 μ*M* of the respective peptide for 2–3 h at 37°C. Following centrifugation for 10 min at 13,000 rpm, serial sections were incubated with the preabsorbed serum.

For alarin immunofluorescence (IF), cryosections (10 μm) were air-dried for 15 min at room temperature, fixed in 4% formaldehyde for 10 min, washed with 1× PBS, and blocked with 3% normal goat serum in PBS for 30 min. The sections were incubated overnight at 4°C with the alarin antibody diluted 1:100 in 3% normal goat serum in PBS. After several rinses with PBS, the sections were incubated for 1 h at room temperature with Cy2-linked goat anti-rabbit antibody (1:100, Amersham Pharmacia, Buckinghamshire, UK). After several washes with PBS and distilled water, the sections were mounted with Aqua Polymount (Polysciences, Warrington, PA) and examined with a fluorescence-equipped microscope. Paraffin sections (4 μm) were deparaffinized, rehydrated, and heated to 90°C for 15 min in 0.01 *M* citric acid buffer (pH 6.0). Sections were washed with 1× PBS, blocked with 3% normal goat serum in PBS for 30 min, and then treated as the cryosections.

## Results

### *Differential Splicing of the* GALP *Gene*

Using GALP primers spanning exons 2–5 for RT-PCR, we detected the expression of *GALP* full length mRNA in different ganglioneuroma tissues (Fig. 1A). In addition, a smaller PCR fragment was observed in ganglioneuroma tissues and a human neuroblastoma cell line (SMS-KAN) (Fig. 1A). Sequencing of this PCR product (~280 bp) revealed differential splicing of *GALP* mRNA with the exclusion of exon 3 (Fig. 1B).

The skipping of exon 3 of G*ALP* mRNA results in a frame shift leading to a novel peptide sequence and a stop codon after 49 amino acids (Fig. 1C). The signal sequence of prepro-GALP and the first 5 amino acids of the mature GALP peptide are still present, followed by 19 amino acids, which do not show homology to any other protein found in public sequence data bases. Because the N-terminal part of the precursor protein is encoded by exon 2, the proteolytic cleavage site of GALP is conserved. Therefore, proteolytic processing should result in a 25-amino-acid-long neuropeptide (GenBank accession no. AY329637 [Fig. 1C]). We termed the novel peptide alarin because of the N-terminal alanine and the C-terminal serine. Searching the human and murine EST data bases (http://www.ncbi.nlm.nih.gov/BLAST) for this 25-amino-acid peptide sequence did not reveal any corresponding EST clones or significant homology to other peptides.

### *Alarin-LI in Neuroblastic Tissues*

To determine the cell type expressing the alarin mRNA variant in the neuroblastic tumor tissues, a polyclonal antiserum to synthetic human alarin 6-24 was generated. This region of the peptide was chosen to avoid cross-reactivity to the first five amino acids of the peptide that are identical with the first five amino acids of GALP. The antiserum was purified via the immobilized immunogenic peptide, and the specificity of the alarin antibody was tested by dot blot analysis.

Immunofluorescence (IF) analysis of paraffin sections revealed granular cytoplasmic alarin-LI in ganglia of a ganglioneuroma tissue (Fig. 2A). High-magnification microscopic examination of IF-positive ganglionic cells in ganglioneuroma tissues indicates a vesicular distribution of alarin, a feature expected of a neuropeptide.

To ensure the specificity of the immunostaining, preabsorption of the antiserum with synthetic alarin

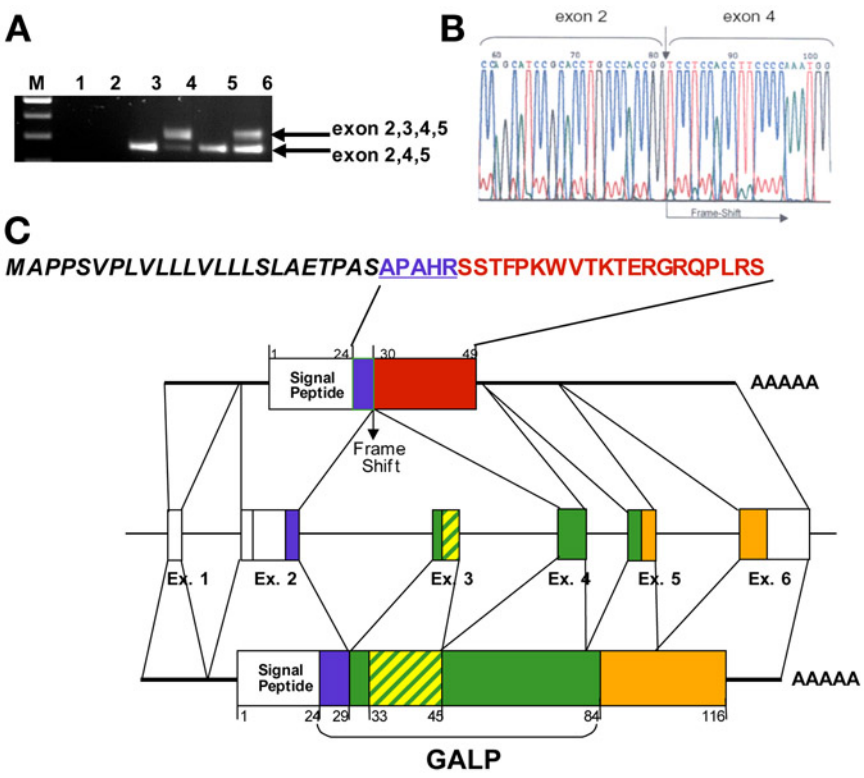


Fig. 1. Differential splicing of *GALP* mRNA **(A,B)** leads to a frame shift creating the novel peptide alarin **(B,C)**. **(A)** RT-PCR using primers spanning exons 2–5 of human *GALP* mRNA. M, 100-bp ladder; lane 1, negative control; lane 2, undifferentiated neuroblastoma tissue (5 yr, male, stage 1); lane 3, ganglioneuroblastoma tissue (13 mo, male, stage 3); lane 4, ganglioneuroma tissue (4 yr, female, stage 1); lane 5, human neuroblastoma cell line SMS-KAN; lane 6, human basal ganglia. **(B)** Sequence of smaller PCR product. **(C)** Schematic drawing of *GALP* gene and splice variants: Blue boxes indicate amino acids identical to mature GALP and alarin. The underlined amino acid sequence corresponds to the mature alarin peptide. Yellow/green boxes indicate the homology of GALP with galanin. Numbers indicate amino acid positions of the precursor peptides.

peptide (3 μ*M*) abolished the staining, whereas preabsorption with GALP 1-32 (3 μ*M*), GALP 1-60 (3 μ*M*) and galanin (3 μ*M*) did not change the staining pattern (Fig. 2). The unpurified preimmune serum also did not show staining of ganglionic structures (Fig. 2F).

The analysis of other samples of neuroblastic tumors revealed that all ganglioneuroma tissues ($n = 5$) showed alarin-LI-positive ganglia. Also in ganglioneuroblastomas ($n = 5$) ganglionic cells were alarin-LI positive. Furthermore, alarin-LI was observed in gangliocytes in all differentiated neuroblastomas ($n = 5$ [Fig. 3]). Poorly differentiated neuroblastomas and undifferentiated tumors displayed scarce alarin-LI-positive gangliocytes ($n = 10$). Undifferentiated neuroblasts did not display alarin-LI (Fig. 3J).

To further ensure the specificity of alarin-LI, RNA was isolated from consecutive sections of selected tissues used in immunohistochemistry studies and analyzed by RT-PCR. Only tissue sections showing positive alarin-LI also carried the alarin mRNA splice variant (Fig. 3A–I). No alarin splice product was seen in tissue sections lacking alarin-LI (Fig. 3J,K). Chromogenic, as well as IF, immunohistochemistry was successful in detection of alarin-LI-positive cells in cryostat and paraffin sections (Figs. 2 and 3).

## Discussion

Here, for the first time, we report differential splicing of the *GALP* gene, which leads to the novel neuropeptide alarin. This peptide does not show homology to other neuropeptides and is not found in EST data bases. There might be several reasons why the alarin splice variant has not been detected by other investigators. First, expression of *GALP* has been investigated by immunohistochemistry, and the antibody directed toward amino acids 1–10 might not cross-react with the first five amino acids of alarin; even if

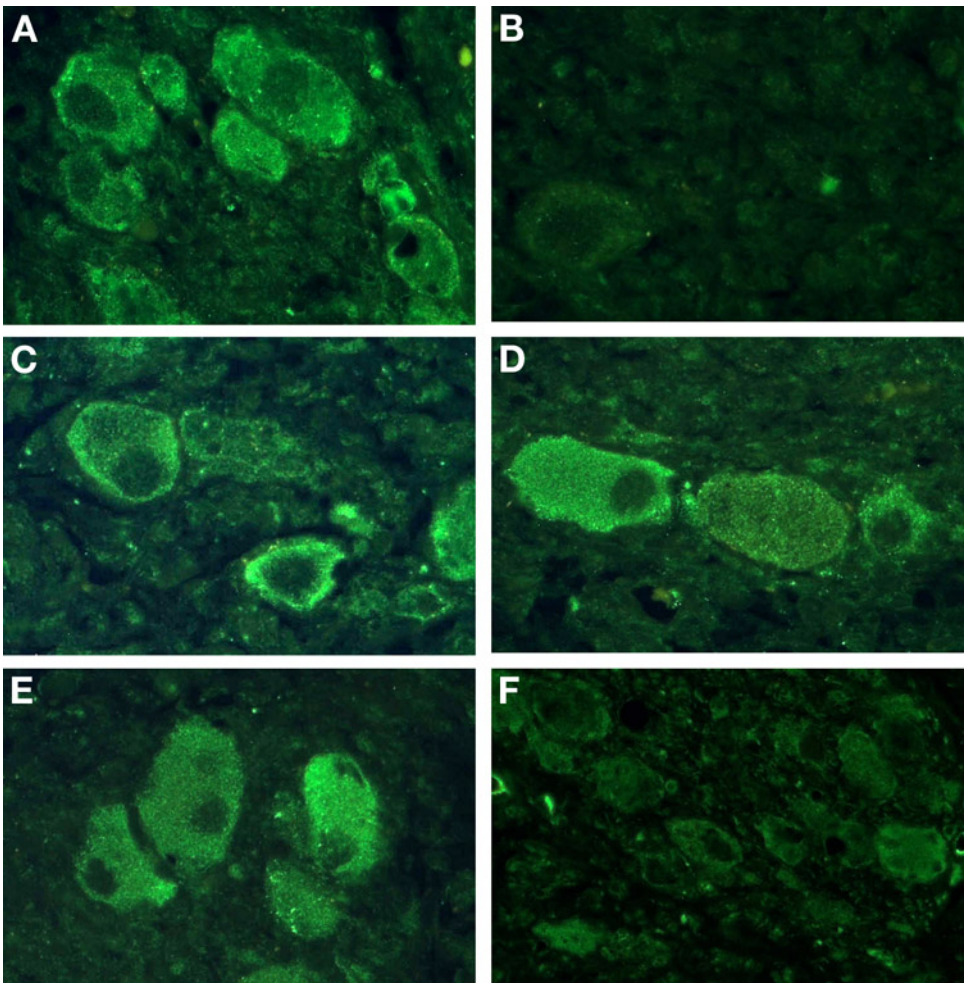


Fig. 2. IF showing alarin-LI in gangliocytes of a ganglioneuroma tissue (stage 1, female, 4 yr) stained with affinity-purified alarin polyclonal antibody (1:100 **[A–E]**). Preabsorption with 3 μ*M* synthetic alarin 6-24-Cys peptide abolished staining in ganglia **(B)**, whereas preabsorbtion of the antibody with 3 μ*M* GALP 1-32 **(C),** 3 μ*M* GALP 1-60 **(D),** and 3 μ*M* galanin **(E)** did not abolish the staining. Preimmune serum (1:200) showed a lack of ganglionic staining **(F)**.

it does, no discrimination could have been visible between the two peptides. Second, *in situ* hybridization also will detect alarin and *GALP* mRNA if a probe is used that spans several exons. Therefore, data generated, especially by *in situ* hybridization with *GALP* mRNA probes, also could have detected alarin mRNA and have to be re-evaluated using GALP- and alarin-specific antibodies or exon-specific probes. Third, expression of the alarin splice variant might be restricted only to a small cell population in certain tissues. Therefore, alarin mRNA might have not been spotted by pepdidomics or the analysis of EST libraries. Finally, read through of sequencing reactions of GALP EST clones might not have been extensive enough to detect the absence of exon 3.

The prepro-alarin precursor protein translated from the *GALP* mRNA missing exon 3 still harbors the signal peptide sequence and proteolytic processing sites of GALP (Ohtaki et al., 1999). Therefore, cells might employ the same processing machinery for both precursor proteins. The lack of the galanin receptor–binding domain indicates that signaling of the alarin peptide is mediated by different receptors. The type of receptor and the physiological activity of alarin have to be identified in future studies.

Other neuropeptide genes also employ differential splicing to generate different peptides from one gene. For example, the calcitonin gene–related peptide (*CGRP*) gene is alternately spliced to produce calcitonin, a hormone involved in calcium homeostasis in non-neuronal cells, and CGRP, a potent vasodilator neuropeptide (Rosenfeld et al., 1983). Calcitonin mRNA is produced by the splicing of exons 1–4, and CGRP comprises exons 1, 2, 3, 5, and 6. This

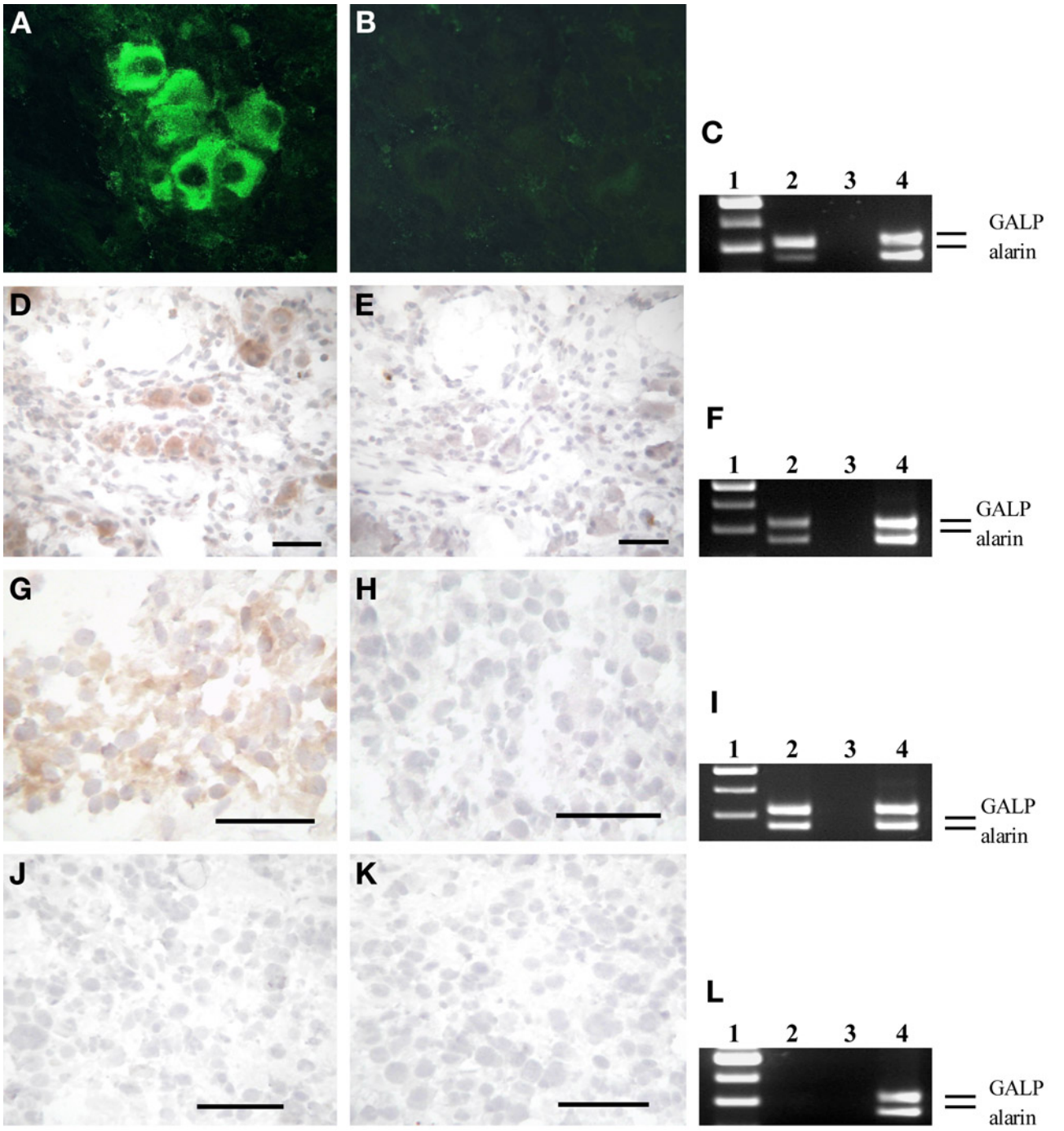


Fig. 3. Alarin-LI staining and RT-PCR of cryostat sections of ganglioneuroma (4 yr, female, stage 1 **[A–C]**), ganglioneuroblastoma (13 mo, male, stage 3 **[D–F]**), differentiated neuroblastoma (6 yr, male, stage 3 **[G–I]**), and undifferentiated neuroblastoma (17 yr, female, stage 4 **[J–L]**). **(A,D,G,J)** Staining with affinity-purified alarin (6-24) antiserum (1:200); **(B,E,H,K)** staining preabsorbed with 3 μ*M* synthetic alarin 6-24-Cys peptide. **(I–L)** RT-PCR of consecutive sections with GALP primers spanning exons 2–5. **(C,F,I,L)** Lanes 1, 100-bp ladder; lanes 2, respective tumor tissues; lanes 3, negative control; lanes 4, positive control, human basal ganglia. Scale bar = 50 μm.

regulation is executed by neural-specific inhibition of splicing to exon 4, which encodes calcitonin. The prepro-tachykinin (*PPT-A*) gene encodes several tachykinin peptides (substance P, neurokinin A, neuropeptide P, and neuropeptide K) with distinct pharmacological properties (Harmar et al., 1990; Helke et al., 1990). The mechanisms accounting for the expression of several tachykinins from the *PPT-A* gene include alternative post-transcriptional splicing and post-translational processing. The differential expression of splice variants of a gene might occur in distinct tissues and organs (Sirand-Pugnet et al., 1995; Wang et al., 1999) and might be regulated developmentally (Enigk and Maimone, 1999) or vary within one tissue type.

In the galanin system differential splicing has been described only for avian and goldfish galanin mRNA (Kohchi and Tsutsui, 2000; Unniappan et al., 2003). The physiological significance of the multiple forms of galanin mRNA in these organisms is unknown. Here, for the first time, we report a splice variant of the *GALP* gene. Up to now it is not clear if non-pathological peripheral tissues express *GALP* and alarin mRNA. Our speculation that alarin is another novel neuropeptide is supported by our findings that mRNA of human basal ganglia also contains alarin mRNA (Figs. 1 and 3). Especially interesting will be the analysis of brain regions known to express *GALP* mRNA. In addition, future studies should indicate if alarin is secreted by neuronal cells and if it has a physiological function via specific neuropeptide receptors.

In the present study, we focused on neuroblastic tumor tissues. Interestingly, alarin-LI was associated with the degree of ganglionic differentiation in the neuroblastic tumors investigated. Gangliocytes irrespective of tumor type were alarin-LI positive. This is in contrast to the expression of δ-like protein, which was only detected in gangliocytes of ganglioneuroma but not of ganglioneuroblastoma and neuroblastoma (Hsiao et al., 2005).

For galanin we were not able to find a correlation with the differentiation of neuroblastic tumors, as galanin peptide concentrations did not correlate with tumor type and degree of differentiation (Berger et al., 2002). However, the presence of galanin receptor–binding sites was associated with the differentiation of the tumor tissues but mainly because of the high density of galanin receptors on Schwannian stroma (Berger et al., 2002, 2005). In contrast, alarin-LI was barely visible in Schwann cell–rich stroma in the tissues investigated here.

In conclusion, differential splicing of the *GALP* gene leads to a novel peptide that, at least in neuroblastic tumors, is specifically expressed in ganglionic nerve cells. Because anti-alarin antibodies stained gangliocytes in all types of neuroblastic tumors, detection of alarin expression could be of clinical value for the identification of differentiated tumor cells in neuroblastomas.

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