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In Vivo Spontaneous Neuronal to Neuroendocrine Lineage Conversion in a Subset of Neuroblastomas

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Neuroblastoma is an embryonal tumor derived from the sympathetic nervous system. Although all neuroblastomas have a neuronal character, a subset of tumors also show evidence of extra-adrenal neuroendocrine differentiation in discrete cell layers. A characterization of the cells of the developing human sympathetic nervous system was performed, identifying growth-associated protein-43, neuropeptide tyrosine, and Bcl-2 as marker genes for sympathetic neurons. Whereas all neuroblastomas express growth-associated protein-43, neuropeptide tyrosine, and Bcl-2, tumors with differentiating cells with neuroendocrine features expressed these genes only in the morphologically immature, proliferating cells. Thus, with neuroendocrine tumor cell differentiation, neuronal marker gene expression vanished and proliferation ceased and was succeeded by expression of chromogranin A/B and insulin-like growth factor-2, markers of neuroendocrine chromaffin differentiation. These tumors appear to provide examples of spontaneous lineage conversion from a neuronal to a neuroendocrine phenotype. (Am J Pathol 1997, 150:107–117)

Neuroblastoma, a tumor in infancy and childhood, is located at any site where sympathetic nervous system (SNS) tissue is found. It is believed that this heterogeneous tumor is derived from clonal expansions of cells arrested during neuronal development, thereby retaining their self-renewal capacity. Neuroblastoma displays morphological and phenotypical characteristics similar to nonmalignant cells of the sympatho-adrenal lineage. The neuronal and neuroendocrine cells of this lineage originate from the neural crest,[1,2] from where they migrate and differentiate to form sympathetic ganglion cells, small intensely fluorescent (SIF) cells, extra-adrenal chromaffin cells of the paraganglia, and sympathetic neurons and chromaffin cells of the adrenal medulla.[1,3]

A remarkable feature of the SNS is the plasticity of its cell components, as demonstrated both *in vivo* and *in vitro*. It is well recognized that nerve growth factor (NGF) is required for the survival and differentiation of sympathetic neurons. Interestingly, in primary cultures, NGF also has the capacity to induce transdifferentiation of terminally differentiated chromaffin cells and SIF cells toward a neuronal phenotype.[1,3–5] *In vivo* experiments have further demonstrated that rat adrenal medullary chromaffin cells differentiate neuronally when NGF is injected during embryogenesis and postnatal life.[6] Tumor-derived neuronal/neuroendocrine cell lines have also been used to investigate SNS cellular plasticity. PC12 cells, a rat pheochromocytoma cell line, differentiate into a sympathetic neuronal phenotype in the presence of NGF.[7] When co-cultured with bovine adrenal medullary endothelial cells, however, PC12 cells mature to chromaffin-like cells.[8] Similarly, the neuroblastoma cell line SMS-KCNR differentiates neuronally upon treatment with retinoic acid whereas a chromaffin-like differentiation is induced by dibuturyl cyclic AMP.[9]

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Neuroblastoma tumors have been suggested to recapitulate the development of differentiating sympathetic neurons and adrenal chromaffin cells of the SNS.[10,11] We have previously reported a subset of neuroblastoma tumors that show a differentiated phenotype similar to neuroendocrine cells of paraganglia and SIF cells.[12–14] These tumors, which would all be classified as differentiated stroma-poor tumors in the Shimada classification,[15] display a lobular arrangement of successive cellular differentiation, in which the cells adjacent to the fibrovascular stroma display undifferentiated neuronal (neuroblastic) morphology. Cells located further away from the fibrovascular stroma appear differentiated and possess neuroendocrine chromaffin characteristics as judged by morphology and intense expression of chromogranin A/B.[12] Lack of phenylethanolamine-*N*-methyltransferase expression demonstrate that these cells have extra-adrenal and not adrenal chromaffin character.[16] Frequently, the cells at the center of these lobular structures appear apoptotic, as is evident by nuclear condensation and DNA fragmentation.[13] Proliferation in these tumors is restricted to the immature cells located close to the fibrovascular stroma, as detected by Ki-67 immunohistochemistry.[12] Previous investigations[12–14,17] have mainly focused on the mature, neuroendocrine cells of these tumors, whereas the biological nature of the immature, proliferating cells bordering the fibrovascular stroma has not been analyzed in detail. In this study, we examined these immature cells further, with the intent to clarify whether they have neuroblast, immature neuroendocrine cell, or undifferentiated neural crest cell characteristics. A comparative phenotypic analysis of the cellular development during human fetal SNS organogenesis has therefore been performed. Based on these data, we conclude that the studied tumors provide examples of SNS plasticity, displaying an *in vivo* cellular transdifferentiation from a neuroblastic to a neuroendocrine phenotype.

Materials and Methods

Tumor, Embryonal, and Fetal Materials

The material comprises 24 neuroblastoma tumors, including 2 ganglioneuromas. Attempts were made to select neuroblastomas displaying evidence of neuroendocrine differentiation, and 15 such tumors were included. These 15 tumors were of stage 1, 2, or 3, most of them diagnosed at an early age (13 of 15 before 18 months of age), with an extra-adrenal primary tumor location (13 of 15) and an N-*myc* copy number of less than 6 (14 of 15). All would be categorized as stroma-poor, differentiating tumors in the Shimada classification,[15] and 11 of these patients survived their disease. For comparative purposes, 5 additional neuroblastomas (stage 3 or 4), 2 ganglioneuromas, and 2 stage 4S neuroblastomas were analyzed. Of the 5 high-stage neuroblastomas, all had undifferentiated histology, 4 were adrenally located, and 4 were N-*myc* amplified. Patients were at varying stages of treatment at the time of tissue acquisition; the majority of patients with stage 3 and stage 4 disease received cytotoxic therapy before the time of tumor biopsy or resection (80%), whereas those with stage 1, 2, or 4S had not (0%). Tumor stage was determined clinically or at the time of surgical biopsy or resection in accordance with international neuroblastoma staging system criteria.[18]

Human fetal tissue was obtained from both elective and spontaneous abortions from 7 to 23 weeks developmental age. Ethical approval was obtained from the local ethical committee of Karolinska Hospital, Stockholm, Sweden (approval Dnr93–216). Normal embryonal and fetal anatomy was confirmed by one of the authors before use in this investigation. Due to the relative scarcity of tissues available, specimens were categorized into three groups: 7 to 8 weeks, 11 to 13 weeks, or 19 to 23 weeks developmental age. Two to five specimens were available for study within each group. The majority of tissues were cross sections through the chest or abdomen in early developmental fetuses and cross-sectionally oriented organ tissue blocks at the later developmental ages. Postnatal adrenal glands, retrieved from 21 autopsies, ranging in age from birth to 4 years of age, were analyzed.

Immunohistochemistry

All tumor and fetal material was fixed in 4% buffered formaldehyde and embedded in paraffin, and sections of 5 μm were secured to glass slides (Superfrost). Immunohistochemistry was performed as described by Hoehner et al.[13] Alkaline-phosphatase-conjugated secondary antibody at 1:40 dilution (Sigma Chemical Co., St. Louis, MO) was used in all cases, and as chromogen, Fast-red-TR-salt (Sigma) was used. Slides were counterstained with hematoxylin. Primary antibodies employed were monoclonal anti-Bcl-2 antibody (MAb 124, Dakopatts, Glostrup, Denmark; specificity tested by Western blotting[19]) at 1:100 dilution, monoclonal anti-neuron-specific-enolase (anti-NSE) antibody (DAKO-NSE, H14, Dakopatts; specificity tested by Western blotting[20]) at 1:50 dilution, monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) antibody (PC-10, Boehr-

Table 1. *Summary of Marker Gene Expression in Normal Embryonic and Fetal Sympathetic Nervous System Tissues and Neuroblastomas*

	TH at developmental age (weeks)			NSE at developmental age (weeks)			Bcl-2 at developmental age (weeks)			GAP-43 at developmental age (weeks)			NPY at developmental age (weeks)			IGF-2 at developmental age (weeks)		
Protein/gene	7–8	11–13	19–23	7–8	11–13	19–23	7–8	11–13	19–23	7–8	11–13	19–23	7–8	11–13	19–23	7–8	11–13	19–23
Embryonic/fetal tissue																		
Sympathetic ganglia	+	+	+	+	+	+	+	++	++	+	++	++	+	++	++	−	−	−
Paraganglia	+	++	++	+	+	+	−	−	−	−	−	−	−	−	−	+	++	++
Adrenal neuronal cells	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Adrenal chromaffin cells	+	++	++	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+

	TH	NSE	Bcl-2	GAP-43	NPY	IGF-2
Neuroblastomas						
Nonlobular tumors*	+	+	+	+	+	−(+)†
Lobular:immature cells‡	+	+	+	+	+§	−
Lobular: mature cells‖	++	+	−	−	+§	+(−)¶

TH, NSE, and Bcl-2 production was analyzed by immunohistochemistry whereas *in situ* hybridization was used to detect GAP-43, NPY, and IGF-2 expression. Gene expression and protein levels were assessed semiquantitatively as follows: +, expression; ++, intense expression; −, no expression detected.

*Expression varying from uniform to scattered positivity.

†Vast majority of tumors negative.[12]

‡Cells adjacent to thin fibrovascular stroma with densely stained nuclei and scant cytoplasm.

§Transient increase of expression with tumor cell differentiation (see Figure 6B).

‖Cells with pale nuclei with distinct nucleoli and large cytoplasm. ¶Vast majority of tumors positive.[12]

inger Mannheim, Mannheim, Germany; specificity tested by Western blotting[21]) at 1:20 dilution, and monoclonal anti-tyrosine hydroxylase (anti-TH) antibody (Boehringer Mannheim; specificity tested by Western blotting[22]) at 1:75 dilution. In addition to the antibody specificity tests by Western blotting, tissue cell type specificity was established in this and previous studies for each antibody using normal human fetal tissues.[12–14,17]

In Situ *Hybridization*

Growth-associated protein-43 (GAP-43), neuropeptide tyrosine (NPY), and insulin-like growth factor-2 (IGF-2) transcripts were detected by *in situ* hybridization as previously described.[23] ^{35}S-Labeled riboprobes were generated by *in vitro* transcription, using an 800-bp *Hin*dIII/*Eco*RI GAP-43 cDNA and a 680-bp *Hin*dIII/*Pst*I IGF-2 cDNA cloned into pGem-3, and a 290-bp *Eco*RI-*Sma*I NPY cDNA cloned into pBluescript as templates. Antisense RNA probes with specific activities of approximately 250 Ci/mmol were generated, and sense probes from the same plasmids, with similar specific activities, were employed as negative controls. Hybridization was performed at 56°C overnight, and sections were washed stringently before RNAse treatment. After exposure for 7 days for IGF-2, 10 days for NPY, and 14 days for GAP-43, sections were developed and counterstained with hematoxylin.

Results

Characterization of Embryonal and Fetal SNS Cells

The neuronal and neuroendocrine cells forming the SNS were phenotypically characterized during human embryonic and fetal development at developmental weeks 7 to 23. Cells were identified by morphology and location, aided by marker gene expression detected by immunohistochemistry and *in situ* hybridization. Markers employed include TH, NSE, GAP-43, NPY, Bcl-2, and IGF-2. The SNS cells studied were neurons of the sympathetic ganglia, chromaffin cells of the paraganglia, and adrenally located sympathetic neurons and chromaffin cells. Neuroblasts/neurons of sympathetic ganglia and of the adrenal medulla are here classified as neuronal cells, whereas chromaffin cells of paraganglia, SIF cells, and chromaffin cells of the adrenal gland are termed neuroendocrine. The results of this characterization are summarized in Table 1. As evidenced by positive PCNA immunohistochemistry, all examined embryonic and fetal SNS tissues, between weeks 7 and 23, contained proliferating neuronal and neuroendocrine cells (not shown).

In embryonal tissue, morphologically undifferentiated SNS cells were detectable in the retroperitoneal area from week 7 (earliest stage studied) onward. These cells expressed TH and NSE.[12,14] Even

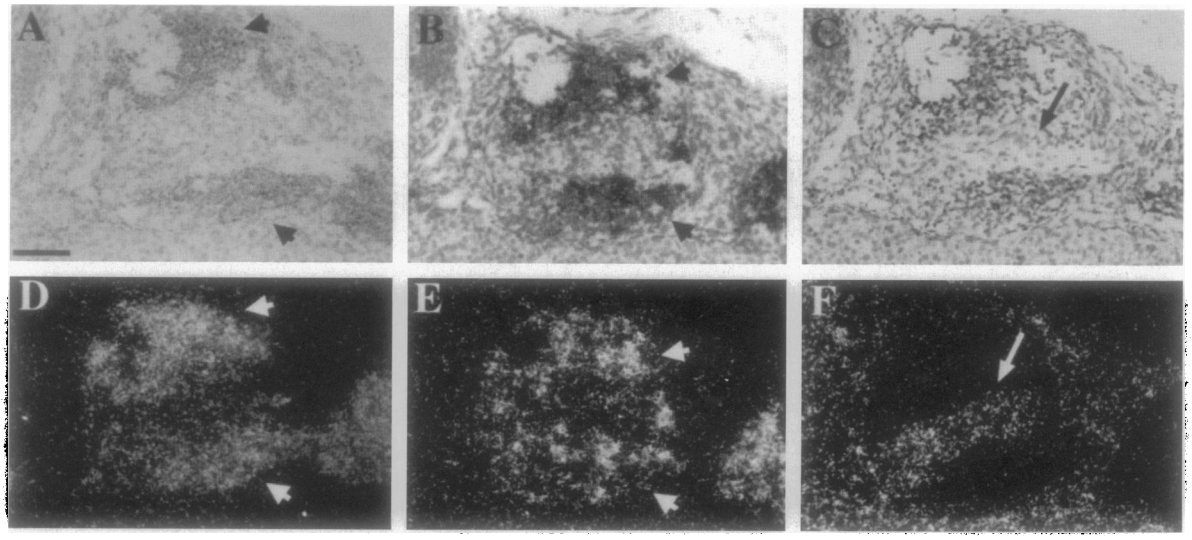

Figure 1. *GAP-43, NPY, and IGF-2 expression detected by* in situ *hybridization in human embryonic sympathetic nervous system precursor cells in the retroperitoneal area at developmental week 7.* **A** *to* **C:** *Bright field.* **D** *to* **F:** *Dark field. Intense GAP-43* (**A** *and* **D** *and NPY* (**B** *and* **E**) *expression in a distinct subset of cells is indicated by the* **short arrows**. *In contrast, IGF-2 was expressed in an adjacent, morphologically different group of cells* (**C** *and* **F, arrows**). *Scale bar, 80 μm.*

though sympathetic neuronal cells were morphologically primitive at this early stage and not yet organized into distinct ganglia, neuronal and neuroendocrine cell types could be distinguished by expression of the marker genes GAP-43, NPY, and IGF-2. GAP-43, which is expressed exclusively in neuronal cells,[24] was mainly found in a distinct subset of cells, and most of the GAP-43-positive cells also appeared to express NPY (Figure 1, A–D). In contrast, IGF-2, which is a sympathetic neuroendocrine marker,[12] was expressed in a morphologically different subset of cells adjacent to the GAP-43-positive cells (Figure 1, C and F). However, there was some GAP-43 and NPY positivity in the region of IGF-2-positive cells, which could suggest that the IGF-2-positive cells at this early developmental stage also express low levels of neuronal marker. At later fetal stages (weeks 11 to 13 and 19 to 23), when the sympathetic ganglia and paraganglia are formed, GAP-43 SNS expression was exclusively localized to neuronal cells of the sympathetic ganglia and the adrenal gland (Figure 2, A, C, and D). This expression pattern was, within the SNS, indistinguishable from that of Bcl-2 and NPY (Figure 3, D and E; Table 1), except that NPY could not be detected in late (weeks 19 to 23) fetal adrenal ganglion cells, which is in accordance with previously published data.[25] As internal positive controls, sensory neurons of the dorsal root ganglia and enteric neurons of the bowel were identified and found to express both GAP-43 and Bcl-2, and intense NPY expression was detected within the central nervous system (not shown). No expression of GAP-43, NPY, or Bcl-2 was detected in extra-adrenal chromaffin cells of paraganglia (Figures 2A and 3, D and E) or in adrenal chromaffin cells as exemplified by GAP-43 (Figure 2, C and D). These structures and cells did, however, express TH and NSE (Figure 3, A–C; NSE in adrenal not shown), whereas phenylethanolamine-*N*-methyltransferase was expressed in adrenal chromaffin cells only.[16] As highly malignant neuroblastomas are often located to the adrenal gland and frequently become clinically overt several years after birth, we searched for an adrenally located postnatal sympathetic neuronal cell type. In 5 of 21 postnatal adrenal glands (age 0 to 4 years) GAP-43-positive sympathetic neurons were conclusively found (Figure 4A). These GAP-43-expressing cells, which were rare postnatally, were morphologically distinguishable from surrounding chromaffin cells. In consecutive sections, they also stained for Bcl-2, confirming their neuronal character (Figure 4B).

In contrast to GAP-43, NPY, and Bcl-2, which are neuronal markers, IGF-2 was intensely expressed in embryonic and fetal chromaffin cells of paraganglia, whereas no IGF-2 expression was detected in sympathetic neuroblasts or neurons at any stage of development analyzed[12] (Figures 1, C and F, and 2B; Table 1). At all fetal and embryonal stages studied, TH expression was more intense in paraganglia, as exemplified in Figure 3B, than in neuronal cells of sympathetic ganglia and of the adrenal medulla. As indicated in an earlier study,[12] fetal SIF cells show similar IGF-2 expression as paraganglionic cells; however, in the sections included in this study, SIF cells were not identified with certainty.

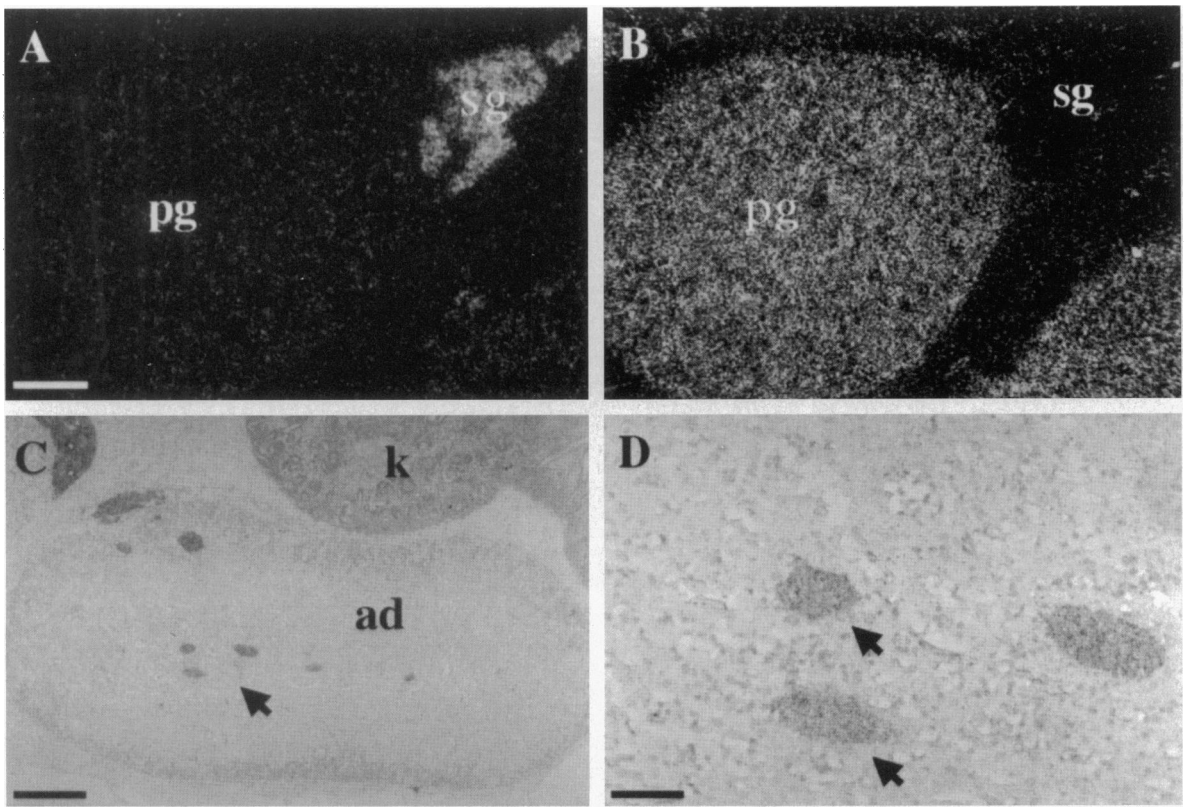

Figure 2. A *and* **B:** *Dark-field view of GAP-43 and IGF-2* in situ *hybridizations in consecutive sections to Figure 3, showing paraganglia (pg) and sympathetic ganglia (sg) at developmental week 13. Neuronal marker GAP-43* **(A)** *intensely stains sympathetic ganglia, in contrast to IGF-2* **(B)**, *which is expressed in paraganglia. Scale bar, 100 μm.* **C:** *GAP-43 expression in human fetal adrenal gland (ad; developmental week 13) and kidney (k). Positive hybridization signal in nests of neuroblasts is indicated by the* **short arrow. D:** *High power view of* **C.** *Scale bars, 400 μm* **(C)** *and 50 μm* **(D)**.

Neuronal and Neuroendocrine Differentiation in Neuroblastoma

In those neuroblastoma tumors with previously described progressive *in situ* neuroendocrine differentiation,[12–14] the immature, small, and nuclear-dense cells bordering the fibrovascular stroma were intensely GAP-43 positive and proliferating as shown by PCNA positivity (Figures 5, A, B, and D, and 6A). These cells also expressed Bcl-2.[13] With progressive neuroendocrine tumor cell differentiation, proliferation ceased; ie, cells became PCNA negative, and GAP-43 and Bcl-2 expression vanished. No or little GAP-43 expression was detected in morphologically differentiated tumor cells (Figure 6A). As reported previously, the more differentiated cells have an abundant cytoplasm, a large nucleus with diminished basophilia, and a visible nucleolus (Figure 6D). These cells express chromogranin A/B but not phenylethanolamine-*N*-methyltransferase[12,14,16] and were intensely IGF-2 positive (Figures 5C and 6C). NPY was expressed in a patch-like pattern, most intensely in cells adjacent to or slightly overlapping the IGF-2-positive cells (Figure 6B). NPY expression was not detected in the immature cells bordering tumor fibrovascular stroma, or in the most mature, neuroendocrine cells (Figure 6B). TH immunoreactivity was least intense in the GAP-43-positive cells, with progressively increasing expression with advancing maturation, supporting the conclusion that IGF-2 and chromogranin-A/B-expressing cells mature along a chromaffin lineage.[12,14] Although the tumor cells presented and illustrated here show a clear progressive conversion from a neuronal to a neuroendocrine phenotype, it is important to emphasize that, in some tumors and in other areas of the tumors described above, the spatial separation of different SNS phenotypes was not as distinct. Tumor cells co-expressing GAP-43 and IGF-2 can also be found, which is similarly of interest considering that we could not with certainty identify such a co-expression during normal embryonic development.

This study focuses on those low-stage neuroblastoma tumors composed of cells with both neuronal and neuroendocrine phenotypes. It is generally

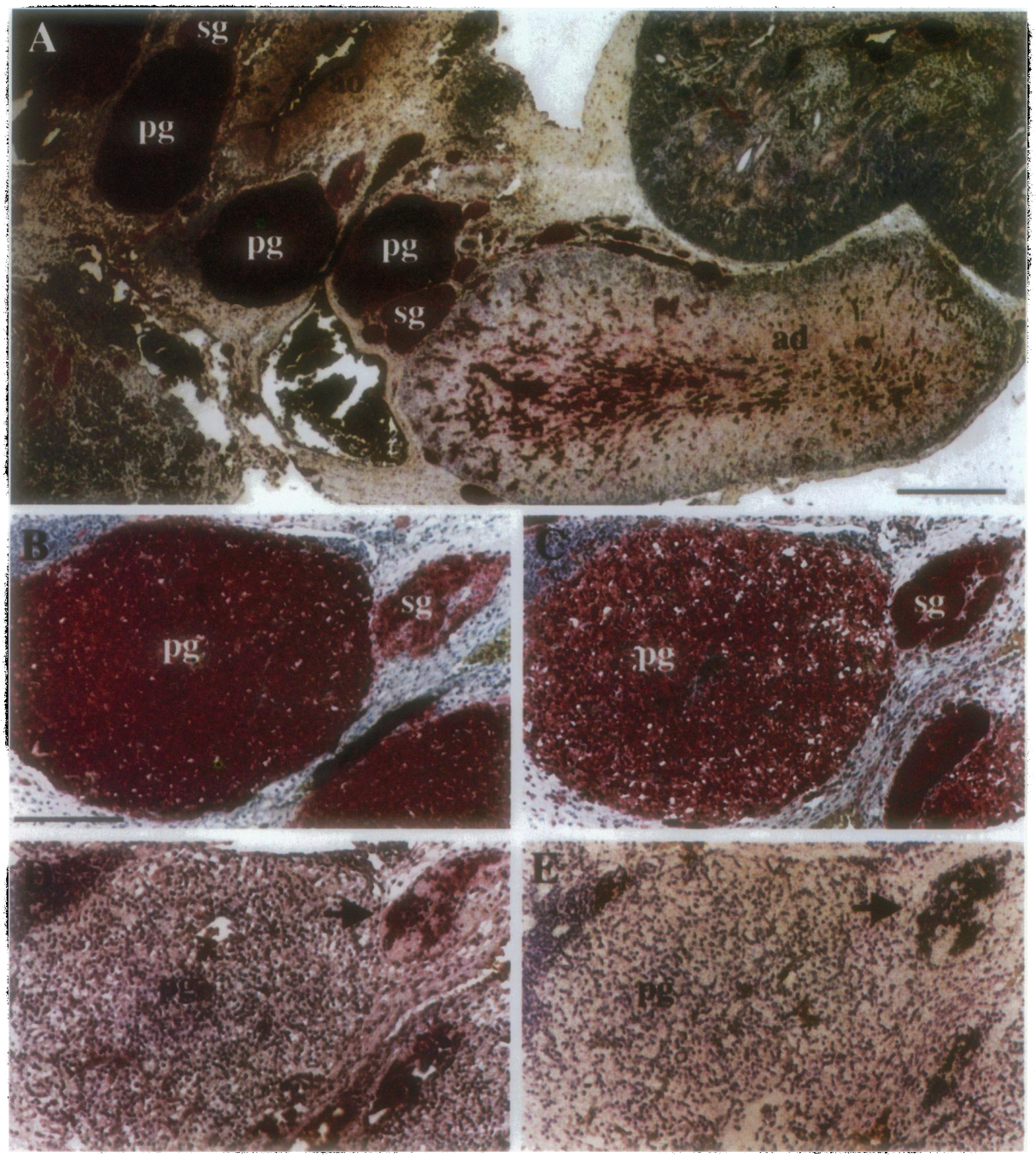

Figure 3. *Immunohistochemical* (**A** *to* **D**) *and* in situ *hybridization* (**E**) *characterization of the developing sympathetic nervous system in consecutive sections of a 13-week human fetus. Tyrosine hydroxylase* (**A**, *overview*; **B**, *close-up*) *and neuron-specific enolase* (**C**) *are expressed in both paraganglia* (*pg*) *and sympathetic ganglia* (*sg*), *whereas Bcl-2* (**D**) *and NPY* (**E**) *are expressed exclusively in sympathetic neurons* (**arrows**). *ao, aorta; k, kidney; ad, adrenal. Scale bars, 500 μm* (**A**) *and 170 μm* (**B**).

taken for granted that neuroblastoma tumors, including those that are of high stage, are sympathetic and arrested early during neuronal development.[10,14,26] To verify that neuroblastomas and ganglioneuromas in general express neuronal genes, this was checked in a few cases. All morphologically undifferentiated neuroblastomas tested displayed a randomly scattered pattern of NSE-, GAP-43-, and Bcl-2-expressing cells (Table 1). Ganglioneuroma tumor cells display evidence of a mature, differentiated morphology. These large cells with pale large nuclei with a distinct nucleolus and abundant cytoplasm were intensely GAP-43 positive (Figure 7A) in accordance with a neuronal phenotype. NPY was also

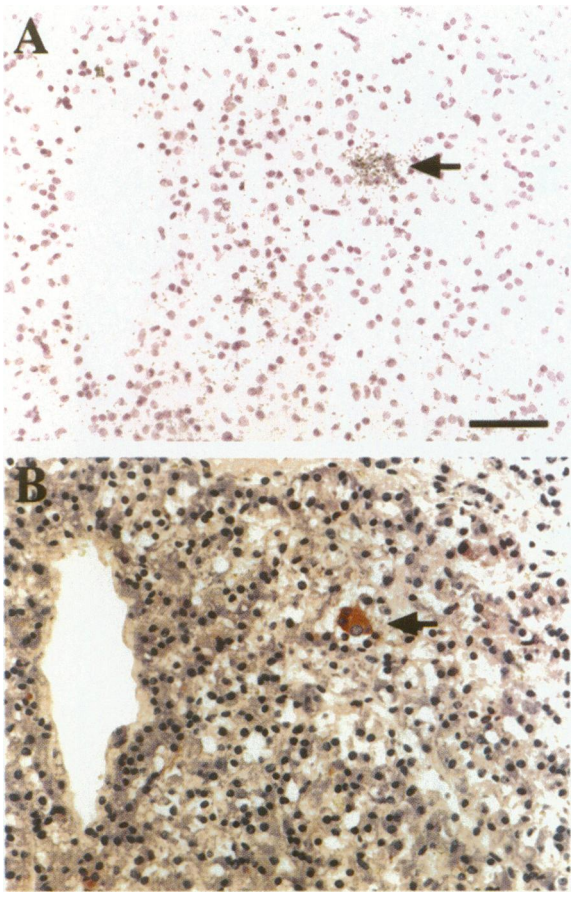

Figure 4. *Cross section of a human adrenal gland retrieved from an autopsy case of a 1-year-old child. A sympathetic neuron* (arrow) *expressing GAP-43* (A) *and Bcl-2* (B), *morphologically distinguishable from surrounding cells, is shown. Scale bar, 50 μm.*

expressed in ganglioneuromas, although, fewer tumor cells expressed NPY than GAP-43 (Figure 7B). IGF-2-expressing cells were not detected in any of the ganglioneuromas analyzed.[12] Thus, ganglioneuroma tumor cells showed characteristics mimicking mature neurons of both the adrenal medulla and sympathetic ganglia.

Discussion

Previously, we and others have reported that a subset of neuroblastomas display neuroendocrine features,[11–14] which appeared paradoxical in view of our own finding that all neuroblastomas express marker genes, such as GAP-43, Bcl-2, and the neuronal *src*-splice variant pp60$^{c\text{-}srcN1}$,[16] which, within the SNS, are restricted to the neuronal lineage. Data presented in this report provide an explanation to this apparent paradox; ie, in neuroblastoma tumors with neuroendocrine features, there is a mixture of neuroblastic and neuroendocrine cells frequently forming discrete layers in parts of the tumors. The PCNA expression profile (Figure 5) and previous Ki-67 studies[12] have demonstrated that it is the tumor cells with a neuronal and morphologically immature phenotype that proliferate, not the neuroendocrine cells. This strongly suggests that these tumors are clonal and are not derived from two disparate cell types and that neuronal tumor cells spontaneously convert into an overt neuroendocrine phenotype. Furthermore, by employing more than one marker gene to demonstrate neuronal and neuroendocrine differentiation, respectively, we conclude that it is unlikely that the neuroendocrine characteristics (morphology, IGF-2, TH, and chromogranin expression, and down-regulation of neuronal gene expression) seen in these tumors are due to ectopic expression of neuroendocrine markers.

The conclusion that some low-stage neuroblastoma tumors contain cells of two SNS lineages relies entirely on the lineage specificity of the marker genes used. GAP-43 is a neuronal protein, of importance during axonal outgrowth and synapse formation. The expression of this gene peaks during neuronal development (early postnatal life in mouse[27]), is elevated during neuronal regeneration, but decreases in fully differentiated neurons. NPY protein is localized to neuronal synapses, and NPY-expressing cells are found both in the central and in the peripheral nervous system. Expression increases with advancing differentiation.[28,29] Bcl-2 protein inhibits apoptosis of a number of cell types, including sympathetic neurons.[30] To verify that these three gene products indeed are expressed in sympathetic neurons, and not in sympathetic neuroendocrine cells, a characterization of the developing neuronal and neuroendocrine cells of the SNS during human embryogenesis was performed. This identified GAP-43, Bcl-2, and NPY as neuronal marker genes in the SNS (Figures 1, A, B, D, and E, 2A, and 3, D and E, and as summarized in Table 1). Similarly, IGF-2 and high expression of TH were established to be neuroendocrine markers in the SNS (Figures 1, C and F, 2B, and 3B). The distinction between neuronal and neuroendocrine SNS cells by expression of these marker genes was confirmed at all developmental ages studied. However, at the earliest stages, ie, developmental week 7, there might be some NPY and possibly also GAP-43 expression in the IGF-2-positive cells, whereas IGF-2 expression was never found in regions of high GAP-43 expression. When employing expression of these genes as markers to distinguish neuronal from neuroendocrine cells in neuroblastoma, at least two different forms of low-stage neuroblastomas could be envisaged, one form

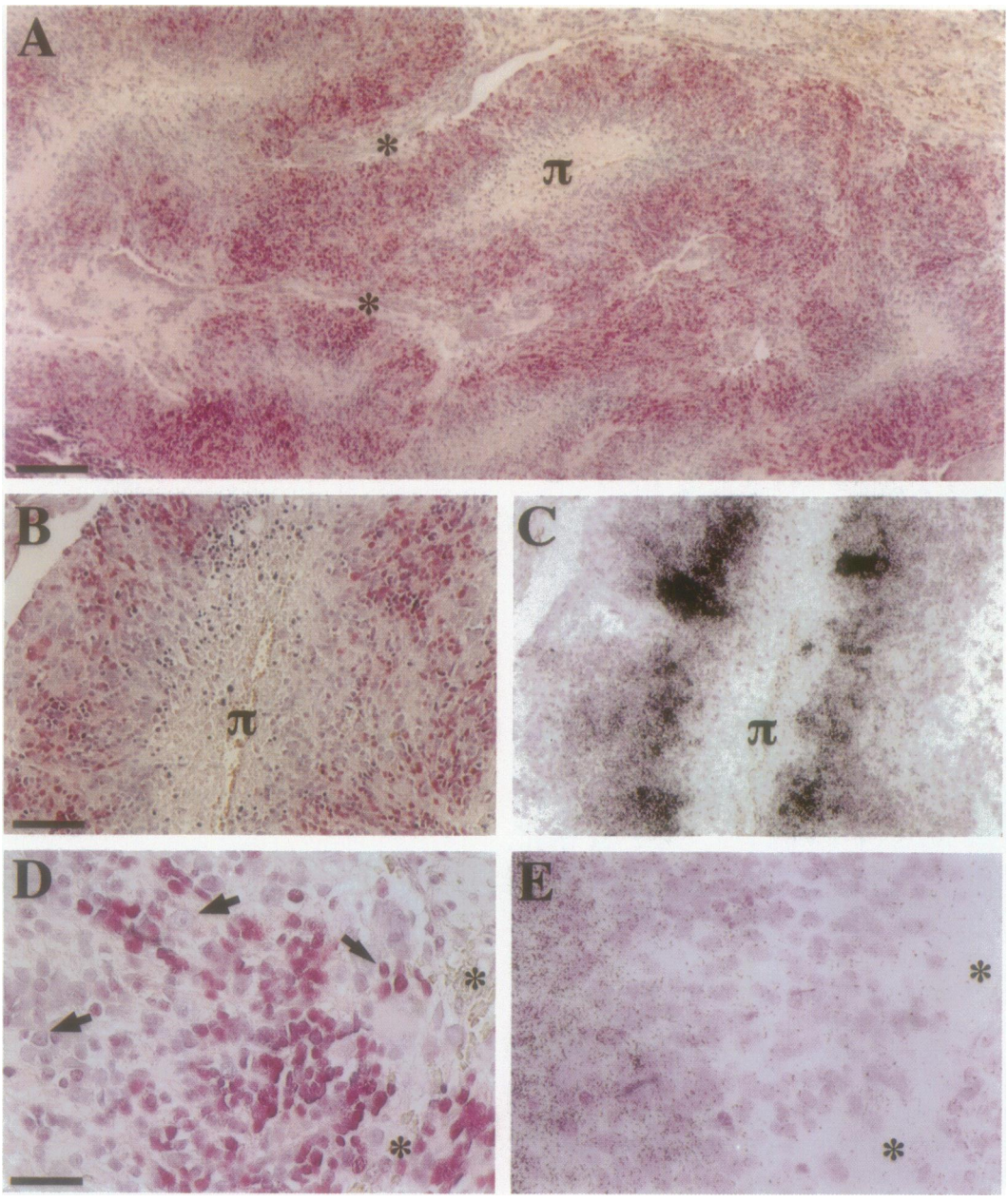

Figure 5. *PCNA immunohistochemistry visualizing layers of proliferating cells in a low-stage neuroblastoma with lobular structures* (**A**). *The morphologically immature cells adjacent to the fibrovascular stroma* (*) *are PCNA positive* (**B** *and* **D**), *whereas the differentiated cells bordering the cell-free zone* (π) *express IGF-2* (**C** *and* **E**), *detected by* in situ *hybridization and visualized in bright field. In* **D**, *an immature PCNA-positive cell is indicated by a* **thin arrow**, *whereas morphologically differentiated cells are shown by* **thick arrows**. *Note that the sections are counterstained with hematoxylin and that cells indicated by* **thick arrows** (**D**) *are PCNA negative. Scale bars, 150 μm* (**A**), *75 μm* (**B**) *and 25 μm* (**D**).

described here that shows spontaneous neuronal to neuroendocrine extra-adrenal chromaffin differentiation and another form that shows spontaneous neuronal differentiation, where ganglioneuroma could be an example of the end-stage of such tumor cell differentiation.

As mentioned above, NPY is a neuronal marker with increasing expression with cellular maturation. In the subset of tumors analyzed here, NPY-expressing cells were located between GAP-43- and IGF-2-positive cells, which might reflect an initial neuronal maturation of these tumor cells before they convert to a neuroendocrine, IGF-2- and chromogranin-A/B-expressing phenotype. Furthermore, the change from a neuronal to a neuroendocrine phenotype as seen in these tumors appears to include cells in transition that have a mixed neuronal/neuroendocrine phenotype, ie, expressing GAP-43 and NPY as well as

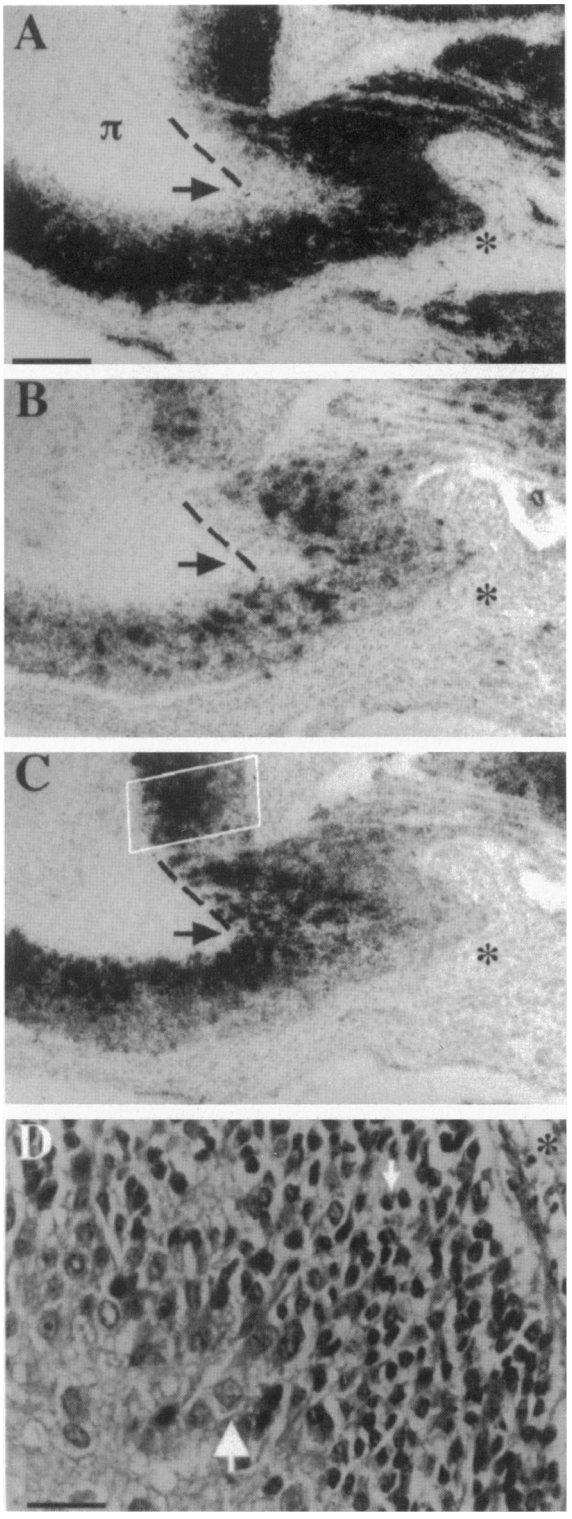

Figure 6. *GAP-43, NPY, and IGF-2 expression, as shown by* in situ *hybridization, in a low-stage neuroblastoma with progressive differentiation. GAP-43* (A) *is primarily expressed in the cells bordering the fibrovascular stroma* (*), *in contrast to IGF-2* (C), *which is expressed in a group of more differentiated cells. NPY* (B) *expression is restricted to fewer cells in the cell layer in between, partially overlapping the GAP-43 and IGF-2 expression.* Black arrows *and* hatched lines *indicate the border for IGF-2-positive cells. The area indicated by* π *is a cell-free zone* (see also Figure 5B). D: *Magnification of the framed in area in* C *in which a consecutive section was H&E stained. Note signs of increasing morphological differentiation* (large white arrow) *as cells become larger and paler and possess visible nucleoli, compared with immature neuroblastic cells* (small white arrow). *Scale bars, 125 μm* (A) *and 25 μm* (D).

IGF-2. Although such mixed phenotypes have not been observed during normal development, our data presented in Figure 1 could be taken as an indication that such cells do exist during a limited period of development. That these lobular tumors have cells with a mixed phenotype further supports the conclusion that these two different tumor cell types have the same cellular origin and are not the products of two distinct sets of events leading to malignancy.

As described earlier, the plasticity of neuroendocrine cells of the sympatho-adrenal cell lineage is well documented. Chromaffin cells and SIF cells can transdifferentiate to a neuronal phenotype *in vivo* and *in vitro* under appropriate conditions. The ontogeny of the different sympatho-adrenal derivatives has been analyzed intensely, and explanatory models have been presented.[31,32] Of interest to the findings presented here are two models discussed by Anderson[31]: a classical branched model, in which a progenitor cell can diverge into any of the different SNS cell types, and a linear model, in which the differentiation of a progenitor cell to a sympathetic neuron involves transition through immature chromaffin and SIF cell stages. Neuroendocrine cells would in the linear model represent transitional stages of differentiation, stages at which cells can be arrested by environmental signals, such as glucocorticoids. The branched model involving transdifferentiation requires dedifferentiation of one cell type before conversion to another type, which is not required in a linear model. Whereas transdifferentiation of chromaffin cells toward a neuronal cell type has been demonstrated in many reports,[3–5] the opposite, ie, neuronal cells converting into chromaffin cells, is not as well recognized.[33] However, this is what seems to occur in the low-stage neuroblastomas described in this report. This conversion does not appear to involve dedifferentiation, considering that the neuroblastic/neuronal tumor cells appear to mature slightly (increasing NPY expression when GAP-43 expression decreases) before the cells convert into a neuroendocrine phenotype. Thus, we conclude that our tumor data fit a slightly modified linear model of sympatho-adrenal differentiation in which chromaffin progenitor cells initially have neuronal characteristics but later mature into a distinct chromaffin phenotype. Whether this is a phenomenon restricted to these neuroblastoma tumor cells or whether it re-

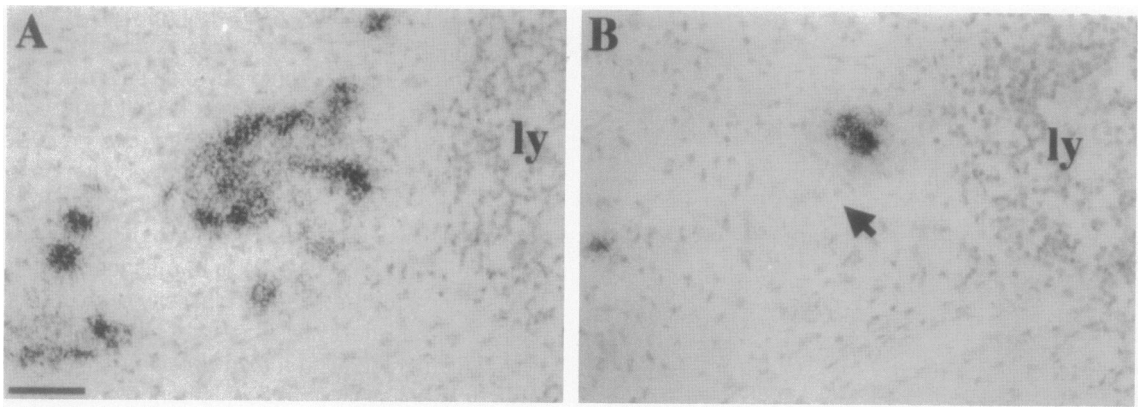

Figure 7. *GAP-43* (**A**) *and NPY* (**B**) *expression detected by* in situ *hybridization in a ganglioneuroma. Note that in these consecutive sections not all GAP-43-positive cells (morphologically neuron-like with large cytoplasms and nuclei) co-express NPY. The* arrow *indicates a cluster of GAP-43-expressing cells that do not express NPY. ly,-lymphocytes. Scale bar, 50 μm.*

flects normal ontogeny of SNS neuroendocrine cells is an open question, as embryonic tissues earlier than week 7 are not available to us. In the developing human SNS, a neuronal and neuroendocrine lineage separation apparently occurs before developmental week 7 as co-expression of neuronal and neuroendocrine markers in SNS cells was not found with certainty at this age.

The lineage conversion we detected in the subset of neuroblastomas described here is not only of interest from an ontological point of view but might also be of clinical relevance. Previous results[13] indicate that the fully differentiated neuroendocrine cells in neuroblastoma tumors undergo an apoptotic cell death *in situ*, thus resembling the involution of paraganglionic and SIF chromaffin cells during early postnatal life. Interestingly, the differentiated neuroendocrine tumor cells also express trkA and trkC.[17] However, we were unable to detect any neurotrophin expression or activity in those tumors, which might suggest that the neuroendocrine differentiation and/or apoptotic cell death are caused by neurotrophin shortage. Adrenal chromaffin cell differentiation is regulated by glucocorticoids.[34,35] It is not known whether extra-adrenal chromaffin differentiation is regulated by similar mechanisms or whether neuroendocrine-differentiating neuroblastomas require glucocorticoids during their maturation. Examination of the glucocorticoid receptor expression in this subset of neuroblastoma tumors would clarify this, and if the receptor is expressed, glucocorticoids might push immature, neuroblastic neuroblastomas along a neuroendocrine differentiation lineage and serve as a new modality to encourage neuroblastoma tumor regression.

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