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Neurotrophin and Neurotrophin Receptor Proteins in Medulloblastomas and Other Primitive Neuroectodermal Tumors of the Pediatric Central Nervous System

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Primitive neuroectodermal tumors (PNETs) of the central nervous system (CNS) are poorly understood childhood neoplasms, and medulloblastomas are the most common pediatric PNETs. Neoplastic cells in medulloblastomas and other PNETs resemble progenitor cells of the developing central nervous system, but they also may exhibit the molecular phenotype of immature neurons or glia. As neurotrophins play a role in regulating differentiation, proliferation, and cell death in the normal developing central nervous system, and recent evidence suggests that neurotrophins may influence the behavior of medullablostomas, we studied 29 PNET biopsy samples (27 of which were posterior fossa medulloblastomas) by immunohistochemistry using antibodies specific for each of the major high affinity neurotrophin receptor proteins, ie, TrkA, TrkB, and TrkC. A subset of these tumors also was examined by Western blot. Immunoreactive TrkA, TrkB, and TrkC were observed in neoplastic cells in 8 (27%), 18 (62%), and 14 (48%) of these PNETs, respectively. Additional immunohistochemical studies of a subset of these PNETs using antibodies to neurotrophins that primarily activate TrkB and TrkC, ie, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5, showed that immunoreactive brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 were detected in 22, 9, and 19% of these PNET biopsies, respectively. Finally, 19 pediatric brain tumors other than these PNETs also were studied here, and they expressed these neurotrophins and their receptors to a variable extent. The demonstration here that neurotrophins and their cognate receptor proteins are expressed in PNETs as well as in other pediatric brain tumors may imply that signal transduction pathways mediated by neurotrophins and/or their receptors influence the induction or progression of these common childhood neoplasms. (Am J Pathol 1996, 148:929–940)

Primitive neuroectodermal tumors (PNETs) of the pediatric central nervous system (CNS) are composed of morphologically undifferentiated tumor cells that resemble neuroectodermal progenitor cells of the developing CNS.[1–4] Posterior fossa medulloblastomas (MBs) are the most common PNETs, but pineoblastoma, retinoblastoma, cerebral neuroblastoma, and olfactory neuroblastoma also are included in this group of neoplasms.[1–4] Although morphologically undifferentiated in most cases,[1–13] PNETs contain populations of neoplastic cells that express markers

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of CNS progenitor cells or markers of neurons, glia, and/or neuroendocrine cells.[5–9,11–18] Although the biological significance of neuronal or glial differentiation in MBs and related PNETs is not fully understood, the clinical behavior of PNETs has been shown to correlate with morphological evidence of neuronal *versus* glial differentiation[19,20] and with the expression of molecular markers of neurons and/or glia.[21] However, the expression of markers of neuronal or glial differentiation in PNETs may be surrogates for the activation of signal transduction pathways by one or more neurotrophic factors that influence the level of neural differentiation and the behavior of PNETs. Indeed, several neurotrophins and their cognate receptors have been detected in PNETs.[22–25]

Neurotrophins are a family of related trophic factors with pleiotrophic effects on CNS cells (for recent reviews and additional citations, see Refs. 26–31). The classic neurotrophin is nerve growth factor (NGF), but several other related neurotrophins have been cloned and sequenced. These include brain-derived neurotrophic factor (BDNF), NT3, NT4/5, and NT6.[26–31] Although neurotrophins bind to the low affinity NGF receptor (p75LNGFR), it is not clear what role p75LNGFR plays in neurotrophin-mediated signal transduction cascades.[27] However, NGF may promote the survival of normal and neoplastic neurons by binding to p75LNGFR and blocking apoptosis.[27,32] In contrast, the better understood functions of neurotrophins appear to be mediated by one or more members of a family of high affinity neurotrophin receptor tyrosine kinases homologous with a previously described oncogene called tropomyosin receptor kinase, or Trk.[26–30] Currently, three major classes of high affinity neurotrophin receptors are recognized, ie, Trk (also known as TrkA), TrkB, and TrkC. However, several alternatively spliced forms of these receptor proteins exist, and the functional role of these variants is under intense investigation. Each neurotrophin binds preferentially to a given Trk receptor to initiate signal transduction.[26–30] For example, NGF binds to TrkA (to promote the differentiation, survival, or proliferation of sensory dorsal root ganglion neurons, neuroepithelial stem cells, etc), whereas BDNF binds to TrkB (to promote the differentiation, survival, or proliferation of motor and sensory neurons, cerebellar granule cells, sensory ganglion precursor cells, etc), and NT3 binds preferentially to TrkC (to promote the differentiation, survival, or proliferation of sensory and motor neurons, cerebellar granule cells, peripheral nervous system precursors, etc). The biological effects of NT4/5 and NT6 are not as well delineated at this time, but NT4/5 binds to both TrkA and TrkB.[31,33]

The binding of neurotrophins to their cognate receptors has pleiotrophic effects on CNS cells that are just beginning to be clarified.[26–30] For example, normal developing granule cells sequentially respond to BDNF followed by NT3 to promote lineage commitment (BDNF) and the subsequent maturation (NT3) of these cerebellar neurons, respectively.[34] The expression of multiple neurotrophins and neurotrophin receptors by immature and adult neurons suggests that neurons may respond to one or more neurotrophins through paracrine or autocrine loops.[28,35] Furthermore, neurotrophins may induce differentiation or regulate cell death or apoptosis in neural and non-neural tumors.[26,27,32]

The precise contribution of neurotrophins to the induction and progression of MBs and related CNS PNETs is uncertain, but neurotrophins might play a role in these events as neurotrophins regulate the proliferation, differentiation, and programmed cell death (apoptosis) of neuroectodermal cells committed to the neuronal lineage, and the aberrant regulation of these important developmental processes may be a central event in the pathogenesis of these tumors.[26–30,36] Indeed, a recent paper demonstrated that both NT3 mRNA and TrkC mRNA were expressed in MBs and that the levels of TrkC mRNA were linked to a more favorable outcome.[25] Thus, to explore further the potential role of neurotrophins and their cognate receptors in the biology of PNETs at the protein level, we probed 29 pediatric PNETs (including 27 MBs) of the CNS using antibodies to TrkA, TrkB, TrkC, p75LNGFR, BDNF, NT3, and NT4/5.

Materials and Methods

Tissue Samples

Forty-eight children with CNS tumors were included in this study. Although the focus of this study was MBs and related PNETs, other pediatric brain tumors also were examined here for comparison with the PNETs (see Tables 1 and 2). The samples included 27 classic MBs and 2 other CNS PNETs (Table 1) in addition to 4 gangliogliomas, 11 glial tumors, and 4 other brain tumors (Table 2). All patients were treated at the Children's Hospital of Philadelphia between 1989 and 1994. Fresh biopsy samples obtained at the time of surgery were fixed with 70% ethanol containing 150 mmol/L NaCl for 24 to 36 hours and then embedded in paraffin as described.[16,17] In addition, adjacent tissue samples

Table 1. *Immunohistochemical Staining of 29 PNETs Using Antibodies to Neurotrophins and Their Receptors*

					TrkAex		TrkAin		TrkBex		TrkBin		TrkBTr	TrkCex	TrkCin	PanTrk					
Case	Age (years)	Sex	Differentiation	Material	254–3	out	256–3	in	201–3	out	306–1	in	271–1	out	in	MCtrk	Etrk16	BDNF	NT3	NT4/5	LNGFR
1	1	F	N and G	P	–	–	+++	–	+++	+/–	–	–	+++	–	–	+++	+++	–	–	–	–
2	7	M	N and G	P	++	++	++	–	++	++	+/–	–	++	++	++	–	–	++	++	+	+
				F	++	+	++	ND	++	+	ND	ND	++	+	ND	–	ND	+	ND	+	ND
3	6	M	N and G	P	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
				F	+/–	ND	++	ND	++	ND	ND	ND	+/–	++	ND	++	ND	++	ND	++	ND
4	2	M	N and G	P	–	–	+	–	–	–	–	–	–	+/–	–	–	–	–	–	–	–
				F	+/–	ND	–	ND	+/–	ND	ND	ND	+	ND	ND	+/–	ND	ND	ND	ND	ND
5	7	M	N	P	+	+	+	+	+++	–	–	–	–	–	–	–	–	–	–	–	ND
6	9	M	N and G	P	++	–	++	–	++	–	ND	–	–	+	–	–	–	–	–	–	–
7	8	M	N	P	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–
8	7	M	N and G	P	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–
9	12	M	N and G	P	++	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–	+++
10	16	F	G	P	–	–	+/–	–	–	–	–	–	–	++	–	+/–	–	+/–	–	+	–
11	14	F	N	P	–	–	+/–	–	+	–	–	+	–	–	–	–	–	–	–	–	–
12	6	F	N	P	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–	–
				F	–	ND	–	ND	+/–	ND	ND	ND	–	ND	ND	–	ND	ND	–	ND	ND
13	1	M	N and G	P	–	–	–	–	–	–	–	–	–	+++	–	–	–	+/–	–	–	–
14	6	M	N	P	–	–	–	–	–	–	–	–	–	++	–	–	ND	+/–	–	–	–
15	4	F	N and G	P	–	–	–	–	–	–	–	ND	–	–	–	–	–	++	–	–	–
16	9	M		P	+/–	+/–	+/–	–	+	+	ND	–	+/–	+++	–	ND	ND	++	–	+++	ND
17	8	M	N and G	P	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	+/–
18	10	F	N	P	–	+	+/–	+	+/–	–	–	–	–	++	–	–	ND	+/–	–	–	–
19	1	M	N and G	P	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	+/–
20	3	F	N and G	P	–	++	–	–	++	–	–	–	–	++	–	–	ND	–	+	–	–
21	5	F	N and G	F	–	ND	–	ND	++	ND	ND	ND	–	+	ND	–	ND	ND	ND	–	ND
22*	3	F	E	F	–	ND	–	ND	++	ND	ND	ND	++	ND	ND	+/–	ND	ND	ND	ND	ND
23	4	M	N	F	–	ND	–	ND	++	ND	ND	ND	–	–	ND	–	ND	–	ND	–	ND
24**	5	F	G	F	–	ND	–	ND	+/–	ND	ND	ND	+/–	–	ND	–	ND	+	ND	–	ND
25	9	M	N and G	F	–	ND	–	ND	–	ND	ND	ND	–	–	ND	–	ND	–	ND	ND	ND
26	10	M	N	F	–	ND	–	ND	+/–	ND	ND	ND	–	–	ND	–	ND	+	ND	–	ND
27	8	M	N and G	F	++	ND	++	ND	+/–	ND	ND	ND	++	++	ND	++	ND	–	ND	++	ND
28	12	F	N	F	–	–	+/–	ND	+	++	ND	ND	+/–	ND	ND	–	ND	ND	ND	–	ND
29	8 month	F	N and G	F	+/–	+	–	++	++	–	–	ND	–	++	–	ND	ND	–	ND	++	ND

F, female; M, male; ND, not done.

Immunoperoxidase staining results from the 29 PNETs examined here using antibodies to neutrophins and their receptor proteins. This summary includes immunoperoxidase data on frozen and/or paraffin samples of these tumors. Also listed here are the age and sex of the patients. All the tumors were posterior fossa MBs, except for one temporal (22*) and one frontal (24**) lobe PNET. The scoring for each antibody reflects the approximate area (as a percentage) of each tumor sample that contained positively labeled tumor cells according to the following scoring system: + = <5%, ++ = 5–50%, +++ = 50–100%. Some tumors were negative (–) or showed equivocal (+/–) staining. The presence or absence of neuronal (N), glial (G), and/or ependymal (E) differentiation by morphological and/or immunohistochemical criteria in each of these PNETs is shown in column 4. The 6th column indicates which tumors were examined using frozen (F) and/or paraffin (P) samples.

The antibodies used here are listed above each column of results. The antibodies recognize extracellular (ex) or intracellular (in) domains of TrkA (TrkAex and TrkAin, respectively), TrkB (TrkBex and TrkBin, respectively) or TrkC (TrkCex and TrkCin, respectively) or truncated TrkB (TrkBTr). The code names of the antibodies are listed below each of these headings as follows: Antibodies 254-3 (also known as anti-$TrkA_{out(SB)}$) and $TrkA_{out}$ (designated "out" in the Table) to the extracellular domain of TrkA; Antibodies 256-3 (also known as anti-$TrkA_{in(SB)}$) and $TrkA_{in}$ (designated "in" in the table) to the intracellular domain of TrkA; Antibodies 201-3 (also called anti-$TrkB_{23-26}$) and $TrkB_{out}$ (designated "out" in the table) to the extracellular domain of TrkB; Antibodies 306-1 (also called anti-$TrkB_{606-619}$) and $TrkB_{in}$ (designated "in" in the table) to the intracellular portion of TrkB; Antibody 271-1 to TrkBTr (ie, TrkB with a truncated intracellular domain); Antibody $TrkC_{out}$ (designated "out" in the table) to the extracellular domain of TrkC; Antibody $TrkC_{in}$ (designated "in" in the table) to the intracellular domain of TrkC; Antibodies MCtrk and Etrk16 that recognize TrkA, TrkB, and TrkC (ie, PanTrk antibodies); Antibody BDNF to BDNF; Antibody NT3 to NT3; Antibody NT4/5 to NT4/5; and LNGFR to p75LNGFR.

The differences in the results summarized here and in Table 2 using frozen sections versus ethanol fixed paraffin sections probably reflect regional heterogeneity of the tumor cells in the adjacent tumor samples, whereas differences in the results obtained by two different antibodies to the same receptor protein may be ascribed to several different factors including differences in the affinity of the different antibodies and the topography of the epitopes recognized by these antibodies.

from several of the biopsies also were snap-frozen and stored at −70°C until examined as frozen sections.[8,9,16,17] In addition, postmortem CNS tissue samples without any evidence of disease were obtained as positive controls and were processed in the same manner described above. The diagnosis for each brain tumor was determined primarily by morphological criteria[2,4,19] supplemented by data from immunohistochemical studies performed with antibodies to glial fibrillary acidic protein, neurofilament proteins (NFPs), and synaptophysin as described.[7–9,16,17,21]

Immunohistochemistry and Western Blot Studies

Sections of each of the brain tumors were cut at a nominal thickness of 6 μm for immunohistochemistry using both paraffin-embedded and frozen tissues. Frozen sections were also fixed in 70% ethanol for 15 minutes after sectioning. Immunohistochemical staining was performed by the peroxidase anti-peroxidase and avidin-biotin complex methods with or without microwave pretreatment as described.[7–9,16,17,21–23,37–39] Although we have

Table 2. *Immunohistochemical Staining of 19 Brain Tumors other than PNETs Using Antibodies to Neurotrophins and Their Receptors*

		TrkAex		TrkAin		TrkBex		TrkBin		TrkBtr		TrkCex	TrkCin	PanTrk				
Case	Histology	254–3	out	256–3	in	201–3	out	306–1	in	271–1	272–1	out	in	MCtrk	BDNF	NT-3	NT4/5	LNGFR
30	Ganglioglioma	+	−	+	−	−	+	+/−	−	−	−	−	+/−	−	+	−	−	++
31	Ganglioglioma	−	−	−	−	+	−	+/−	−	−	−	++	−	−	++	−	−	−
32	Anaplastic Ganglioglioma	−	−	−	−	−	−	+/−	−	−	+/−	−	−	ND	−	−	−	+++
33	Gangliglioblastoma	++	−	+	−	ND	++	++	−	+	−	++	+	ND	−	−	−	++
34	Astrocytoma	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	−	−
35	Astrocytoma	−	−	−	−	++	−	+/−	−	−	−	−	+/−	−	+/−	−	−	−
36	Astrocytoma	−	−	−	−	+/−	+	+/−	−	−	−	++	−	ND	++	−	−	−
37	Anaplastic astrocytoma	−	−	−	−	−	ND	−	−	−	−	++	+/−	ND	++	−	−	−
38	Glioblastoma	−	−	+	−	+++	++	+/−	−	−	−	+/−	+++	−	−	+	−	++
39	Glioblastoma	+	++	+/−	++	++	++		−	−	−	+++		+	−	−	−	−
40	Glioblastoma	−	+	−	−	+/−	++	−	−	+/−	−	−	−	−	++	+?	+	+
41	Oligodendroglioma	−	++	++	−	++	+	++	++?	++	−	++	−	−	+	−	−	++
42	Ependymoma	−	−	ND	−	−	+++	−	−	++	−	+++	−	−	−	−	−	+++
43	Anaplastic ependymoma	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	−	−
44	Oligo-astrocytoma	−	−	+/−	−	−	+	−	−	−	−	++	−	−	−	−	−	−
45	Meningioma	−	−	−	−	+/−	ND	−	−	−	−	−	−	−	−	−	−	−
46	Choroid plexus papilloma	−	−	−	−	+	+	−	−	+	−	+++	−	−	+	−	−	−
47	Atypical teratoid/ rhabdoid tumor	ND	−	−	−	+++	++	+/−	−	−	−	−	−	ND	+/−	++	+	−
48	Atypical teratoid/ rhabdoid tumor	−	−	−	−	+++	++?	+/−	−	−	−	++	+/−	ND	−	−	−	++

ND, not done.

Immunoperoxidase staining results of 19 pediatric brain tumors other than PNETs using antibodies to neurotrophins and their receptors. This table summarizes the immunoperoxidase studies conducted on paraffin samples of 19 different brain tumors other than PNETs. Positivity is scored as in Table 1, ie, − = negative; +/− = equivocal; + = <5%, ++ = 5–50%, +++ = 50–100%.

The antibodies used here are listed above each column of results. The antibodies recognize extracellular (ex) or intracellular (in) domains of TrkA (TrkAex and TrkAin, respectively), TrkB (TrkBex and TrkBin, respectively) or TrkC (TrkCex and TrkCin, respectively), or truncated TrkB (TrkBTr). The code names of the antibodies are listed below each of these headings as follows: Antibodies 254-3 (also known as anti-$TrkA_{out(SB)}$) and $TrkA_{out}$ (designated "out" in the Table) to the extracellular domain of TrkA; Antibodies 256-3 (also known as anti-$TrkA_{in(SB)}$) and $TrkA_{in}$ (designated "in" in the table) to the intracellular domain of TrkA; Antibodies 201-3 (also called anti-$TrkB_{23-36}$) and $TrkB_{out}$ (designated "out" in the table) to the extracellular domain of TrkB; Antibodies 306-1 (also called anti-$TrkB_{606-619}$) and $TrkB_{in}$ (designated "in" in the table) to the intracellular portion of TrkB; Antibody 271-1 to TrkBTr (ie, TrkB with a trunacted intracellular domain); Antibody $TrkC_{out}$ (designated "out" in the table) to the extracellular domain of TrkC; Antibody $TrkC_{in}$ (designated "in" in the table) to the intracellular domain of TrkC; Antibodies MCtrk and Etrk16 that recognize TrkA, TrkB, and TrkC (ie, PanTrk antibodies); Antibody BDNF to BDNF; Antibody NT3 to NT3; Antibody NT4/5 to NT4/5; and LNGFR to p75LNGFR.

noted minor differences in the results obtained by the peroxidase anti-peroxidase and avidin-biotin complex methods, we have found that the immunoreactivity of a wider variety of different polypeptides is better preserved in ethanol-fixed *versus* aldehyde-fixed paraffin-embedded tissue and that microwave pretreatment is not generally required for the detection of most proteins in ethanol-fixed tissues.[39] Furthermore, most antigens are detected equally well in frozen and ethanol-fixed paraffin sections. Western blot studies were performed on frozen tissue samples, cultured cells, and recombinant proteins as described.[40–42] To probe tumors by Western blot, the samples were homogenized in Laemmli sample buffer, boiled for 10 minutes, and centrifuged at 100,000 $\times$ g, and approximately 25 μg of soluble tissue extract were loaded in separate lanes for analysis by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Nitrocellulose replicas of these gels were prepared for Western blot study, and antibody-labeled protein bands were visualized by chemiluminescence (DuPont, Wilmington, DE) as described.[40]

Antibodies

Several different previously characterized monoclonal antibodies (MAbs) and polyclonal antibodies to individual neurotrophin receptors (ie, TrkA, TrkB, and TrkC) as well as antibodies to each of three different neurotrophins (ie, BDNF, NT3, and NT4/5) were used in this study.[40–50] The immunogens used to generate, screen, and characterize these antibodies included synthetic peptides as well as recombinant full-length, truncated or fusion proteins, and the essential features of these antibodies (including the ability of these antibodies to detect their cognate antigens by immunochemical and immunohistochemical methods) have been documented extensively in several recent publications.[40–50] These antibodies are listed in Tables 1 and 2, and representative Western blot data on several of these antibodies are illustrated in Figures 1 to 3. Finally, absorption control experiments for the immunohistochemical studies were performed on selected antibodies using recombinant proteins or peptides as described.[40–50]

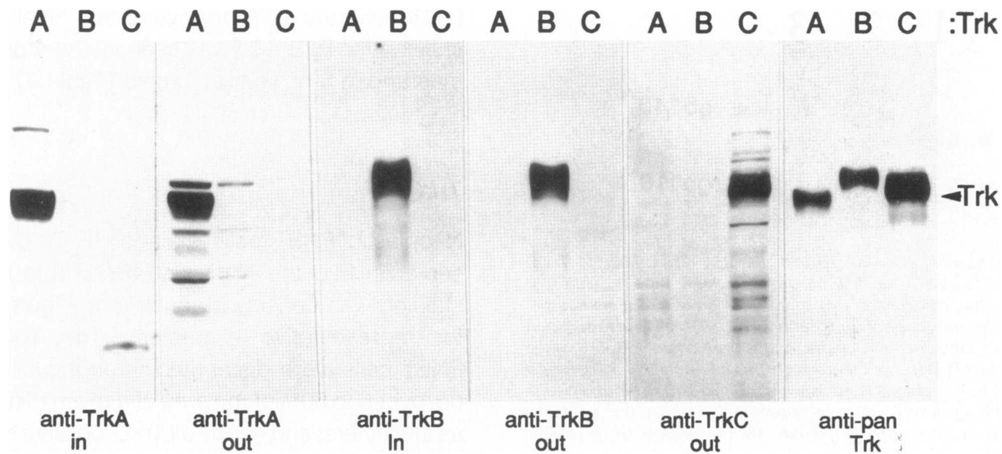


Figure 1. *Specificity of antibodies for different Trk receptor proteins. Different anti-Trk receptor antibodies (identified below each panel) were used to generate the Western blots shown here. Approximately 40 ng of Trk proteins* (lane A, *human TrkA;* lane B, *mouse TrkB; and* lane C, *rat TrkC) expressed in Sf9 cells were electrophoresed in 7.5% SDS-polyacrylamide gels, transferred, and probed with each Trk antibody as described in the text. Note that none of the ant-Trk antibodies, except the anti-pan Trk antibody, cross-react with other Trk receptor proteins.*

Several other well characterized antibodies also were used in the immunohistochemical analysis of the tumors studied here. These antibodies included 1) Me20.4, a mouse MAb to p75NGFR,[51] which was purchased from the American Type Culture Collection (Rockville, MD); 2) 2.2B10, a rat anti-glial-fibrillary-acidic-protein MAb[52]; 3) NF-L, a rabbit antibody to the low molecular weight (NF-L) NFP[53]; 4) HO14, a rat MAb to a phosphorylated form of the middle molecular weight (NF-M) NFP[54]; 5) RMdO9, a mouse MAb to a nonphosphorylated form of NF-M and the high molecular weight (NF-H) NFP[53–55]; 6) RMO24, a mouse MAb to a phosphorylated form of NF-H[54]; 7) SY38, a mouse MAb to synaptophysin (Boehringer-Mannheim, Indianapolis, IN)[56]; and 8) MIB-1, a mouse MAb to Ki-67, which is expressed throughout all phases of the cell cycle, but not in G_0 (Dako Corp., Carpinteria, CA).[57] All of these antibodies also have been used in previous immunochemical and immunohistochemical studies of normal and neoplastic human CNS tissue samples and/or cell lines.[7–9,16,17,21–23,37–39]

Results

TrkA

Positive staining for TrkA was observed in neoplastic cells in 8 of the 29 PNET cases (27%) studied here using several different antibodies to this neurotrophin receptor (Table 1). Labeled tumor cells were distributed over regions that represented approximately <5% to >50% of the total area of a given MB or other PNET. Positive staining was punctate or finely granular, and TrkA-positive tumor cells and tumor neuropil (ie, processes not contiguous with a

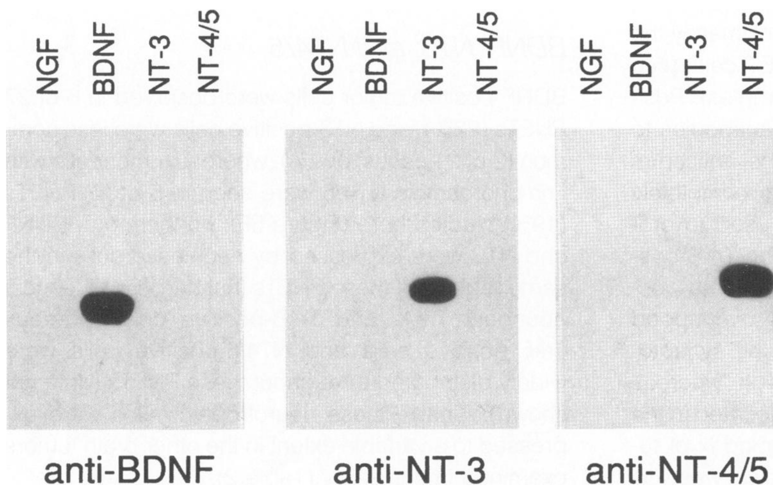


Figure 2. *Specificity of antibodies to BDNF, NT3, and NT4/5. Antibodies to BDNF, NT3, and NT4/5 (identified below each panel) were used to generate the Western blots shown here. Approximately 100 μg of each neurotrophin (kindly donated by Genentech, South San Francisco, CA) shown in the figure were electrophoresed in 15% SDS-polyacrylamide gels, transferred, and probed with each antibody as described in the text. Note that each of the antibodies is specific for a given neurotrophin.*

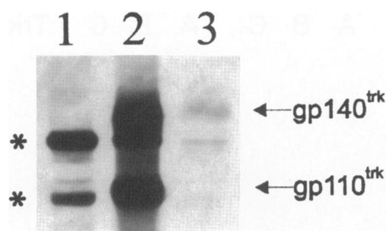


Figure 3. *Immunoblots produced with the 254–3 antibody to TrkA. The samples loaded in each lane are as follows:* **lane 1**, *wild-type NIH3T3 cell line, which does not express TrkA protein to show nonspecific low molecular weight bands;* **lane 2**, *NIH3T3 cell line transfected with rat TrkA;* **lane 3**, *tumor biopsy sample from case 29. Note that the transfected NIH3T3TrkA cell shows both 140-kd and 110-kd Trk bands and that the tumor sample demonstrates the 140-kd Trk band. Finally, although 254–3 detects some nonspecific bands in the wild-type NIH3T3 cells, these nonspecific bands are not present in the tumor biopsy sample.*

tumor cell in the plane of section examined) were seen (see Figures 4 and 5 for representative examples). Many of these regions also were positive for synaptophysin, but NFP-positive tumor cells did not often occur in these same regions (data not shown). The Ki-67 labeling index of these TrkA-positive areas was usually <1%, and TrkA was not observed commonly in areas with a high (>30%) Ki-67 labeling index (data not shown). In control studies performed with one of the antisera to the intracellular domain of TrkA (ie, the antiserum known as 256–3 or anti-$TrkA_{in(SB)}$), positive TrkA staining was completely eliminated by absorption with purified full-length TrkA derived from *Escherichia coli* (data not shown). Finally, immunoreactive TrkA also was detected in neoplastic cells in 2 of 4 gangliogliomas and in 3 of 11 glial tumors (Table 2).

TrkB

Tumor cells immunolabeled with the TrkB antibodies were observed in 18 of 29 PNETs (62%; Table 1). The TrkB staining also was finely granular and was observed in tumor cells located in approximately the same regions as the TrkA-positive PNET cells (see Figures 4 and 5 for representative examples). Positive staining for TrkB using one of the antibodies to the extracellular domain of TrkB (ie, the antiserum known as 201–3 or anti-$TrkB_{23-36}$) was completely absorbed by the peptide immunogen used to produce this antibody (data not shown). The TrkB-positive areas were somewhat larger than the TrkA-positive areas, but both of these areas overlapped extensively with one another and with the synaptophysin-positive areas (data not shown), whereas NFP-positive tumor cells were not often located in the TrkB-positive areas. The Ki-67 labeling index of tumor cells in the TrkB-positive areas also was low (<1%) like the TrkA-positive areas. Finally, positive staining for TrkB also was seen in 4 of 4 gangliogliomas and in 8 of 11 glial tumors (Table 2).

TrkC

Neoplastic PNET cells stained for TrkC were observed in 14 of the 29 PNETs (48%; Table 1), and this staining was densely granular (see Figures 4 and 5 for representative examples). The TrkC-positive PNET cells were distributed throughout the tumors (ie, over 5 to 50% of the area of a given PNET) as well as in clusters, and nearly all TrkC-positive tumor cells were seen in regions with a high Ki-67 labeling index. Furthermore, the TrkC-positive area did not overlap extensively with the synaptophysin-, TrkA-, and TrkB-positive area of these PNETs, but NFP-positive tumor cells were abundant in the TrkC-positive regions. Finally, several other different types of pediatric brain tumors also contained TrkC-positive tumor cells (Table 2).

p75LNGFR

As described earlier,[23] the expression of p75LNGFR is not common in PNETs, and immunoreactive p75LNGFR was observed in only 2 of the 18 PNET samples (11%), but p75LNGFR immunoreactivity was seen in 42% of the other brain tumors examined here (Tables 1 and 2). Notably, the distribution of tumor cells in PNETs with p75LNGFR immunoreactivity did not co-localize with the staining pattern of any of the neurotrophins (see below) or with the cells that were positive for TrkA, TrkB, and TrkC (data not shown).

BDNF, NT3, and NT4/5

BDNF-positive tumor cells were observed in 6 of 27 PNETs (22%), and NT3-positive cells were less common (2 of 21 cases, or 9%), whereas tumor cells with immunoreactive NT4/5 were seen in 5 of 27 PNETs (19%) studied here (Figure 5F). Furthermore, BDNF and NT3 were expressed by neoplastic cells in the same regions of these PNETs that harbored the most abundant TrkA- and TrkB-positive cells, whereas TrkC-positive cells and NT4/5-positive cells were widely distributed throughout these PNETs (data not shown). Finally, these neurotrophins also were expressed to a variable extent in the other brain tumors examined in this study (Table 2).

Western Blot Studies

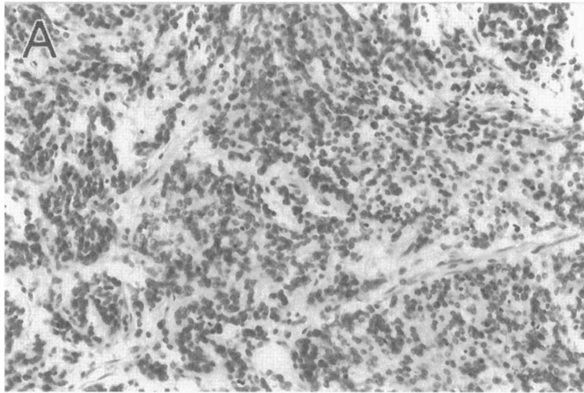
Western blot studies to verify the specificity of the antibodies to the neurotrophins and neurotrophin receptors studied here (see Figures 1 and 2 for representative data) were complemented by performing similar studies on adjacent portions of selected PNET samples from which residual unfixed frozen tissue was available. In Western blots performed using $TrkA_{254-3}$, two TrkA-positive bands (p140 and p110) were observed in extracts of the NIH3T3 cells transfected to express full-length TrkA (Figure 3). Similar studies were performed on extracts of 4 frozen PNET samples, and one of the 4 cases showed $gp140^{Trk\ A}$ (Figure 3) identified by two of the anti-TrkA antisera (ie, the antibodies known as 254-3 or $antiTrkA_{out(SB)}$ and 256-3 or anti-$TrkA_{in(SB)}$). Notably, this PNET also contained TrkA-positive tumor cells by immunohistochemistry. The limited availability of unfixed frozen PNET biopsy samples precluded more extensive studies of the other three PNETs by Western blot.

Discussion

The sequence of events leading to the induction and progression of MBs and related PNETs in the postnatal brain of children remains to be elucidated, but recent insights into the basic cell biology of these tumors are consistent with the notion that they may result in part from a disruption of normal developmental programs that regulate the proliferation, differentiation, and apoptotic death of residual neuroectodermal progenitor cells in the immature brain.[36,38] Although the clonal expansion of increasingly malignant tumor cells in nascent MBs and PNETs undoubtedly results from the accumulation of genetic lesions, the failure of residual neuroectodermal cells to differentiate or undergo apoptosis in the postnatal brain could provide the cellular substrate for these genetic lesions. Given the central role that neurotrophins are thought to play in regulating cell proliferation, cell differentiation, and cell death in the developing CNS,[26–30,32] it is plausible to hypothesize that neurotrophins could play a contributory role in the pathogenesis of MBs and PNETs.

This hypothesis is supported by recent evidence that mRNAs encoding neurotrophins and their cognate receptors are found in biopsy samples of MBs.[25] Nonetheless, although the detection of mRNA encoding a given polypeptide is suggestive of the expression of the corresponding translation product, the importance of the present study is that it directly demonstrates the presence of several different neurotrophins and neurotrophin receptor proteins in MBs and related PNETs. Thus, it is possible that normal or aberrant neurotrophin-mediated signaling mechanisms may be activated in PNETs and that the activation of these neurotrophin receptors may influence the clinical behavior of these pediatric brain tumors. Indeed, although our data are preliminary, they suggest that the Trk expression may correlate with neuronal differentiation in PNETs as defined by immunohistochemistry using antibodies to neuron-specific proteins such as NFPs. Furthermore, neurotrophin receptors may be aberrantly expressed in MBs as TrkA was detected in a few MBs here, but TrkA mRNA and protein are not found in the normal developing or adult cerebellum where MBs arise.[26,28,40] Thus, we speculate that one or more neurotrophins may influence processes such as proliferation, differentiation, and/or cell death in PNETs through normal or aberrant signal transduction pathways that involve one or more neurotrophins and/or their cognate receptors. For example, our data suggest that the presence of a given neurotrophin and its cognate receptor in specific regions of tumors could be functionally significant and modulate the differentiation or proliferation rate of tumor cells in these regions in a meaningful way. However, additional double-labeling studies are needed to address this issue in more detail. Although we have conducted less extensive analyses of several different types of primary pediatric brain tumors other than PNETs in the present study, nonetheless, our preliminary data on this series of CNS neoplasms suggest that similar speculations also may apply to at least a subset (eg, astrocytomas and other gliomas) of this group of neoplasms.

Although MBs are prototypical PNETs that make up approximately 20% of all brain tumors in children,[1–3,21] advances in conventional therapy after tumor resection have resulted in modest improvements in the treatment of PNETs, which remain one of the most malignant brain tumors in childhood.[1–3,10,19–21,58,59] Despite evidence suggesting a correlation between the presence or absence of neuronal and/or glial differentiation in PNETs and survival,[19–21] the ability of neoplastic cells in PNETs to undergo differentiation may be a surrogate marker for the activation of neurotrophins or other trophic factors in these tumors, and it remains to be determined whether differentiation or the expression of one or more neurotrophins and their cognate receptors will predict a favorable prognosis in PNETs. However, antibody probes to study the expression of trophic factors and their receptor proteins have become available only recently, and very little information is available on this aspect of the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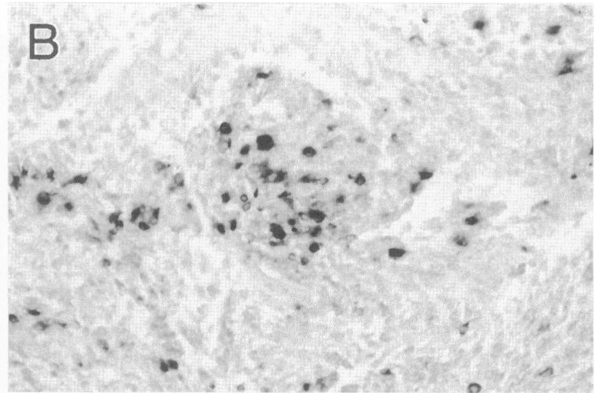
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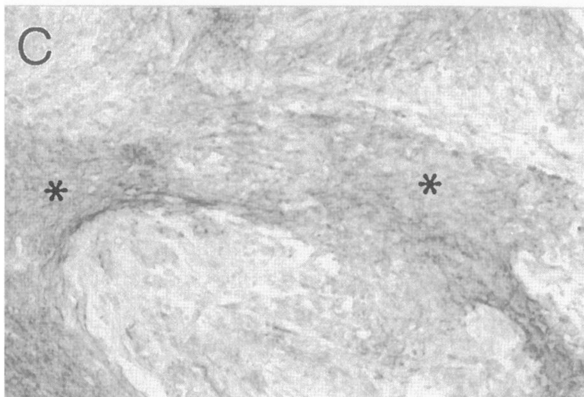
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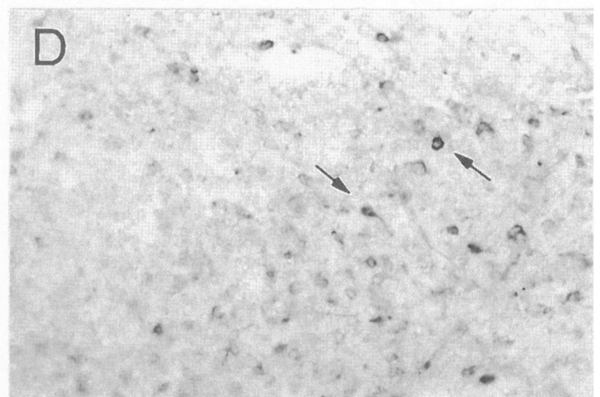
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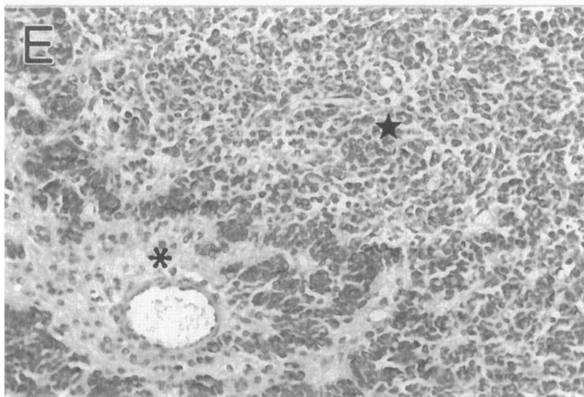
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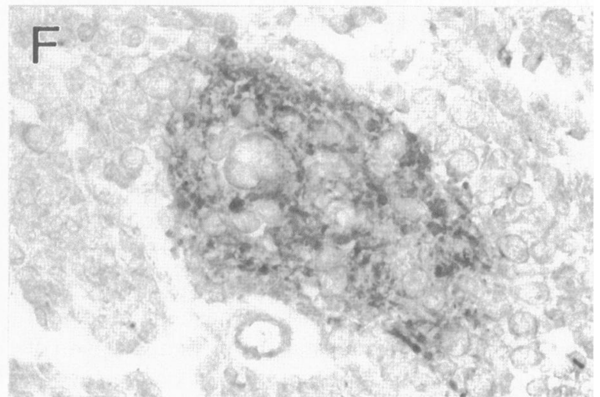
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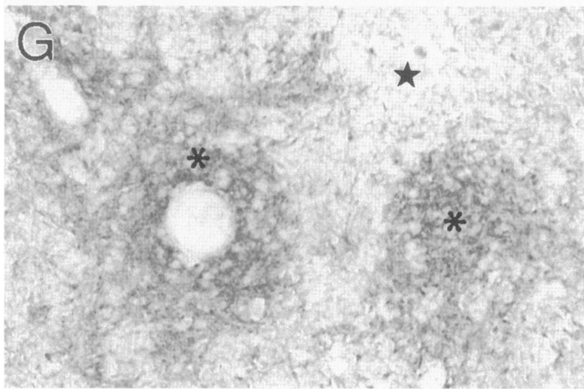
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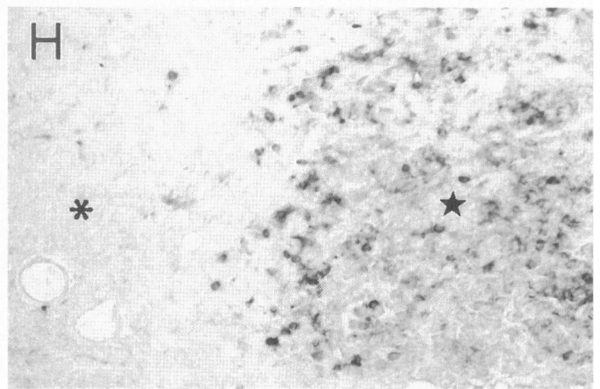
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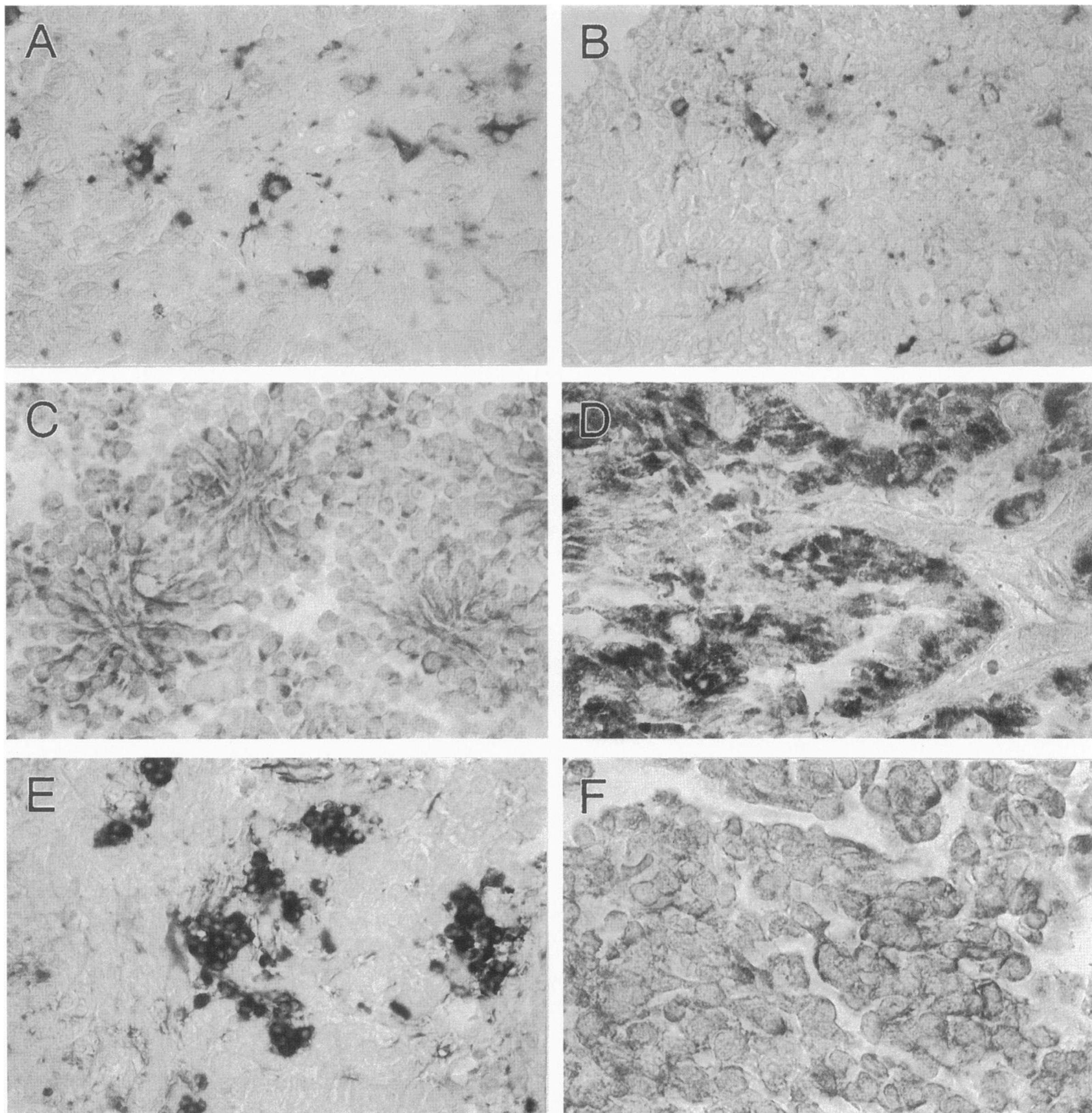

Figure 5. *Immunostaining of four different MBs (cases 2, 12, 16, and 18 in Table 1) using different anti-Trk and anti-neurotrophin antibodies.* **A** *and* **B**: *Small numbers of tumor cells are stained with both the anti-TrkA-out* (**A**) *and the anti-TrkA-in* (**B**) *antibodies in the same MB biopsy (case 18). Magnification, ×40.* **C**: *The 201–3 antibody stains tumor cells forming Homer Wright rosette structures (case 12). ×60.* **D**: *Most of the tumor cells in this MB (case 1) are labeled with the 271–3 antibody to truncated TrkB. ×40.* **E**: *The TrkC-out antibody stains a cluster of tumor cells in this MB (case 2). ×40.* **F**: *The anti-NT4/5 antibody homogeneously stains tumor cells in this MB (case 16). ×60.*

biology of MBs and PNETs at this time.[22–25] Furthermore, only a few preliminary studies of the mechanisms whereby neurotrophins might influence the biology of MBs and PNETs have appeared,[23,24,60] and it is not yet clear which neurotrophins might play a key role in regulating the proliferation, differentiation, and cell death of these tumors.[35,61] However, in view of the growing evidence that the levels of Trk A

Figure 4. *Trk immunostaining in two different MBs (cases 7 and 20 in Table 1).* **A** *to* **D**: *Case 20.* **E** *to* **H**: *Case 7.* **A**: *Histology shows typical MB. H&E; magnification, ×20.* **B**: *A small number of tumor cells (less than 5%) are stained with the Trk A-out antibody. ×30.* **C**: *The 201–3 antibody against the extracellular domain of TrkB shows neuropil staining. ×20.* **D**: *The TrkC-out antibody stains cell bodies* (**upper arrow** *on the right*) *as well as processes in the neuropil of this MB* (**lower arrow** *on the left*)*. ×30.* **E**: *H&E staining demonstrates two different patterns of tumor growth within this specimen, ie, a perivascular growth pattern* (**asterisk**) *and a sheet of undifferentiated tumor cells* (**star**)*. ×20.* **F**: *The 254–3 antibody primarily stains perivascular tumor cells. ×60.* **G**: *The 201–3 antibody also primarily stains perivascular tumor cells* (**asterisk**)*, whereas other tumor cells are negative or variably stained by this antibody* (**star**)*. ×20.* **H**: *In contrast to antibodies 254–3 and 201–3, the TrkC-out antibody primarily stains tumor cells* (**star**) *outside the region of the perivascular structure* (**asterisk**)*. ×20.*

mRNA expressed in neuroblastomas closely correlate with a better prognosis,[62,63] it will be important to investigate further the potential role of neurotrophins and other CNS trophic factors in the induction and progression of CNS PNETs. The increasing availability of antibody probes to detect the expression of CNS trophic factors and their receptor proteins as well as the development of a number of different cell culture and animal model systems for experimental studies of MBs and PNETs[18,36] should accelerate the pace of understanding of these important childhood brain tumors.

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