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Neuronal colocalization of mRNAs for neurotrophins and their receptors in the developing central nervous system suggests a potential for autocrine interactions

(low-affinity nerve growth factor receptor/TrkA/TrkB/paracrine action/*in situ* hybridization)

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ABSTRACT **Development and survival of neurons in the central nervous system are dependent on the activity of a variety of endogenous neurotrophic agents. Using combined isotopic and nonisotopic *in situ* hybridization histochemistry, we have found that subsets of neurons within the developing forebrain coexpress the mRNAs for both neurotrophins (nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3) and their receptors ($p75^{NGFR}$, TrkA, and TrkB). The colocalization of mRNA for neurotrophin receptors and their ligands in presumptive neurotrophin target neurons suggests the potential for autocrine and paracrine mechanisms of action during development. Such mechanisms may ensure the onset of differentiation and survival of specific subsets of neurons prior to and following target innervation.**

Neuronal survival and differentiation are regulated by many endogenous and target-derived neurotrophic factors. Members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3), are important examples of such ligands (1). The neurotrophins act via a family of transmembrane receptors to regulate multiple cellular functions, including protein phosphorylation, gene induction, and cellular differentiation (1, 2). The earliest identified member of the neurotrophin receptor family, $p75^{NGFR}$, binds all members of the neurotrophin family with low affinity and, by itself, cannot explain either high-affinity binding or the biological effects of the neurotrophins (2). Subsequent research has identified protooncogenic members of a family of receptor tyrosine kinases, TrkA (3), TrkB (4, 5), and TrkC (6), which can account for high-affinity binding of NGF, BDNF, and NT-3, respectively (2). While TrkA itself appears to have low affinity for NGF (3), coexpression with $p75^{NGFR}$ results in high-affinity NGF binding (7), suggesting a synergistic role for $p75^{NGFR}$ with the Trk family of receptors.

Previous studies have suggested the possibility of cellular colocalization of the neurotrophin receptors with their ligands, based on the observed overlap in developmental and regional patterns of neurotrophin and receptor mRNA expression. For example, the expression of NGF and $p75^{NGFR}$ mRNAs appear to overlap developmentally in many central nervous system regions (8, 9) and in peripheral somatic targets (10). Others (6, 11) have suggested the possibility of regional overlap in the expression patterns of other neurotrophins (BDNF and NT-3, for example) and their receptors, raising the possibility that autocrine/paracrine regulatory influences may be characteristic of the neurotrophin family in general. These potential interactions do appear to have a functional basis. For example, developing chicken sensory ganglia appear both to be the source of BDNF and to be responsive to it (12). Similarly, hippocampal neurons of the dentate gyrus appear to synthesize and to be developmentally regulated by NT-3 (13). Autocrine mechanisms may induce patterns of autonomously regulated gene expression, permitting cell survival and differentiation prior to and even subsequent to the availability of target-derived survival and differentiative factors.

Using combined isotopic and nonisotopic *in situ* hybridization, the present studies investigated whether the mRNAs for both the neurotrophin receptors and their ligands were coexpressed by individual neurons within regions of the developing forebrain which are targets of the neurotrophins and/or sites of neurotrophin synthesis. Furthermore, since the available evidence suggests that NT-3 may be a common ligand for both TrkA and TrkB (2), we also investigated the extent to which NT-3 mRNA colocalized with mRNA for their receptor-specific ligands, NGF and BDNF, respectively. We focused on the second week of rodent postnatal development, since previous studies have reported maximal expression of neurotrophins and their receptors in the rodent brain during this period (14–19).

MATERIALS AND METHODS

Preparation of Tissue Sections. Paraformaldehyde-fixed brain tissue from rats (Sprague–Dawley; Zivic–Miller) and mice (RIII; our colony) of both sexes at postnatal days 9–10 (P9–P10) ($n = 11$) was obtained and prepared as described (20). Coronal brain sections were cut serially at 10–14 μm on a cryostat, thaw-mounted onto slides [coated with 3-aminopropyltriethoxysilane (Fluka)], air dried, and processed immediately.

Oligonucleotide Probes for Neurotrophin Receptors. *$p75^{NGFR}$*. This previously characterized (21, 22) oligonucleotide is a 46-base sequence from the region encoding the putative membrane-spanning domain of chicken $p75^{NGFR}$ (21), with 80% identity to nt 807–852 of the rat homologue (23). Northern analysis indicates that this probe hybridizes to a single transcript (3.7 kb) in P6 mouse cortex. *TrkA*. This oligonucleotide is a unique 60-base sequence complementary to nt 208–267 in the putative extracellular domain of the human cDNA sequence (24), corresponding to the first exon of rodent *trkA* mRNA [overall homology with the human sequence, >80% (25, 26)]. Northern analysis indicates that this probe hybridizes to a single transcript (3.2 kb) in P6–P14 mouse cortex. This oligonucleotide has no significant homology (<30%) with any other known nucleotide sequences, including those coding for other members of the receptor tyrosine kinase family. *TrkB*. This oligonucleotide is a unique 60-base sequence complementary to nt 1241–1300 within the extracellular domain of the mouse cDNA clone (27). There is

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin 3; P*x*, postnatal day *x*.
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no significant homology (<28%) between this probe and any other known nucleotide sequences, including those coding for other members of the receptor tyrosine kinase family and specifically for TrkA. Northern analysis indicates that this probe hybridizes to multiple transcripts (9.0, 8.0, and 2.0 kb) in P4–P14 mouse cortex.

Oligonucleotide Probes for Neurotrophins. *NGF*. This oligonucleotide is a previously characterized (28) 51-base sequence, spanning nt 868–918 of the rat cDNA clone (29). Northern analysis indicates that this probe hybridizes to a single transcript (1.35 kb) in mouse salivary gland. *BDNF*. This oligonucleotide is a previously characterized (18) 50-base sequence encompassing nt 746–795 of pig BDNF cDNA. This region has 90% identity with rodent BDNF sequences (30). Northern analysis indicates that this probe hybridizes to two transcripts (4.0 and 1.6 kb) in mouse heart. *NT-3*. This oligonucleotide is a previously characterized (18) 50-base sequence encompassing nt 667–717 of the rat NT-3 clone. Northern analysis indicates that this probe hybridizes to a single transcript (1.2 kb) in mouse salivary gland.

Combined Isotopic and Nonisotopic *in Situ* Hybridization Histochemistry. Combined *in situ* hybridization was carried out with a variation of previously described protocols (20, 23, 31–34), using two oligonucleotide probes, one 3′-end-labeled with [α-[^{35}S]thio]dCTP and the other, with digoxigenin-dUTP (20). Briefly, sections were dehydrated and rehydrated, rinsed in 1× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7.2), and prehybridized (for details, see refs. 20 and 33) for 2 hr. Sections were first hybridized with the probe which had the higher melting temperature [T_m (35); calculated according Baldino *et al.* (36)]. Slides were washed at 10–15°C below the T_m for that probe, in 0.1× SSC, for 30 min and then hybridized to the second probe. The sections were then washed at a stringency appropriate to the second probe (10–15°C below the T_m for this probe, in 0.1× or 0.2× SSC). After a final overnight wash (1× SSC, 40°C), sections were exposed to alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) at a 1:500 dilution. Slides were first processed for the alkaline phosphatase-linked color reaction (for the detection of the digoxigenin-labeled probe) and then coated with autoradiographic emulsion (Ilford, L4 or K5) and processed for the detection of the ^{35}S-labeled oligonucleotide probe. Ilford emulsion was used in order to avoid positive chemography induced by the alkaline phosphatase product (37). Hybridization with ^{35}S-labeled probes was considered specific where the accumulation of exposed silver grains exceeded 5 times background (38).

Probe and Method Specificity. Probe and method specificity has been extensively documented (20, 23, 33, 34). Briefly, "sense" sequences did not hybridize to tissue under the hybridization conditions used. Hybridization with an experimental probe was blocked by a larger (90-base), overlapping, complementary oligonucleotide. Pretreatment with RNase A prevented hybridization. Alternative oligonucleotide sequences to the same mRNA exhibited similar patterns of hybridization. Slides hybridized with digoxigenin-labeled but not ^{35}S-labeled probes and processed for alkaline phosphatase-linked immunohistochemistry were apposed to x-ray film or dipped in L4 emulsion. No exposure above background was observed over the alkaline phosphatase color reaction product, indicating that the double *in situ* hybridization methodology does not induce positive chemography. Northern analysis indicated that each of the oligonucleotide probes hybridized to transcripts of the appropriate size. Thermal stability analysis (36) of the unique probes (TrkA and TrkB) indicated hybridization to a single mRNA species of the appropriate base composition, with a close match between theoretical (TrkA, 70.2°C; TrkB, 59.1°C) and empirically ascertained T_m values (TrkA, 71.1°C; TrkB, 61.5°C).

RESULTS

Colocalization of mRNAs. Cellular colocalization of any two mRNA transcripts was identified by the presence of a blue-black cytoplasmic reaction product (brightfield) with an overlying accumulation of green silver grains (epipolarization). The colored enzymatic reaction product is attributable to hybridization to one transcript with a digoxigenin-labeled probe, while the overlying accumulation of epipolarized grains represents specific hybridization of an ^{35}S-labeled probe hybridized to a second transcript (Fig. 1).

Colocalization of Neurotrophin mRNAs with p75^{NGFR} mRNA. Neurons in the forebrain colocalized p75^{NGFR} mRNA with mRNA for NGF, BDNF, and NT-3 in a regionally specific manner (Table 1). In the cerebral cortex and hippocampus, many neurons expressing p75^{NGFR} mRNA also expressed mRNA for either NGF or BDNF (Fig. 2 *A–C*) or, more rarely, NT-3, but very few neurons of the basal forebrain appeared to coexpress the mRNAs. NGF and BDNF mRNA-containing cells of the basal forebrain appeared to be relatively rare and, when present, were more often observed interdigitated between neurons expressing p75^{NGFR} mRNA. In the lateral striatum, p75^{NGFR} mRNA colocalized with NGF mRNA in a few dispersed neurons. Few p75^{NGFR} mRNA-containing neurons of the hypothalamus (mainly in the arcuate nucleus) expressed either NGF or BDNF mRNA, and little, if any, coexpression was observed in thalamic and amygdaloid nuclei.

Colocalization of Neurotrophin mRNAs with mRNAs for TrkA and TrkB. The mRNA for TrkA appeared to be coexpressed with the mRNA for its cognate ligand (NGF) in brain regions (Table 1) such as the cerebral cortex (Fig. 2 *D*

Table 1. Regional and cellular colocalization of neurotrophin mRNAs with neurotrophin receptor mRNAs

Brain region	p75^{NGFR} with NGF	p75^{NGFR} with BDNF	p75^{NGFR} with NT-3	TrkA with NGF	TrkB with BDNF
Cerebral cortex	+	++	±	++	++
Hippocampal formation	+	++	±	++	+++
Septum/diagonal band	−(±)*	−(±)*	−	±	±
Basal ganglia					
Caudate–putamen	+†	+	−	+	+
Globus pallidus	+	+	−	++	++
Entopeduncular/ subthalamic nuclei	+	++	−	+	++(±)‡
Amygdaloid complex	−	−	−	±	+
Thalamus	−	−	−	±§	++
Hypothalamus					
Anterior	+	±	±	±	+
Lateral	±	±	±	±	++
Ventromedial	±	+	−	+	+
Arcuate/ retrochiasmatic	+	++	−	+	+++
Paraventricular	±	±	−	±	++
Supraoptic	+	+	−	±	+

Regional variations in the neuronal colocalization of neurotrophin receptor and ligand mRNAs were tabulated on an ordinal scale: +++, very strong coexpression (>60% of neurons which express mRNA for one neurotrophin also express the mRNA for its cognate receptor); ++, strong (30–60%); +, moderate (5–30%); ±, few cells coexpressing mRNAs (<5%); −, no colocalizing cells were detected.

*Multiple ordinal rankings indicate interanimal or intraregional variability.

†Colocalizing cells were observed mainly in lateral striatum (caudate–putamen).

‡While a large number of colocalizing neurons were observed rostrally, virtually no BDNF mRNA-containing neurons in the caudal portion of this region appeared to colocalize TrkB mRNA.

§A few colocalizing cells were observed, mainly in anterior thalamus.

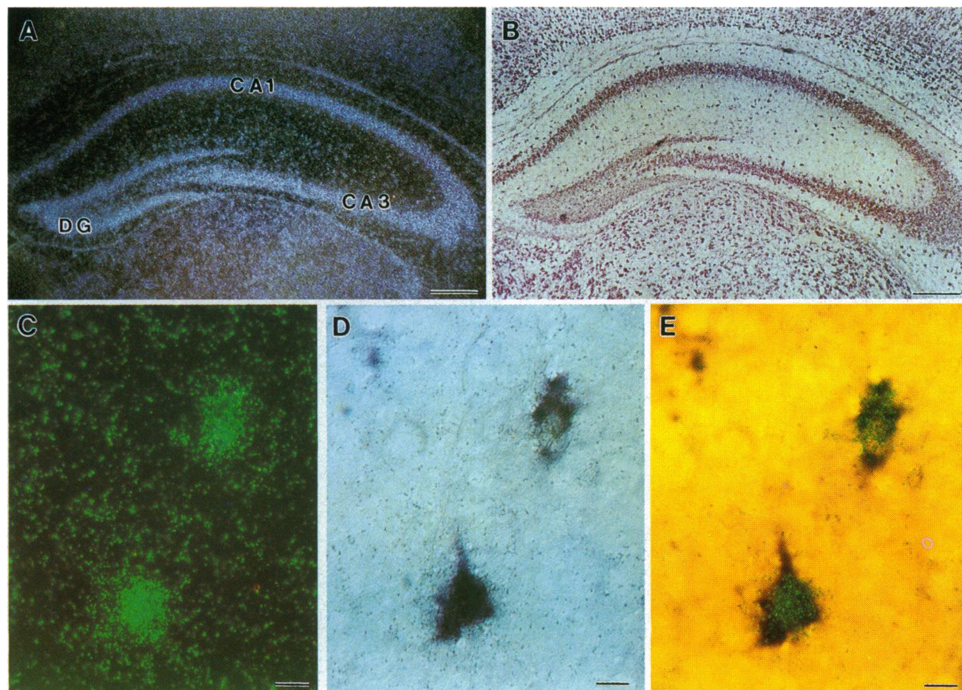

FIG. 1. Colocalization of BDNF and TrkB mRNAs in P9 mouse hippocampus by isotopic/nonisotopic *in situ* hybridization. (*A*) Darkfield micrograph shows distribution of BDNF mRNA (^{35}S-label). (*B*) Brightfield micrograph of the same section shows nonisotopic (digoxigenin) *in situ* hybridization to TrkB mRNA. (*C–E*) Higher magnification of a single tissue section shows two cells in stratum radiatum (CA3) coexpressing BDNF and TrkB mRNAs. Epipolarization (isotopic *in situ*; green grains) was combined with brightfield (nonisotopic *in situ*; blue-black reaction product) photomicrography. BDNF mRNA was visualized by epipolarization (*C*), TrkB mRNA with brightfield (*D*), and colocalization of the two mRNAs within the same two cells by combined brightfield/epipolarization photomicrography (*E*). An orange filter enhanced the contrast between the green silver grains and the underlying blue-black alkaline phosphatase reaction product. DG, dentate gyrus; CA1 and CA3, regions of the hippocampus [cornu Ammonis (Ammon's horn)]. [Bar = 200 μm (*A* and *B*) or 10 μm (*C–E*).]

and *E*) the hippocampus, and the striatum and hypothalamus (arcuate and ventromedial nuclei). Similarly, at this developmental stage, TrkB and BDNF mRNAs appeared to be coexpressed in regions of the forebrain (Table 1) such as the cerebral cortex (Fig. 2 *F* and *G*), striatum (caudate–putamen), globus pallidus, and hippocampus (Fig. 1). Neurons in the hypothalamus, in some regions of the thalamus such as the zona incerta, and particularly in somatosensory relay nuclei such as those in the ventral posteromedial/posterolateral and the ventral mesencephalon also colocalized BDNF and TrkB mRNAs extensively.

Colocalization of mRNAs for Multiple Neurotrophins. Some neurons of the forebrain coexpressed mRNAs for more than one neurotrophin. NGF and NT-3 mRNAs, for example, were coexpressed extensively in regions like the cerebral cortex (particularly the prefrontal area) and olfactory cortex as well as the hippocampus. In the cerebral cortex, a majority of neurons coexpressing the two mRNAs were observed in superficial layers of the cerebral cortex and in the cortical subplate. Few, if any, neurons colocalized the two mRNAs in regions such as the basal forebrain, striatum, globus pallidus, thalamus, and hypothalamus.

BDNF and NT-3 mRNA coexpression was primarily restricted to neurons of the superficial layers of the cerebral cortex (Fig. 2*H*) and the hippocampus (CA2 and CA3). Few, if any, neurons were observed to coexpress the two mRNAs in any other region of the developing forebrain.

DISCUSSION

Overlapping distributions of neurotrophin and receptor mRNAs (6, 8, 10, 11) have suggested the potential for neurotrophin receptor-mediated autocrine and paracrine interactions. Our study directly addresses this potential in the central nervous system, by determining whether individual neurons in regions of the developing forebrain coexpress mRNAs for the neurotrophins and their receptors. During maturation, such mechanisms may regulate survival and differentiation where functional contacts ensuring target-derived neurotrophin availability have yet to be made.

Neuronal coexpression of mRNA for neurotrophin receptors and their cognate ligands appears to be regionally specific. Such colocalizations occurred abundantly in the cerebral cortex and hippocampus, in contrast to the basal forebrain. Thus, whereas the dominant mode of interaction in the basal forebrain may be via paracrine or target-derived trophic mechanisms, autocrine mechanisms may predominate in the developing cerebral cortex and hippocampus. Discrete thalamic efferents (ventral posteromedial/posterolateral) also appeared to exhibit extensive coexpression of neurotrophin (BDNF) and receptor (TrkB) mRNA. The potential ability to synthesize both a trophic agent and its receptor may be important for the survival and differentiation of these thalamic projection neurons, particularly during the synaptic organization and maturation of the cortical plate.

Previous studies have shown that both $p75^{NGFR}$ (9, 15) and NGF (8, 9) are expressed by the developing striatum. We find that $p75^{NGFR}$ and TrkA mRNA-containing neurons colocalized NGF mRNA, mainly in large-diameter neurons of the lateral striatum and globus pallidus. BDNF and TrkB mRNAs were also extensively colocalized in these regions. BDNF, synthesized by the striatum, is reported to enhance the survival of embryonic ventral mesencephalic dopaminergic neurons (39), a population of which are striatal afferents. Interestingly, groups of neurons in the ventral mesencephalon also coexpress TrkB and BDNF mRNAs. This region is also the origin of extensive mesolimbic/mesocortical dopaminergic projections, circuitry that is implicated in the control of working memory (40) and the etiology of cognitive and affective disorders such as schizophrenia (41). Potential

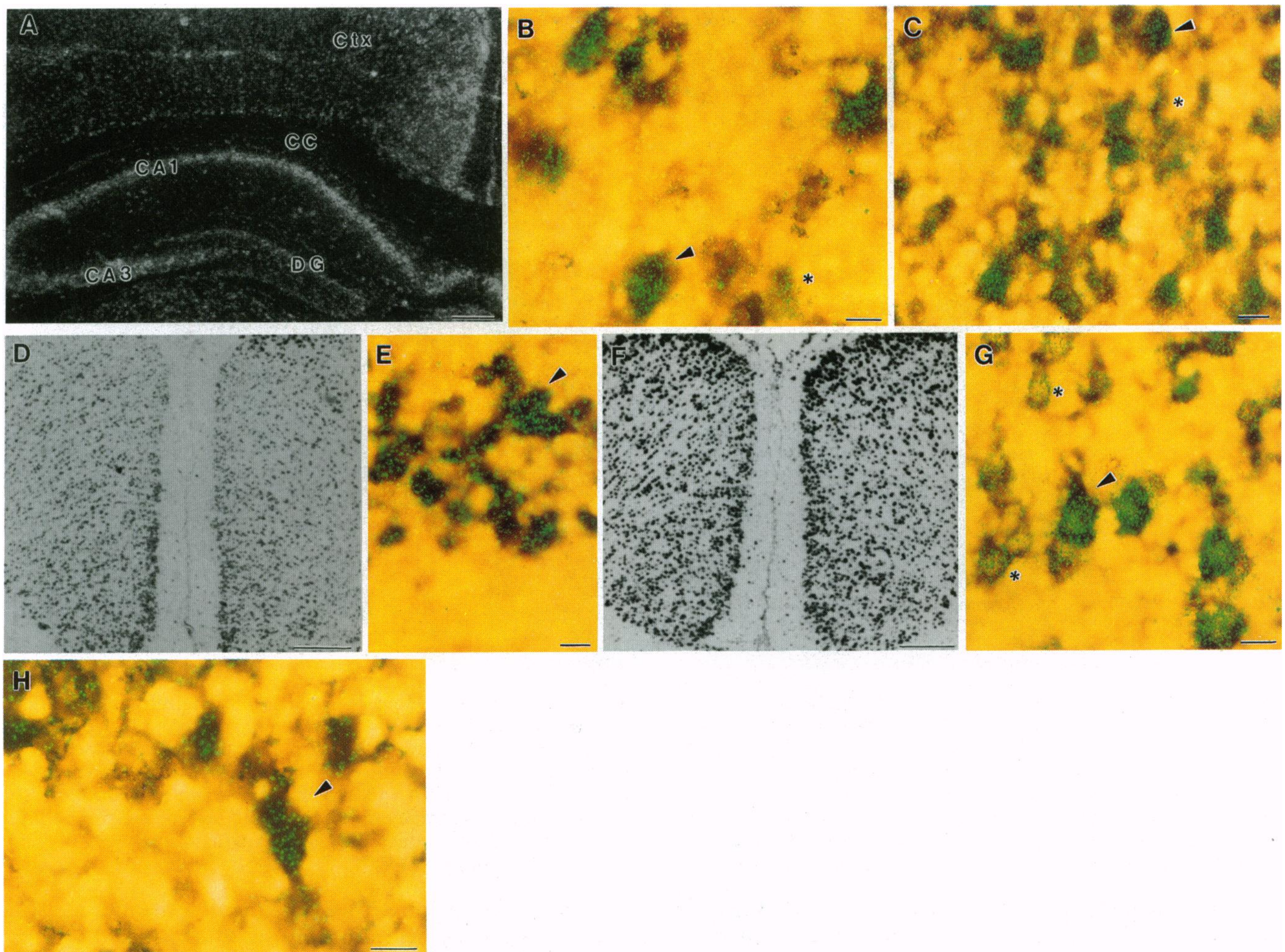

FIG. 2. Coexpression of the mRNAs for the neurotrophins and their receptors in neurons of the developing rodent forebrain (*A* and *D–G*, P9 mouse; *B*, *C*, and *H*, P10 rat). (*A–C*) Distribution of $p75^{NGFR}$ mRNA in the cerebral cortex (*A*) and colocalization (arrowheads) of $p75^{NGFR}$ (green grains) mRNA in the cerebral cortex with NGF mRNA (color product) (*B*) and BDNF mRNA (color product) (*C*). Asterisks indicate expression of $p75^{NGFR}$ mRNA alone. (*D–G*) Colocalization of mRNAs for the neurotrophins (green grains) and the *trk* gene products (color product). Shown are the distribution of TrkA mRNA in the cerebral cortex (*D*) and colocalization of TrkA mRNA (*E*) with NGF mRNA (*F*); distribution of TrkB mRNA in the cerebral cortex and colocalization of TrkB mRNA with BDNF mRNA (*G*) (asterisk, expression of BDNF alone); and colocalization of mRNA for BDNF (blue product) with NT-3 mRNA (green grains) in the cerebral cortex (*H*). Ctx, cerebral cortex; CC, corpus callosum; DG, dentate gyrus. [Bar = 200 μm (*A*, *D*, and *F*) or 10 μm (*B*, *C*, *E*, *G*, and *H*).]

autocrine/paracrine loops could have important consequences for the development of target forebrain regions, and, an alteration in these developmental mechanisms may be one predisposing factor in the etiology of some psychoses.

TrkA and TrkB are the specific transducers of NGF and BDNF actions, and some (42–44) but not all (7, 12) of the biological actions of the neurotrophins may be activated independently of $p75^{NGFR}$. These observations indicate the potential for multiple neurotrophin-mediated signaling mechanisms localized to distinct pools of autocrine-responsive cells. Some neurotrophin-containing cells may express only one member of the Trk family whereas others may colocalize $p75^{NGFR}$ as well as one of the Trk receptors. Preliminary evidence (unpublished observations) does support the suggestion that $p75^{NGFR}$ mRNA is colocalized to subsets of TrkA- and TrkB-containing neurons in the developing forebrain. The resulting permutations may lead to cell-specific patterns of neuronal differentiation, even within local regions.

Not every neuron expressing neurotrophin receptor mRNA expressed the mRNA for its cognate ligand(s). Such interneuronal variability indicates that any autocrine mechanism may be cell-, region-, and temporally specific. The distinguishing phenotype(s) of neurons coexpressing ligand and receptor mRNAs is as yet little explored. However, recent work in our laboratory (20, 22, 33) suggests that during restricted developmental stages, one common phenotype of these neurons may be a responsivity to estrogen. Preliminary evidence (45) suggests that estrogen does regulate $p75^{NGFR}$ and TrkA mRNA expression in peripheral neurotrophin targets. Alternatively, it may be that as development proceeds, fewer neurons coexpress neurotrophin receptor and ligand and that no distinguishing phenotype may be associated with those remaining. Indeed, there is some evidence in support of this hypothesis. For example, early in the first postnatal week, virtually all cortical neurons expressing $p75^{NGFR}$ mRNA also appeared to express NGF mRNA (unpublished observations). During the second postnatal week, however, this overlap appeared to be no longer as complete. Perhaps, as differentiation proceeds, fewer neurons remain dependent on local or intracellular availability of neurotrophins for the initiation of differentiation or even survival.

The regional distribution of neurotrophin mRNAs suggests that certain neuronal target populations may express more than one neurotrophin (8, 17–19). This does indeed appear to be the case. The cerebral cortex and hippocampus, for example, expressed NT-3 mRNA in both BDNF and NGF

mRNA-containing neurons. While the functional role of ligand colocalization remains to be investigated, TrkA and TrkB have been reported to bind NT-3 (with a lower affinity) as well as their specific ligands, NGF and BDNF, respectively (2). The existence of two or more members of the neurotrophin family within a single cell may be indicative of stage-specific roles for different neurotrophins, such as early maturation versus later neuronal survival, for example (12).

The local availability of ligand for receptor occupation may be a generalized phenomenon of the developing nervous system. Autocrine/paracrine interactions may permit differentiation and cell survival prior to synaptic maturation. Subsequently, target-derived factors may ensure continued differentiation and survival of neurons that successfully establish appropriate functional connections. For other cells, this relationship may persist, or a temporal decline in the local availability of ligand—and, therefore, receptor occupation—may signal the onset of events leading to cell death, unless sensitivity and dependence are switched to another class of factors.

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