Gene Expression, Vol. 14, pp. 241–250
Printed in the USA.

1052-2166/07 $90.00 + .00
E-ISSN 1555-3884
www.cognizantcommunication.com

REVIEW

The Role of Neurotrophins During Early Development

PAULETTE BERND

Department of Anatomy and Cell Biology and Department of Otolaryngology, State University of New York, Brooklyn, NY, USA

The effects of neurotrophins during the middle and late stages of development are well known. It was previously thought that neurotrophins had no role during early development, but this is not the case and is the subject of this review article. The earliest neurotrophin receptor expressed is that for neurotrophin-3 (NT-3). TrkC is detected in the neural plate and is present in the neural tube. Initially, the distribution of TrkC is homogenous, but it becomes localized to specific regions of the neural tube as the neural tube differentiates. The receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), TrkB, is detected somewhat later than TrkC in the neural tube where it is also differentially localized. In contrast, the NGF receptor, TrkA, was not detected during early development. Both NT-3 and BDNF have been shown to have effects in vitro during early development. NT-3 caused an increase in neurite outgrowth and apoptosis in neural plate explants, and promoted differentiation of progenitors into motoneurons. BDNF increased the number of motoneurons in neural tube explants. These data suggest that NT-3 and BDNF may play a role during early development in vivo.

Key words: Neurotrophin; Trk; p75; Nerve growth factor; Brain-derived neurotrophic factor; Neurulation; Neural plate; Neural tube; Rhombomere;

INTRODUCTION

Following the discovery of nerve growth factor (NGF) (54), the prevailing belief was that it was an important survival and differentiation factor during the middle and later stages of neural development, but had no role during early development. For example, dorsal root ganglia and neural crest-derived cranial sensory ganglia were considered unresponsive to NGF prior to embryonic day 4 or 5 (E4 or E5) in the chick because NGF had no effect on neurite outgrowth from explanted ganglia (14). Likewise, cultured dorsal root ganglia neuronal precursor cells were found to survive in the absence of NGF (21).

NGF is a member of a family of neurotrophins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (5). Data suggest that some members of this family, in addition to NGF, do not have differentiation or survival effects on early neurons. Ernsberger and Rohrer (21) found that BDNF had no effect on neurite outgrowth or survival of dorsal root ganglia neuronal precursor cells, while Ockel et al. (63) reported paradoxically that increased levels of NT-3 in dorsal root ganglia undergoing formation resulted in a decrease, rather than an increase, in the number of neurons.

It has become increasingly apparent, however, that the nervous system is responsive to neurotrophins during its early development, with NT-3 and BDNF as the most effective. The work described in this review will focus on avian embryos because that model is most amenable to research on early development,

Address correspondence to Paulette Bernd, at her present address: Professor of Clinical Pathology & Cell Biology, Columbia University Physicians and Surgeons, 630 West 168 Street, New York, NY 10032, USA. Tel: (212) 305-7572; Fax: (212) 305-6595; E-mail: pb106@columbia.edu

but a discussion of neurotrophin knockout mice is included.

NEUROTROPHIN RECEPTORS

Potentially responsive cells are identified by the expression of receptors. The *trk*A proto-oncogene product (also known as *trk*) is a functional receptor for NGF (33,44), the *trk*B proto-oncogene product is a functional receptor for BDNF and NT-4/5 (29,45, 46,75,77), and the *trk*C proto-oncogene product is a functional receptor for NT-3 (51). The low-affinity p75 receptor acts as a coreceptor during Trk signaling (33).

Minute amounts of all neurotrophin receptors, TrkA, TrkB, TrkC, and p75, have been detected throughout early chicken development from the prestreak stage (stage 1) onward using RT-PCR (86,90). This technique is extremely sensitive and may detect minute amounts of neurotrophin receptors that do not play a physiological role. Furthermore, RT-PCR does not localize the source of the neurotrophin receptors. A less sensitive technique, in situ hybridization, is able to detect and localize *trk* mRNA during early development but results vary considerably from those of RT-PCR; expression of some Trks occurs later during early development while others are not detected at all during early development. This suggests that the temporal sequence detected by in situ hybridization may be a more accurate assessment of the functionality of neurotrophins during early development. Therefore, in situ hybridization was used to determine the distribution of neurotrophins and their receptors in avian embryos from prestreak stages to the forming neural tube.

EXPRESSION PATTERN OF TrkC DURING EARLY DEVELOPMENT

TrkC, NT-3's receptor, is the neurotrophin receptor expressed earliest during chicken development because it appears at the start of neurulation. No *trk*C mRNA was found in prestreak chicken embryos (stage 1) using in situ hybridization. However, *trk*C mRNA was localized in the chicken embryo at stage 4 anterior to Hensen's node and lateral to the notochordal process; this location is the beginning of neural plate formation (4) (Fig. 1A). The staining was restricted to the epiblast layer. Expression of *trk*C mRNA was found in the neural plate throughout its presence in the embryo (4).

As the neural plate begins to invaginate, initiating the formation of the neural tube (stages 6 to 8), *trk*C mRNA was found to be restricted to the neural groove and neural folds, with the exception of the midline; no staining was seen in the underlying mesoderm or endoderm, adjacent ectoderm, or notochord (4,39) (Figs. 1B and 2A). As neurulation proceeds, *trk*C mRNA became differentially expressed in the neural tube. By stage 11, areas of the neural primordium were devoid of *trk*C mRNA; the anterior prosencephalon still expressed *trk*C mRNA, but the remainder of the prosencephalon, the entire mesencephalon, and part of the rhombencephalon (rhombomeres 1 and 4) were negative (4) (Fig. 1C). In areas expressing *trk*C mRNA, cross-sectional analysis revealed a disparity in staining between the dorsal and ventral regions of the neural tube, with *trk*C mRNA expression being considerably higher in the dorsal neural tube (4,39) (Fig. 2B). By stage 15, only the hindbrain exhibited *trk*C mRNA expression and was limited to rhombomeres 3 and 5 (4) (Fig. 1D). Kahane and Kalcheim (39) described the presence of *trk*C mRNA-positive postmitotic neurons on the peripheral (mantle) layer of the neural tube beginning at stage 16 and becoming dense by stage 18 (Fig. 2C). Unlike earlier stages, the expression of *trk*C mRNA in the peripheral (mantle) layer is present along the dorso-ventral extent of the neural tube (39,84), although Williams et al. (84) describe the labeling as being more intense ventrally, while Kahane and Kalcheim (39) found it to be homogeneous.

Therefore, TrkC is present in the anlage of the neural tube and the neural plate, as well as in the neural tube. This receptor is initially expressed homogeneously but becomes heterogeneous as the neural tube differentiates. The restriction of *trk*C mRNA to rhombomeres 3 and 5 in the hindbrain is striking because there is segmental migration of the chicken hindbrain neural crest, resulting in a lack of neural crest cells in the mesenchyme lateral to rhombomeres 3 and 5 (35,57,72). While there is no disagreement that neural crest cells are generated from rhombomeres 3 and 5, two divergent explanations have been given for the apparent lack of neural crest cells lateral to these rhombomeres. The first is that neural crest cells generated from rhombomeres 3 and 5 die and fail to migrate, as evidenced by increased levels of apoptosis in the dorsal midline over rhombomeres 3 and 5 (35,57). The second explanation is that neural crest cells migrate from rhombomeres 3 and 5 but deviate rostrally and caudally as they do so, thereby failing to enter the regions adjacent to those rhombomeres (72). It is intriguing to speculate that the persistent expression of *trk*C mRNA in rhombomeres 3 and 5 is also somehow related to the segmental migration of hindbrain neural crest cells. This seem plausible because the formation and migration of neural crest cells generated from rhombomeres 3 and 5 occurs between stages 9 and 11 (35,57), coincidental with the

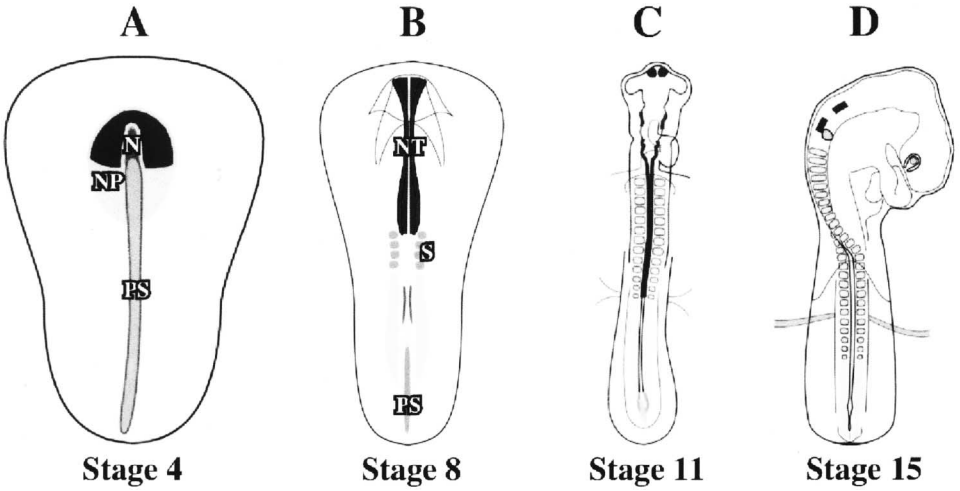

Figure 1. Rostro-caudal expression of *trk*C mRNA in the chicken embryo. At stage 4 (A), *trk*C mRNA expression (indicated by red) is limited to the anterior portion of the neural plate (NP); there is no staining of the primitive streak (PS) or notochordal process (N). *trk*C mRNA is found along the length of the forming neural tube (NT) at stage 8 (B), while at stage 11 (C) it becomes restricted to the anterior prosencephalon, the hindbrain (except for rhombomeres 1 and 4), and the remainder of the neural tube adjacent to somites. By stage 15 (D), *trk*C mRNA is only detected in rhombomeres 3 and 5.

shift from homogeneous to differential expression of *trk*C mRNA.

EXPRESSION OF TrkA AND TrkB DURING EARLY DEVELOPMENT

TrkB (BDNF and NT-4/5's receptor) appears later in early development than TrkC. Unlike TrkC, *trk*B mRNA was not detected in the neural plate of avian embryos using in situ hybridization (90). Jungbluth et al. (37) reported that mRNA for TrkB was first detectable in the chicken hindbrain in the ventral part of rhombomere 2 as well as rostrally and caudally to a lesser degree (with the exception of the floor plate) starting weakly at stage 9 and becoming more pronounced by stage 10 (Fig. 3A). By stage 12, *trk*B

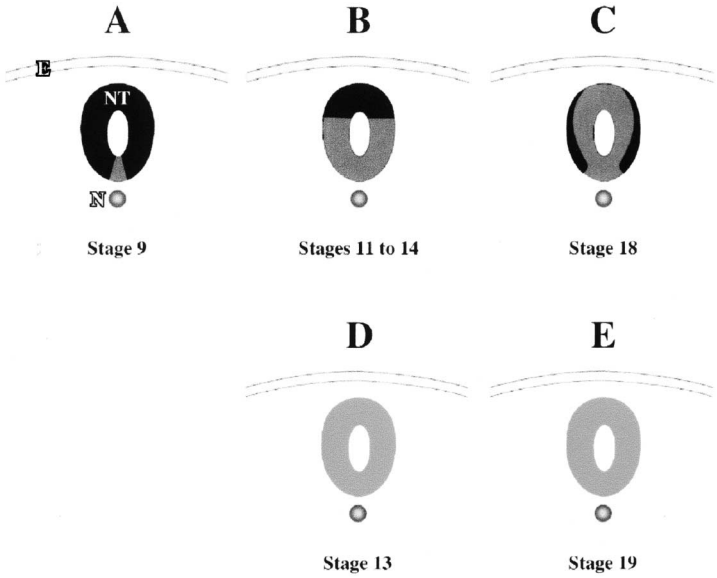

Figure 2. Cross-sectional expression of *trk*C mRNA and NT-3-like immunoreactivity in the chicken embryo. At stage 9 (A), *trk*C mRNA expression (indicated by red) is located throughout the forming neural tube (NT) with the exception of the midline; there is no staining of the ectoderm (E) or notochord (N). From stages 11 through 14 (B), *trk*C mRNA expression is higher in the dorsal neural tube while at stage 18 (C) it is expressed in the peripheral (mantle) layer. At stages 13 (D) and 19 (E), NT-3-like immunoreactivity (indicated by blue) is located throughout the forming neural tube.

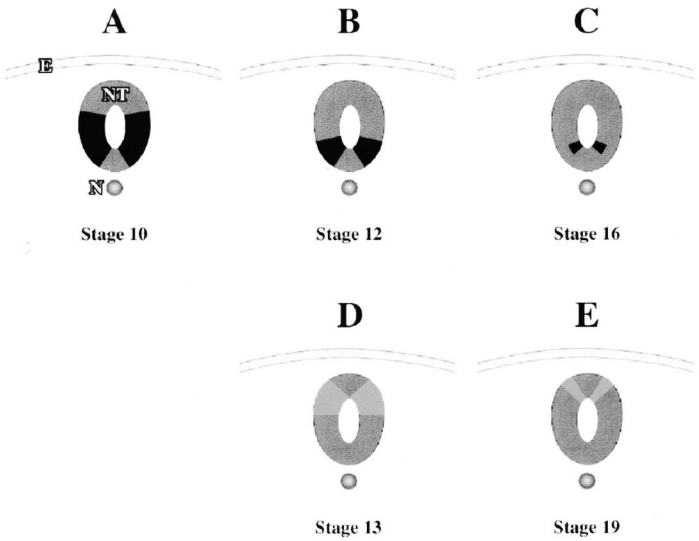

Figure 3. Cross-sectional expression of *trk*B and BDNF mRNA in the chicken embryo. At stage 10 (A), *trk*B mRNA expression (indicated by red) is located in the ventral region of the neural tube (NT) with the exception of the floor plate; there is no mRNA detected in the ectoderm (E) or notochord (N). Its localization becomes more restricted by stage 12 (B) and is limited to the ventricular zone by stage 16 (C). At stages 13 (D) and 19 (E), BDNF mRNA (indicated by blue) is limited to the dorsal part of the neural tube with the exception of the dorsal-most region.

mRNA was detected as stripes on either side of the floor plate starting in the caudal midbrain and extending through the hindbrain into the spinal cord (37) (Fig. 3B); later in development (stage 16) this staining was limited to the ventricular zone (37) (Fig. 3C). The *trk*B mRNA-expressing cells were shown to be motor neuron progenitors (37).

In contrast, the NGF receptor, TrkA, was not detected during early development. *trk*A mRNA was not detected in the neural plate (90) and neither the neural tube nor migrating neural crest cells exhibited NGF binding at stage 19 (76). *trk*A mRNA was first found in developing sensory and sympathetic ganglia; none was found in migrating neural crest cells or dorsal root ganglia until stage 30 (E6) (89). Therefore, it is unlikely that NGF plays a role in early development.

Trk ISOFORMS

In situ hybridization can be used to localize mRNA, but the size of the mRNA cannot be determined unless carefully designed riboprobes are used. Knowledge of the size is important because both full-length (catalytic) isoforms and truncated (noncatalytic) isoforms of TrkB and TrkC have been described in the mouse, rat, and chicken (28,43,60,64). Noncatalytic forms lack either the entire tyrosine kinase domain, or part of it, and appear to be incapable of signal transduction. Their role is unclear.

There is evidence that full-length (catalytic) forms of TrkB and TrkC are present during early development because riboprobes used in in situ hybridization only spanned the tyrosine kinase domain, or expression patterns were the same whether the riboprobe used spanned either the tyrosine kinase or extracellular domains (4,37,55,90). Furthermore, the *trk*C mRNA detected at an early time of development (stages 3 and 6) appears to be translated into functional cell surface receptors as immunoblotting has demonstrated the presence of full-length Trk protein (90) and neurotrophins can induce phosphorylation of Trk on tyrosine residues at a somewhat later time in development (stage 11) (90). This indicates that NT-3, BDNF, and NT-4/5 could have effects during early development because there are full-length Trk receptors present.

EXPRESSION PATTERN OF NEUROTROPHINS DURING EARLY DEVELOPMENT

As described above, evidence from many laboratories indicates that TrkC and TrkB receptors are present during early development. In order to be physiologically relevant, the ligands NT-3 and BDNF or NT-4/5 must be present in the same region or in close proximity to cells expressing TrkC and TrkB. As with receptor expression, the more sensitive RT-PCR technique detected both NT-3 and BDNF mRNA in the embryo as it begins to undergo neurulation (86). However, using the less sensitive in situ hybrid-

ization technique, both NT-3-like immunoreactivity and BDNF mRNA were first detected in the neural tube towards the end of neurulation at approximately stage 13 and the two neurotrophins exhibited different expression patterns (37,67). NT-3-like immunoreactivity was expressed throughout the cross-sectional area of the neural tube, a pattern that persisted through stage 19 (67) (Fig. 2D and E), while BDNF mRNA was restricted to the dorsal part of the neural tube, particularly at older stages, with the exception of the dorsal-most region (37,40) (Fig. 3D and E). In contrast to NT-3 and *trk*C, it is striking that BDNF and *trk*B mRNA are localized in different regions of the neural tube with no apparent areas of overlap (Fig. 3). It is possible that BDNF released in the dorsal part of the neural tube could diffuse and bind to receptors in the ventral half, but this remains to be shown. Given the RT-PCR data, neurotrophins may be expressed earlier in neurulation, but are below the limit of resolution using anatomical techniques.

EFFECTS OF NT-3 AND BDNF DURING EARLY DEVELOPMENT

Both TrkC and TrkB full-length receptors and their ligands are expressed during early development. Evidence is accumulating that this expression is functional and that neurotrophins do have effects on the developing nervous system.

TrkC appears to be the only neurotrophin receptor expressed at significant levels in the neural plate during early neurulation. This suggests that NT-3 is the most likely neurotrophin to influence the cells of the neural plate in vivo, because NT-3 is the only member of the NGF family of neurotrophins that is a ligand for TrkC (45,51). This is in contrast to TrkA and TrkB, which serve as receptors for multiple neurotrophins; NGF, NT-3, and NT-4/5 bind to TrkA (3,12,33,34,44), while BDNF, NT-3, and NT-4/5 bind to TrkB (29,45,46,75,77).

It was shown that in the presence of exogenous NT-3, chicken neural plate explants exhibited a dose-dependent statistically significant increase in the total number of neurites compared to explants maintained under control conditions, as well as a statistically significant increase in the amount of apoptosis (55). An NT-3 antibody blocked these NT-3 effects. In contrast, NT-3 had no effect on the length of neurites.

Both NT-3 and BDNF have been shown to have effects on the neural tube in vitro. Exposure of dissociated neural tube cells acquired from stage 12–13 quail embryos to NT-3 promoted the differentiation of progenitors into motoneurons (1). There was no effect on the overall number of cells or the proportion of motoneurons; NT-3 did not appear to be a survival factor for motoneurons during early development. In contrast, exposure of chicken ventral neural tube explants to BDNF resulted in an increase the number of motoneurons (37). This study did not determine whether this increase was due to an increase in proliferation or an increase in survival.

EFFECTS OF NT-3 AND BDNF ON APOPTOSIS

The phenomenon of programmed cell death of neurons in the peripheral and central nervous system during development is well known (32), but extensive cell degeneration also occurs in the neural plate during neurulation (30,87). The increase in apoptosis by NT-3 (55) was unexpected because neurotrophins are usually classified as neuronal survival factors. This survival effect could be a result of several mechanisms such as increasing proliferation or decreasing apoptosis, and several studies have shown that neurotrophins increase survival of developing neurons by specifically preventing apoptosis (23,38,50,69,78). It has been shown, however, that early embryonic application of NT-3 in ovo results in a marked decrease in neuronal numbers in dorsal root and nodose ganglia of the chicken (63), and treatment with NGF in vitro can induce apoptosis under certain circumstances in the hippocampus, otic vesicle, retina, as well as in glial cells (7,9,24,26,27,81). BDNF has also been shown to induce neuronal apoptosis in the superior cervical ganglion in vitro (2). Metabolic precursors of neurotrophins, proneurotrophins, can also induce apoptosis in motoneurons and smooth muscle (16,53,62,79,82). Therefore, the effect of NT-3 and BDNF in vivo may in fact be due to pro-NT-3 and pro-BDNF.

It appears that the apoptotic effects of neurotrophins and proneurotrophins are mediated by the p75 receptor (6). There is controversy as to whether neurotrophin binding or the extracellular domain of p75 are necessary (23,42,59,69); however, there is consensus that p75's effects on apoptosis are mediated by ceramide and subsequent activation of JNK, p53, and some caspases (7,27,66,68,80). Proneurotrophins bind to p75 with high affinity and, unlike mature neurotrophins, bind weakly to Trk receptors (53). The p75 coreceptor, sortilin, is required for proneurotrophins to induce apoptosis (16,62,79,82). The role of p75 in apoptosis is further supported by targeted deletion of p75 in which mice exhibited increased numbers of sympathetic neurons, putative motoneurons, and cells in the retina (2,25).

NEUROTROPHIN KNOCKOUTS

It is intriguing to speculate that neurotrophins or proneurotrophins may play a role in early development in vivo. Knockout mice provide an excellent opportunity to address that question and one might be tempted to discount this hypothesis in view of the data involving targeted deletion of either neurotrophin receptor or neurotrophin genes (11,13,18–20,22,47,48,52,56,74). In all cases, the mice were born undergoing neurulation and early development of the nervous system; however, in many cases they exhibited profound neurological defects.

Animals with a targeted deletion of NT-3 or TrkC exhibited severe movement and postural defects; their muscles lacked spindles and Golgi tendon organs and Ia afferents (20,22,47). Furthermore, there was a loss of neurons in sympathetic, myenteric, and submucosal ganglia (10,20,22), as well as a loss of sensory neurons in the dorsal root, trigeminal, nodose/petrosal, cochlear (spiral) and geniculate ganglia, and the mesencephalic nucleus of the trigeminal nerve (18, 20,22). More subtle neuronal effects included a change in the morphology of mossy fiber terminals in the hippocampus (65). In addition, effects were also seen in the glia of these knockout animals; there are fewer oligodendrocyte precursors as well as fewer astrocytes and activated microglia (41).

BDNF and TrkB knockout animals had difficulties with coordination and balance, and a loss of sensory neurons in the dorsal root, trigeminal, nodose/petrosal, vestibular and geniculate ganglia, and the mesencephalic nucleus of the trigeminal nerve (11,18,19). Other sensory deficits included the absence of Meissner's corpuscles in hairless skin (31). The coordination defects appear to stem from improper pruning of climbing fiber-Purkinje cell synapses in the developing cerebellum (36). Similar to the NT-3 and TrkC knockouts, these animals also exhibited changes in the morphology of neurons in the hippocampus (65, 83), as well as electrophysiological changes (8). There were also defects in the development of the somatosensory and piriform cortices (58,61). Interestingly, animals with targeted deletions of NT-4 did not exhibit any neurological defects (11,31,56,58).

Unlike animals with targeted deletions of NT-3, TrkC, BDNF, or TrkB, those with targeted deletions of p75 were long-lived and exhibited a milder neurological phenotype. However, the adults exhibited sensory deficits such as hearing loss and a decrease in taste buds (49,71) and the hippocampus exhibited morphological and electrophysiological changes (70, 85,88). Normal oligodendroglial differentiation has also been shown to be dependent upon p75 (17).

Therefore, both the peripheral and central nervous systems are affected by the targeted deletion of NT-3, TrkC, BDNF, TrkB, or p75. The window of vulnerability of these neurons remains to be determined. Given the spectrum of neurons involved, one possibility is that the deficit is upon common precursors of these neurons, such as those in the neural tube. The expression pattern of neurotrophins and neurotrophin receptors in avians suggests that this is plausible. However, it is not known if deletion of neurotrophins or neurotrophin receptor genes would have similar effects on avians. Their pattern of expression may not be the same in avian and mouse embryos so one must cautiously evaluate data from two species.

CONCLUSIONS

The fact that mice with a *trk* or neurotrophin deletion still undergo neurulation and early development does not rule out a role for neurotrophins in this process. Evidence described above suggests that NT-3 is required for initial neurite outgrowth from at least some cells in the neural plate, regulates the number of surviving cells and promotes the differentiation of motoneurons, while BDNF promotes survival and differentiation of motoneurons. Therefore, depletion of NT-3 or BDNF could result in a delay in neurite outgrowth or an excess number of neural plate cells as well as fewer motoneurons, all of which might in turn affect development of the peripheral or central nervous systems. For example, cells destined for a neuronal fate may fail to differentiate; however, the effects of such changes are compatible with further development of the embryo. An alternative explanation is that compensatory mechanisms come into play whereby gene products that do not normally function in neurulation and early development now do so. Finally, it is possible that there is sufficient redundancy so that a targeted deletion in only one neurotrophin or its receptor is not sufficient to interfere with these early processes, although this does not appear to be the case because animals with a targeted deletion of both TrkC and TrkB or NT-3 and BDNF undergo neurulation and are born (15,18,73).

The experiments described above were done in tissue culture and it remains to be determined whether NT-3 and BDNF have similar effects in ovo or in vivo. Negative in situ hybridization data suggest it is questionable if there are sufficient levels of NT-3 present in avian embryos at the beginning of neurulation to affect the neural plate. In contrast, both RT-PCR and in situ hybridization data indicate the pres-

ence of NT-3 and BDNF at the time of motoneuron differentiation. Assuming NT-3 and BDNF have access to developing motoneurons, it seems likely that they would have an effect in ovo. Future experiments in ovo with avians or in vivo with mouse embryos will help us to understand the putative role of neurotrophins during early development.

ACKNOWLEDGMENTS

I am grateful to my former graduate students, Drs. Joycelyn Speight, Lihua Yao, Damin Zhang, and Rong Li, for their scientific contributions to some of the work described in this review. Ms. Inna Rozenberg provided valuable technical assistance.

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