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Neuronal Heterotopias in the Developing Cerebral Cortex Produced by Neurotrophin-4

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Summary

The marginal zone (MZ) of embryonic neocortex is crucial to its normal development. We report that neurotrophin-4 (but not NT3 or NGF), applied to embryonic rodent cortex in vitro or in vivo, produces heterotopic accumulations of neurons in the MZ. Although heterotopia production is TrkB mediated, BDNF is >10-fold less effective than NT4. Heterotopic neurons have the same birth date and phenotype as normal MZ neurons; they are not the result of NT4-induced proliferation or rescue from apoptosis. We suggest that NT4 causes excess neurons to migrate into the MZ and thus may play a role in normal MZ formation as well as in the pathogenesis of certain human cortical dysplasias.

Introduction

Development of the six-layered neocortex depends on a series of precisely timed proliferative, migratory, and maturational events (Rakic, 1975; Allendoerfer and Shatz, 1994; McConnell, 1995). Neocortical cells arise from a proliferative neuroepithelium, the ventricular zone (VZ), located in the wall of the cerebral ventricle (Rakic, 1975). The first postmitotic neurons to appear in the neocortex form a subpial layer, the preplate. Early cortical plate (CP) neurons migrate into the preplate, splitting it into the marginal zone (Layer I) above the CP, and the subplate below (Marin-Padilla, 1971; Luskin and Shatz, 1985). Layers VI–II form within the CP in an inside-out fashion as laminar cohorts, born during restricted gestational periods, that migrate past the deeper cells to more superficial positions (Angevine and Sidman, 1961; Rakic et al., 1974; Caviness, 1982; Bayer et al., 1991). Upon arrival at their destinations in the preplate or CP, neurons differentiate, extending dendritic processes and axons to appropriate targets (O'Leary et al., 1990). Ultimately, a subset of cortical neurons, including a large proportion of those in the marginal zone, undergoes naturally occurring cell death (Finlay and Slattery, 1983; Derer and Derer, 1990; Gadisseux et al., 1992).

The embryonic marginal zone (MZ) is a complex network of neurons, neuronal and radial glial processes, and extracellular matrix (Marin-Padilla, 1971; Pearlman and Sheppard, 1996) that is essential for the orderly formation of subsequent cortical layers (Caviness, 1982). While the neocortical VZ is generally considered to be the source of MZ neurons, recent observations in rodent and man indicate that at least some MZ neurons may arise in subventricular zones in the lateral ganglionic eminence (Anderson et al., 1996, Soc. Neurosci. abstract; De Carlos et al., 1996) and just behind the olfactory bulb (Gadisseux et al., 1992; Meyer et al., 1996, Soc. Neurosci. abstract). These neurons then appear to migrate tangentially, immediately beneath the pia, to reach their destinations in the neocortical MZ. Mitotic figures in or very near the preplate imply that other MZ neurons may be generated locally (Valverde et al., 1995).

Increasing evidence indicates that members of the NGF family of neurotrophins play significant roles in neocortical development. Messenger RNA for NT3 and for its high affinity receptor, TrkC, are present early in cortical development during periods of peak neuroblast proliferation (Maisonpierre et al., 1990; Lamballe et al., 1994), and TrkC expression persists in a distinct laminar pattern in adults (Lamballe et al., 1994). Conversely, BDNF, initially present at low levels, increases as proliferation ceases and cortical differentiation occurs (Maisonpierre et al., 1990). TrkB, the receptor for NT4 and BDNF, is present at high levels during corticogenesis (Klein et al., 1990b); receptor expression is prominent in subsets of cortical neurons and their processes (Cabelli et al., 1996) but is not layer specific (Pearlman et al., 1995, Soc. Neurosci. abstract). Rather, TrkB expression undergoes developmental shifts from full-length to truncated forms in the VZ, intermediate zone, and cortical plate that coincide with the end of neurogenesis and the formation of mature axonal connections (Allendoerfer et al., 1994). Binding of neurotrophins to their receptors and measurements of receptor phosphorylation follow a similar developmental pattern (Allendoerfer et al., 1994; Escandon et al., 1994; Knusel et al., 1994).

Neurotrophins function in a variety of roles throughout the peripheral nervous system (reviewed by Davies, 1994; Snider, 1994). In the central nervous system, neurotrophins promote the differentiation and survival of several neuronal populations including striatal neurons (Ardelt et al., 1994) and cerebellar granule cells (Lindholm et al., 1993). In the neocortex, in vitro evidence suggests that NT3 may regulate the exit of neurons from the cell cycle (Ghosh and Greenberg, 1995), while NT4 and BDNF modulate dendritic differentiation (McAllister et al., 1995). In vivo, exogenous application of NT4 or BDNF to visual cortex prevents the normal formation of ocular dominance columns (Cabelli et al., 1995).

Our work has focused on defining functional roles for neurotrophins during early cortical development. We applied neurotrophins to an organotypic slice preparation (Stoppini et al., 1991; Sheppard et al., 1995) of early neocortex of embryonic mouse. In our slice cultures, tissue cytoarchitecture is highly preserved for several days (Figure 1) and neocortical development, including cell proliferation and migration, occurs in a manner quite comparable to development in vivo (J. E. B., and A. L. P., unpublished data). We also applied neurotrophins to the intact cortex of embryonic rats by intrauterine, intraventricular injections. We find that one member of the neurotrophin family, NT4, produces a dramatic increase in the total number of neurons in the MZ. Excess MZ neurons

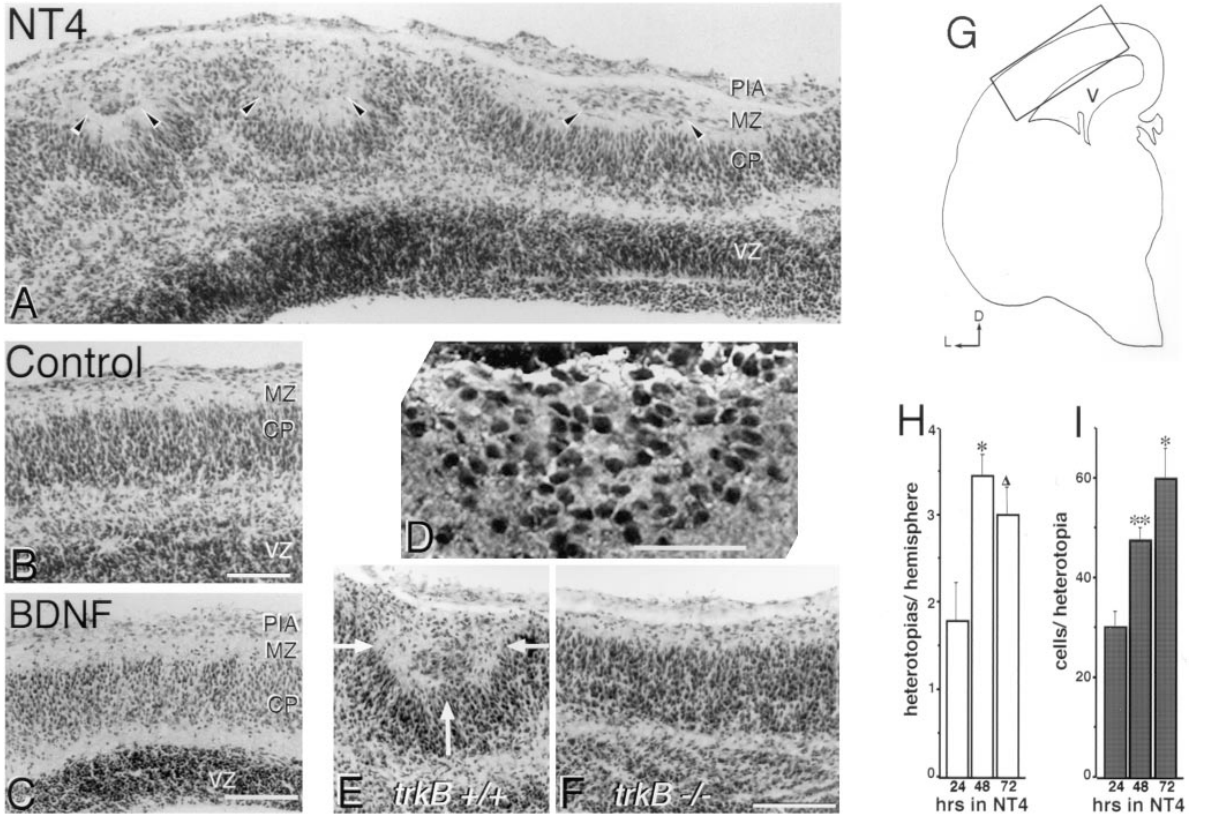


Figure 1. Heterotopic Collections of Neurons Are Produced by NT4 in Organotypic Slices; This Effect Is Mediated by TrkB

(A–C), (E), and (F) Hematoxylin-stained sections from cortical slices (E14 mouse).

(A) After treatment with NT4 (100 ng/ml; 48 hr), collections of cells (heterotopias, arrowheads) in the MZ disrupt the normal cortical architecture.

(B) Heterotopias are not present in control-slices (48 hr) or (C) after BDNF treatment (200 ng/ml; 72 hr).

(D) Laser confocal micrograph of heterotopic cells immunolabeled with an antibody (TuJ1) to a neuron-specific form of β-tubulin.

(E) A heterotopia (arrows) in an NT4-treated slice from a *trkB*$^{+/+}$ mouse.

(F) Heterotopias are not present in NT4-treated slices from *trkB*$^{-/-}$ littermates.

(G) Outline drawing of an s.h. The boxed area represents the region shown in A (V, ventricle; L, lateral; and D, dorsal).

(H) The mean (±SEM) number of heterotopias per s.h. increases from 24–48 hr of NT4 treatment but does not change significantly between 48 and 72 hr.

(I) The mean (±SEM) number of cells/heterotopia increases steadily from 24–72 hr. (H and I) * = $P < .002$; ** = $P < .005$; Δ = $P < .05$; ANOVA, unpaired Student's *t* test comparing means at 48 and 72 hr with 24 hr. Scale bars in (A–C), (E), and (F), 100 μm; (D), 25 μm.

group together to form heterotopic collections that disrupt the underlying cortical architecture. Excess numbers of neurons and neuronal heterotopias in Layer I have been described in humans with primary generalized epilepsy (Meencke and Veith, 1992) and in association with mental retardation (Yamaguchi et al., 1996).

Results

NT4 Produces Heterotopic Collections of Neurons in the Marginal Zone in Organotypic Slices

NT4, BDNF, NT3, NGF, or PBS were applied to cortical slices from embryonic mice (E13.5–E14.5) for 6–72 hr. Microscopic analysis was performed on 18 μm sections taken from the center of each slice. Each slice consists of two hemispheres (slice hemispheres), which are considered independently. A total of 255 slice hemispheres (s.h.) were selected for analysis (see Experimental Procedures).

NT4 produces collections of cells (heterotopias) in the MZ that are identifiable as neurons by immunolabeling for a neuron-specific isoform of β-tubulin (TuJ1; Lee et al., 1990) (Figures 1A and 1D). These heterotopias are evident 20–24 hr after exposure to NT4 (100 ng/ml, 10 of 14 s.h.; Figures 1H and 3A–3D). The number of heterotopias per s.h. increases nearly 2-fold between 24 and 48 hr, then remains relatively constant (Figure 1H); the size of each heterotopia, based on the number of cells per cluster, increases 2-fold between 24 and 72 hr of NT4 treatment (Figure 1I). At 72 hr, heterotopias are largest in lateral neocortex where they contain twice the number of cells as collections located more medially (average number of cells/heterotopia laterally = 87.2 ± 11.4; medially = 40.3 ± 6.9; $P = .0028$). The percentage of MZ cells that are TuJ1 positive is not significantly different after NT4 treatment (95% of 812 cells ± 1.5% SEM), compared with control conditions (94% of 423 cells ± 1.1% SEM). Many of these cells also express microtubule-associated protein 2 (MAP-2; not illustrated), a neuron-specific marker that appears with neuronal maturation (Crandall et al., 1986). However, the

percentage of MZ cells that are MAP-2 positive after 48 hr of NT4 treatment (63.4% ± 3.9% SEM) is lower than in controls (88% ± 2.5% SEM; $P = <.0001$). Taken together, these results demonstrate that during in vitro application of NT4 in early cortical development, there is continuous addition of immature neurons to the MZ and that this accumulation of neurons is greatest in lateral regions of the neocortex.

BDNF Is Much Less Effective than NT4 in Increasing the Number of MZ Neurons

NT4 and BDNF are preferred ligands for the high affinity receptor tyrosine kinase TrkB (Klein et al., 1989, 1991), which also interacts with NT3 (Ip et al., 1993). To determine the relative sensitivity of embryonic neocortex to each of these TrkB ligands, we exposed neocortex to various doses of NT4, BDNF, or NT3 in the slice preparation, where controlled amounts of neurotrophin can be delivered continuously. MZ heterotopias are evident in vitro 48 hr after exposure to doses of NT4 as low as 20 ng/ml (not shown). In contrast, no heterotopias are produced with 10-fold higher doses of BDNF (200 ng/ml) for 72 hr (Figure 1C), although heterotopias are produced with extremely high doses (1 mg/ml; not shown). No heterotopias are evident in slices treated with NT3 (100 ng–2 mg/ml) or NGF (100 ng/ml; not illustrated).

NT4 Effects Are Mediated by TrkB

To demonstrate that the induction of abnormal collections of neurons by NT4 is mediated by TrkB, the high affinity receptor for NT4 and BDNF, we applied NT4 to cortical slices from mice homozygous for a null mutation in the *trkB* gene. These mice do not express TrkB (Klein et al., 1993; Snider, 1994). Heterotopic collections of neurons do not form in cortical slices from *trkB*$^{-/-}$ animals (Figure 1F) but are present in slice cultures from heterozygous (not shown) and wild-type littermates (Figure 1E).

Heterotopic Collections Also Occur after Application of NT4 In Vivo

To determine whether the effects produced by NT4 in the cortical slice cultures represent the effects of NT4 on the developing neocortex in vivo, we used intrauterine, intraventricular injections to deliver neurotrophins to embryonic neocortex (Figure 2). Embryonic rats were used instead of mice because of their larger size. Two consecutive injections (Henderson et al., 1994) (5 μl; 580 μg/ml) of NT4, BDNF, NT3, or PBS were administered at E16 (cortical development at this stage is comparable to E14 mouse) and E18. Embryos were harvested at E19. For each brain (n = 64), consecutive coronal sections (18 μm) were collected from the entire region located between the anterior and posterior poles of the lateral ventricles. Every fourth section (72–80 μm apart) was viewed after hematoxylin staining.

All of the brains treated with NT4 contain striking abnormalities. The most prevalent finding, in 23 of 24 brains, is a dramatic increase in the number of cells throughout the entire MZ. These cells form a dense band over the hemispheres that is thickest in the anterior (not shown) and lateral regions of the neocortex (Figures 2A and 2E). Analysis of additional, sagittally sectioned brains confirmed this distribution, showing that the bands of cells in the MZ extend rostrally to just behind the olfactory bulb and posteriorly to the occipital poles (not shown). Elliptical collections of cells (Figure 2F) are present in the MZ in 25% of NT4-treated brains (6 of 24) in the posterior dorsomedial neocortex. Round collections of cells (Figure 2B) that distort the underlying CP are found in 67% of NT4-treated brains (16 of 24) in the MZ in posteromedial (retrosplenial) cortex.

None of the BDNF-treated brains (n = 17) contain disruptions of the magnitude of those found after administration of NT4. However, subtle, isolated MZ abnormalities are evident in five of the brains, including small patches of increased cells in extreme anterolateral cortex (two of five), a small collection of cells in anteromedial (cingulate) cortex (two of five), and a small collection of cells overlying retrosplenial cortex (one of five). In the remaining BDNF-treated brains (n = 12), as well as in those injected with NT3 (n = 7) or control solutions (n = 18), no abnormalities were noted (Figure 2D) except those due to injury from the injection (Figure 2C).

Ectopic collections of neurons, attributed to a disruption of the external glial-limiting membrane, occur in Layer I in rodents following a postnatal puncture wound (Rosen et al., 1992). In our study, 57 of the 59 embryonic brains that we injected contained one (31 of 59) or two (26 of 59) lesions resembling a puncture-wound ectopia. No lesions were evident in uninjected brains (n = 4). The injury-induced lesions that we observed (Figure 2C) are uniformly associated with a break in the pial surface and consist of a columnar mass of cells that extends up to, and frequently through, the pial opening. In contrast, the accumulations of cells in the MZ produced by NT4 are consistently found under an intact pial membrane and occur at sites distant from the injection. To demonstrate that the abnormalities seen after NT4 exposure are not due to injury produced by the needle during intraventricular injections, we injected a subset (n = 8) of the NT4-treated brains at E16 and E18 in only one hemisphere; in all of them, we found the same dramatic effects of NT4 in the uninjected hemisphere.

NT4 Does Not Induce Neuronal Proliferation in the MZ

Recent observations suggest that proliferation of at least some of the earliest cortical neurons may occur outside the VZ, within the preplate (Valverde et al., 1995). Additionally (in the PNS), neurotrophins induce proliferation in cultured neural crest cells (Kalchiem et al., 1992) and in sympathetic neuroblasts (DiCicco-Bloom et al., 1993). To examine the possibility that NT4 may be mitogenic to MZ neurons, we exposed cortical slices to NT4 (n = 24 s.h.) or control (n = 19 s.h.) for 6–7 or 20–24 hr, then pulse labeled them with bromodeoxyuridine (BrdU) to label cells synthesizing DNA before and during the formation of the earliest heterotopic neuronal collections (Figures 3A and 3B). Additional slices were cultured with BrdU from the outset to label all cells entering S phase within the first 10 hr (n = 5 s.h.) or 24 hr (n = 22 s.h.) of exposure to NT4. Sections from these slices were immunolabeled for BrdU and for another marker of proliferation, cyclin A. Despite normal incorporation of BrdU in endothelial cells and in the VZ (Takahashi et

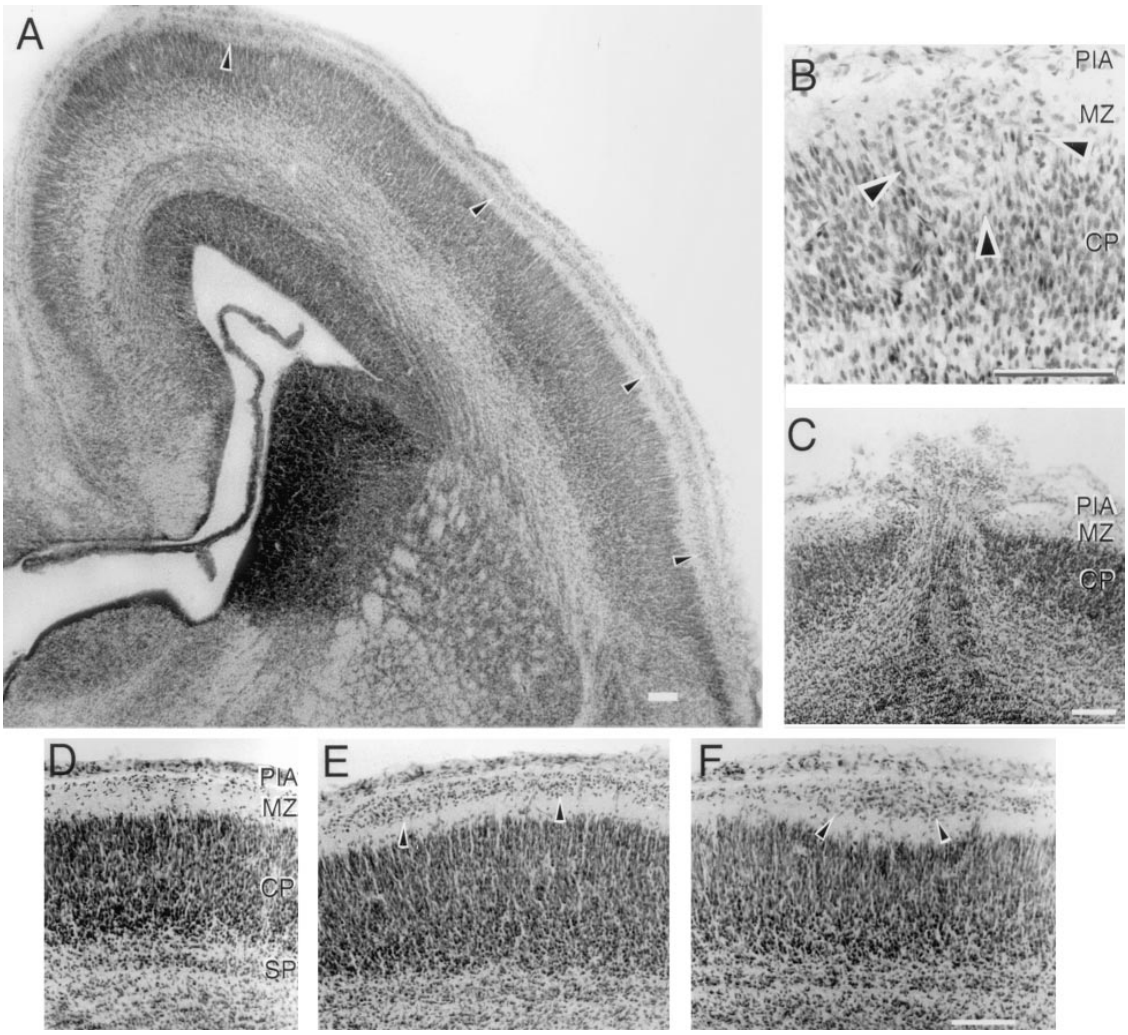


Figure 2. Heterotopic Collections of Neurons Are Produced by NT4 In Vivo

Hematoxylin-stained coronal sections (18 μm) from embryos (E19 rat) injected intraventricularly at E16 and E18 with BDNF (D) or NT4 ([A], [B], [E], and [F]).

(A and E) Increased cells in the MZ after NT4: These cells form a dense band (arrows) that is thickest laterally (to the right) and anteriorly (not shown).

(F) Elliptical collections are evident posteriorly in dorsal cortex, and (B) round collections of cells that indent the underlying CP are found in extreme posteromedial cortex. These are quite different from the (C) puncture-wound ectopias at the injection site in 97% of injected brains. CP, cortical plate; MZ, marginal zone; and SP, subplate. Scale bars, 100 μM.

al., 1992), cells in the MZ are BrdU negative, with (Figure 3B) and without added NT4 (not shown) and are negative when immunolabeled for cyclin A (Figures 3C and 3D). Thus, MZ neurons are not proliferating in response to NT4.

Heterotopic Collections of Neurons Are Composed of MZ Neurons

We labeled MZ cells with BrdU at their time of origin to determine whether cells within the NT4-induced collections are generated at the same time as normal MZ cells. In cortical slice cultures (E14 mouse; Figures 4A–4D), a single injection of BrdU (given to the pregnant mouse at E11–E11.5) labels normal MZ cells (Figure 4B) as well as cells within the NT4-induced heterotopias (Figure 4D). Similarly, the excess neurons evident in the MZ in vivo, after intraventricular administration of NT4 in the embryonic rat, are labeled in S phase at the same time as preplate neurons (E13 in the rat; Figures 4I and 4K). TuJ1 immunoreactivity verifies that nearly all of the BrdU-labeled MZ cells are neurons in NT4 (97.5% ± 0.4% SEM) and control conditions (96% ± 1.1% SEM; $P = 0.14$). Most of these BrdU-labeled neurons also immunolabel for MAP-2 (NT4 = 75% ± 3.2% versus controls = 90% ± 2.8% SEM; $P = .012$). However, as demonstrated after NT4 application in vitro, the proportion of immature neurons in the MZ after administration of NT4 in vivo is greater than in controls.

We counted the total number of neurons labeled with BrdU at E13 in the MZ after NT4 application in vivo and

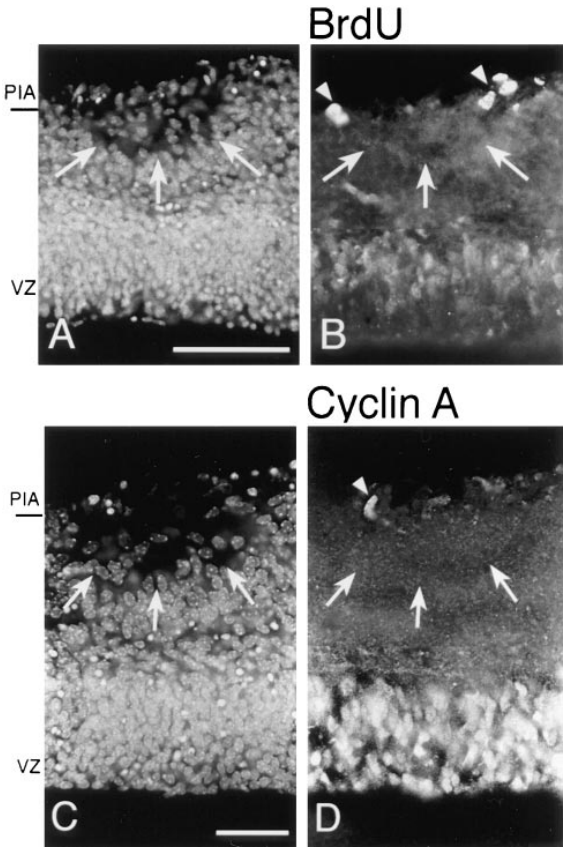


Figure 3. Neurons Are Not Proliferating in the MZ in Response to NT4

Cortical slice cultured for 24 hrs with NT4, then pulsed with BrdU for 4 hr.

(A and B) and (C and D) A pair of adjacent sections immunolabeled for BrdU (B) or cyclin A (D). Normally proliferating cells are labeled in the VZ and pia (arrowheads).

(A and C) Counterstain with bisbenzimide demonstrates small clusters of cells in the MZ (arrows) that indent the CP.

(B and D) These cells are negative for both markers of cell division (arrows). Scale bars in (A) and (B), 100 μm; in (C) and (D), 50 μm.

found a >2-fold increase in the number of preplate-derived neurons throughout the entire MZ (Figure 4E). This NT4-induced increase in MZ neurons is greatest anteriorly (283% of controls; $P = .0002$). Additionally, since the MZ and subplate (SP) layers are derived from a single preplate layer (Marin-Padilla, 1971; Luskin and Shatz, 1985), we also counted the total number of E13 BrdU-labeled cells in the subplate and found no significant difference between NT4 (3247 ± 194 SEM) and control conditions (PBS = 2667 ± 304 SEM; $P = .13$). Therefore, the accumulation of neurons in the MZ after NT4 does not occur at the expense of the SP.

Our in vitro and in vivo cell birth-dating results indicate that NT4 induces an accumulation of excess MZ neurons born at the same time as normal MZ neurons. Neurons within these NT4-induced accumulations also express the calcium-binding protein, calretinin, and are thus phenotypically similar to a major subtype of MZ cells, the Cajal-Retzius (CR) neurons (Figures 5A and 5B) (Ramon y Cajal, 1890; Ogawa et al., 1995).

NT4-Induced Heterotopias Are Not Due to an Abnormal Migration of Cortical Plate Neurons

To determine whether an abnormal migration of early CP cells into the MZ contributes to the accumulation of neurons in the MZ after NT4 treatment, we used BrdU to label layers V and VI as they are being generated in the VZ (Caviness, 1982; Bayer and Altman, 1991). In our slice cultures, BrdU-labeled CP cells migrate to the top of the CP normally in the presence or absence of exogenous NT4 (Figures 5C and 5D) but are not present within the heterotopic collections (asterisk, Figure 5D). However, in areas where the normal architecture of Layer I has been severely disrupted by heterotopia formation, CP neurons appear to move out between the heterotopias, filling the space between CP and pia (arrowhead, Figure 5D; Figure 1A), which is vacated when MZ neurons cluster into abnormal collections.

To determine whether CP neurons contribute substantially to the increased numbers of neurons in the MZ after NT4 administration in vivo, we counted the number of BrdU-labeled cells found in the MZ after intraventricular injections of NT4 or PBS at E16 and E18. In both conditions, a single injection of BrdU at E15 (rat) heavily labels CP cells as well as a cohort of MZ cells, consistent with the overlap in birth dates between cortical layers (Bayer and Altman, 1991). However, the number of E15 BrdU-labeled cells in the MZ is not significantly different between the two conditions (NT4 = 1071 ± SEM 90 versus control = 882 ± 83; $P = .1555$). Thus, NT4 does not cause CP neurons to enter the MZ in vivo or in vitro.

Rescuing Neurons from Cell Death Does Not Induce Heterotopia Formation

In keeping with the classic neurotrophin hypothesis (Levi-Montalcini, 1976), NT4 might produce excess MZ neurons by rescuing cells in the MZ from naturally occurring cell death (apoptosis). We examined this possibility in several ways. First, we used two different methods to detect the cut ends of DNA that occur in apoptotic cells to look for evidence of cell death in the embryonic murine neocortex (E14). We also looked for cell death in cortical slices, cultured in the presence or absence of NT4. Finally, we applied a selective inhibitor of the interleukin-1β converting enzyme (ICE) family of proteases to block cell death in order to determine whether rescue from apoptosis produces accumulations of neurons in the MZ similar to the collections produced by NT4.

We used the Klenow fragment of DNA polymerase I as well as terminal deoxytransferase to label apoptotic cells in frozen tissue sections and observed a similar pattern of end labeling in all conditions. Very rare apoptotic cells are present in the neocortex of E14 mice (not illustrated), and none are evident in the preplate nor the MZ. In contrast, labeled cells are present in the pia, and there are scattered dying cells in the embryonic hippocampus and ganglionic eminence. The same distribution of apoptotic cells is present in sections from lightly fixed cortical slices (E14 mouse) examined at the start of culture experiments (Figures 6A and 6B).

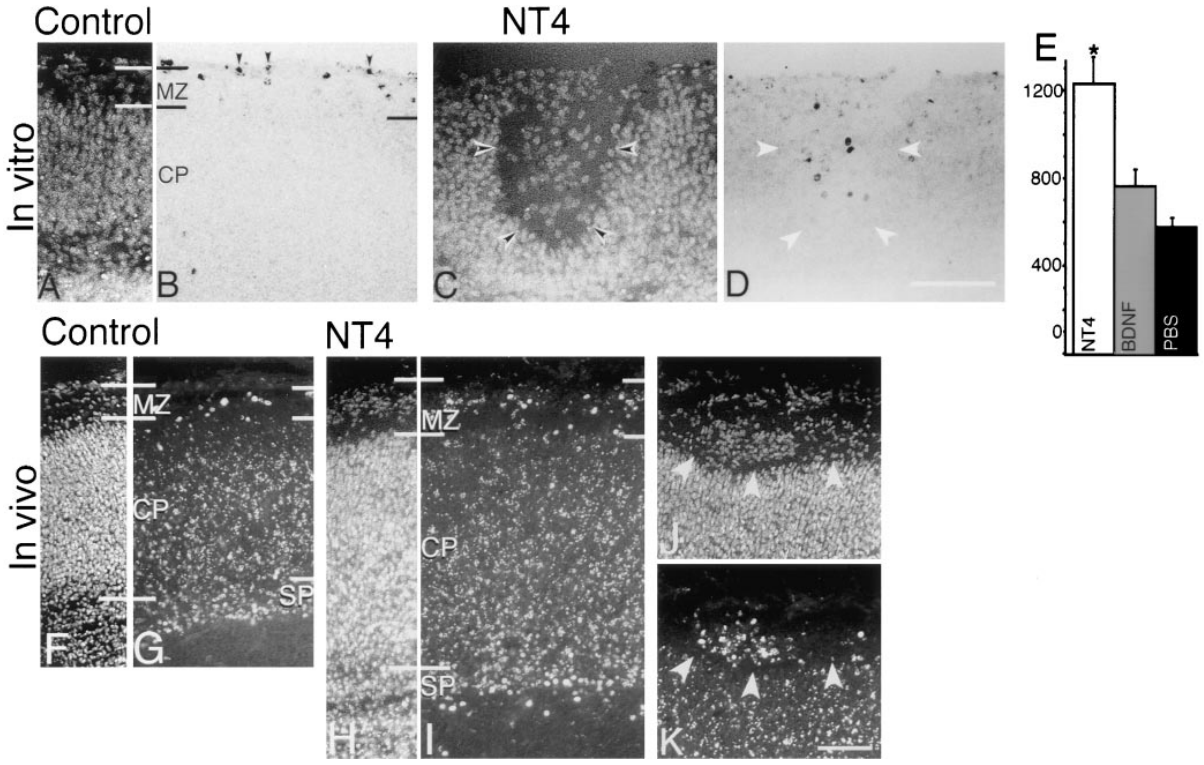


Figure 4. Heterotopic Neurons Are Cogenerated with Preplate Cells

(A–D) In vitro: sections from cortical slices (E14 mouse) cultured 48 hr in control ([A] and [B]) or NT4-containing media ([C] and [D]). An injection of BrdU to the pregnant dam at E11–E11.5 labels normal MZ cells (B) and occasional pial cells ([B], small arrowheads) as well as several cells within the NT4-induced MZ heterotopias (enclosed by arrowheads in [D]).

(A and C) Bisbenzimide counterstain.

(E–J) In vivo: sections from embryonic rat brain (E19) after intraventricular injection of PBS ([F] and [G]) or NT4 (H–K) on E16 and E18, immunolabeled for BrdU ([G], [I], and [K]) and counterstained with bisbenzimide ([F], [H], and [J]).

(E) The total number of labeled cells (mean ± SEM) in the MZ, in vivo after a single BrdU injection (E13 rat), increases >2-fold after NT4 treatment compared to control conditions (*P = .0006) but does not increase significantly after BDNF.

(F) BrdU, administered at the time that preplate cells are generated in the rat (E13), labels neurons within the normal MZ and SP.

(I and K) The same BrdU injection also labels cells in the collections of cells in the MZ of NT4-injected littermates. Scale bars in (A–D) and (F–K), 100 μm.

After 24 (not illustrated) and 48 hr in culture (Figures 6C–6E), end labeling demonstrates dying cells in control slices (arrows, Figures 6C and 6E). Scattered apoptotic cells are evident in the VZ, subventricular zone (SVZ), and intermediate zone, while very few are present in the CP. Numerous labeled cells are present in the pia, but only rarely are labeled cells evident in the MZ. The distribution of apoptotic cells is very similar in slices treated with NT4 (Figures 6H and 6I), suggesting that NT4 does not rescue cortical neurons from apoptosis.

Boc-aspartyl(Ome)-fluoromethlyketone (BAF), an inhibitor of ICE-like proteases including those in the CPP32 family, rescues sympathetic neurons from apoptosis induced by NGF deprivation (Deshmukh et al., 1997). We applied BAF (100 μM) to cortical slice cultures and observed a marked reduction in end-labeled cells in neocortex in control and NT4-treated slices (Figures 6F, 6G, 6J, and 6K), indicating that BAF has rescued these cells from apoptosis. However, rescue by BAF does not produce collections of neurons in the MZ (Figure 6G) and does not impair the formation of NT4-induced collections (Figure 6K). Thus, rescue of MZ cells from death is unlikely to account for the heterotopic collections in the MZ produced by NT4.

Discussion

We used an organotypic slice preparation and intrauterine, intraventricular injections to examine the effects of neurotrophins during early neocortical development. Our results demonstrate a novel effect of NT4 on the developing neocortex. Application of exogenous NT4, in vitro and in vivo, causes an excess number of neurons to accumulate in the MZ; these neurons form abnormal collections (heterotopias) that increase in size and distort the underlying cortical architecture.

The TrkB Receptor Distinguishes between Its Two High Affinity Ligands, NT4 and BDNF

The growth of cortical dendrites is differentially influenced by the two high affinity ligands for TrkB, NT4 and BDNF (McAllister et al., 1995). Our results indicate that a set of cortical neurons distinguishes between these two neurotrophins even at very early stages of cortical

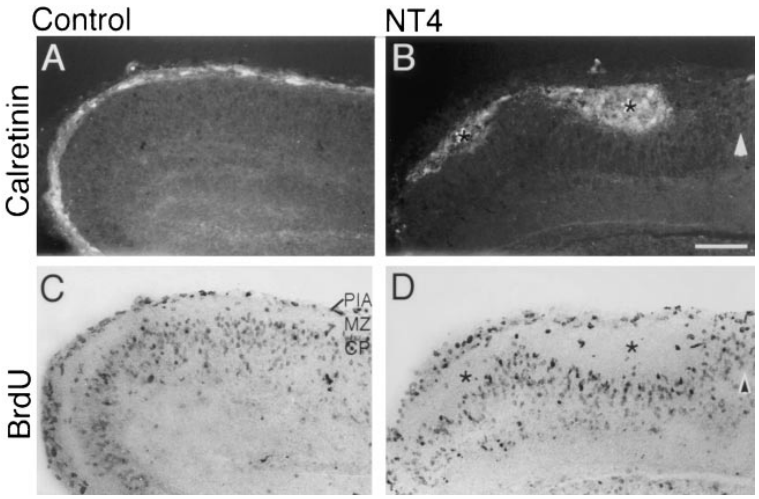


Figure 5. Heterotopic Neurons Are Not Displaced Cortical Plate Cells

(A–D) Cortical slice cultures (E14 mouse) after 48 hr in control ([A] and [C]) or NT4-treated ([B] and [D] conditions; single sections from each slice are shown, double immunolabeled for calretinin and BrdU. Calretinin-positive cells are normally in the MZ in control (A) and BDNF-treated conditions (not shown) and are also found within heterotopic collections after NT4 treatment (B). Early CP cells identified by cell birth dating with BrdU ([C] and [D]) surround the heterotopias but are not present in them (asterisks, [D]). CP cells extend to the pial surface in regions where the MZ has been vacated by heterotopia formation ([B] and [D], arrowhead). In treated and control conditions, occasional BrdU-labeled capillary endothelial cells or late-generated MZ cells whose birth dates overlap with the earliest CP cells are evident in the MZ, and BrdU-labeled cells are also present in the pia. Scale bar (A–D), 100 μm.

development. The induction of heterotopic collections by NT4 is mediated by the TrkB receptor since it does not occur in animals lacking *trkB*, but BDNF is much less effective in producing neuronal accumulations.

Impaired diffusion of BDNF is not likely to account for the differential effectiveness of BDNF and NT4. Although impaired diffusion of BDNF, attributed to TrkB expression in the ependymal layer, occurs in adults after intraventricular injections (Yan et al., 1994), it has not been demonstrated in embryos. The widespread effects of NT4 in vivo in our experiments indicate that NT4, which is closely related in size and structure to BDNF, diffuses throughout the cortex after embryonic intraventricular injection. In addition, we applied BDNF to cortical slices in similar doses and with similar culture methods as those previously used to elicit differential dendritic growth by developing cortical neurons (McAllister et al., 1995). Compared to the slices of ferret cortex used in those studies, our slices of mouse neocortex are much smaller in diameter, nearly 40% thinner, and younger developmentally. These factors should improve rather than impede the diffusion of BDNF.

The differential responsiveness of early cortical neurons to BDNF and NT4 may result from variations in the structure of TrkB. There are several splice variants of TrkB, including variants of the extracellular domain (Garner et al., 1996; Strohmaier et al., 1996) and a truncated form that lacks tyrosine kinase activity (Klein et al., 1990a). Variant forms of avian TrkB respond differentially to BDNF or NT4 (Boeshore et al., 1996, Soc. Neurosci. abstract) but thus far have not been reported in mammals. Alternatively, other receptors, such as the low affinity neurotrophin receptor (p75), which binds all members of the NGF family of neurotrophins, could differentially influence the sensitivity or responsiveness of cells to the two TrkB ligands (Ryden et al., 1995).

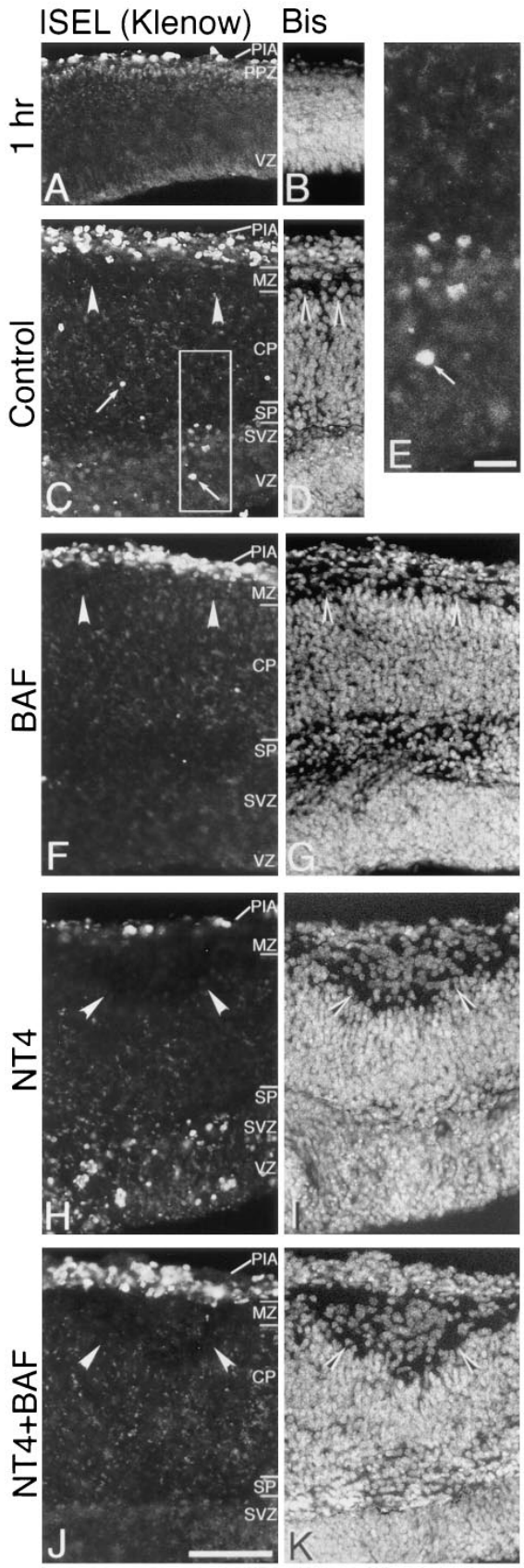
The Pathogenesis of NT4-Induced Heterotopias

We have eliminated several plausible mechanisms by which NT4 could produce accumulations of neurons in the MZ: 1) NT4 does not induce cells to proliferate in the MZ; 2) The collections of neurons in the MZ produced by NT4 are not due to abnormal migration of CP neurons; 3) It is unlikely that NT4 alters the fate of dividing progenitor cells in the neocortical VZ (McConnell, 1995), causing them to generate more MZ cells, since continuous application of BrdU and NT4 to cortical slices for 24 hr (the time required for the first collections to form) fails to label cells within the collections; 4) NT4 does not prevent formation of the subplate or decrease the number of cells within it; 5) Heterotopic neurons in the MZ do not result from the trauma of the intraventricular injection, which produces ectopias with very different morphology (Rosen et al., 1992); and 6) Finally, NT4 is unlikely to be rescuing MZ neurons from apoptotic cell death.

Our end-labeling studies indicate that apoptotic cells in the early embryonic neocortex are very rare, suggesting that there are few if any dying cells for NT4 to rescue. Our findings agree with several prior reports that show dying cells in Layer I postnatally but not during embryonic development (Derer and Derer, 1990; Ferrer et al., 1992; Spreafico et al., 1995). However, these findings have been challenged recently by a modification of terminal transferase–mediated end-labeling (ISEL+) that detects a substantial number of labeled cells (>50%) in embryonic neocortex (Blaschke et al., 1996).

Although the degree to which ISEL+ labeling reflects true apoptosis remains controversial (Takahashi et al., 1996), a role for apoptosis during some stage of cortical development must be reconsidered in light of the finding that the absence of CPP32, a member of the ICE protease family, leads to excess numbers of neurons in the brain (Kuida et al., 1996). The ICE family of proteases is important in the cascade of events leading to apoptosis, including that which occurs in neurons after NGF withdrawal (Deshmukh et al., 1997). Therefore, we used a selective inhibitor of ICE-like proteases (Deshmukh et al., 1997) in the cortical slice preparation to rescue cells from apoptosis and ask whether the rescue of neurotrophin-dependent cells from apoptosis could account for the NT4-induced collections of neurons in the MZ.

Although we find virtually no evidence for apoptosis



in embryonic neocortex or in cortical slices at the start of our experiments, it is evident in slices after 1–2 days in culture. One might expect some degree of inducible cell death in slices as a consequence of trauma, hypoxic–ischemic injury, axotomy, or trophin deprivation in defined media. We were surprised to find that apoptosis is prominent in the proliferative layers of neocortex (VZ–SVZ), either in the presence or absence of NT4, while postmitotic neurons in the CP and MZ are relatively less affected. Nearly all of these dying cells are rescued by BAF, a selective inhibitor of at least two ICE-like proteases including CPP32, but rescue by BAF does not induce the accumulation of neurons in the MZ that occurs with NT4 (Figure 6).

Thus, while we have not excluded the possibility that NT4 could rescue a subset of neocortical cells from death, our evidence indicates that rescue is unlikely to account for the 2-fold increase in MZ neurons produced by NT4. Furthermore, we find a progressive accumulation of postmitotic neurons in the MZ with time in response to NT4 in the slice, demonstrable by an increase in the number of heterotopic collections and the number of neurons in each collection (Figures 1H and 1I). This evidence indicates that neurons are continuously added to the MZ in response to NT4, a phenomenon that cannot be accounted for by rescue of preexisting MZ neurons alone.

What Is the Source of the Excess Neurons That Form NT4-Induced Heterotopias?

Our data support the hypothesis that exogenous NT4 induces an exaggeration of the normal in-migration of MZ neurons: 1) NT4 produces accumulations of neurons that are cogenerated with normal MZ neurons, including

Figure 6. Rescue of Neocortical Cells from Apoptosis Does Not Produce Heterotopias

(A, C, E, F, H, and J) In situ end labeling of apoptotic cells. (B, D, G, I, and K) Bisbenzimide counterstain of the same sections (18 μm).
(A and B) A cortical slice (E14 mouse) after 1 hr in culture. At this stage, the neocortex consists of a VZ and preplate zone (PPZ) dorsally and medially, with a thin CP forming laterally. Although the pia contains many labeled cells, apoptotic cells are extremely rare in the underlying neocortex, consistent with the paucity of labeling found in sections of neocortex from whole brains at the same age (not illustrated).
(C–K) Slices cultured for 48 hr.
(C and D) Control: after 2 days in culture, the CP is much thicker (D) and is present throughout the entire medial–lateral extent of the neocortex; the PPZ has been split into the MZ (arrowheads) and SP.
(C) Scattered apoptotic cells are labeled in the VZ and SVZ, while relatively few are in the overlying CP.
(E) A high power view of the labeled apoptotic cells in the box in (C). Arrows in (C) and (E) point to examples of apoptotic cells.
(F and G) Treatment of slices with BAF (100 μM), an inhibitor of ICE-like proteases, produces a dramatic reduction in dying cells (F) compared with controls (C). However, cells do not accumulate in the MZ in the presence of BAF (G).
(H and I) NT4-treated slices show a pattern of cell death similar to controls, with prominent labeling of apoptotic cells in the VZ and SVZ (H).
(J and K) These cells, dying in the presence of NT4, are rescued by BAF. Heterotopic neuronal collections (arrowheads, [H–K]) are produced by NT4 in the presence (K) or absence (I) of BAF. Scale bars, 100 μm; in (E), 20 μm.

neurons that are phenotypically similar to CR neurons; 2) In the presence of NT4, early-born neurons in different states of maturation continue to accumulate in the MZ even after the CP has begun to form; and 3) The accumulations produced in the MZ by NT4 in vivo extend beneath the pia along the entire cortex and are most extensive in anterior and lateral cortex. Thus, the phenotype of neurons within these collections and their pattern of accumulation after NT4, in vivo, are remarkably similar to that observed in the normal development of the MZ in several mammals, including man.

Although it is widely assumed that most of the neurons in the MZ are formed in the neocortical VZ (Bayer et al., 1991) and move to the preplate either by nuclear translocation or radial migration (Brittis et al., 1995), recent evidence indicates that neurons may also migrate tangentially into the MZ from the proliferative zone of the ganglionic eminence (De Carlos et al., 1996) or from an SVZ just caudal to the olfactory bulb (Gadisseux et al., 1992). In addition, populations of early-born neurons that appear to wait in the upper VZ (Menezes and Luskin, 1994) and the lower intermediate zone (DeDiego et al., 1994) could be affected by NT4.

In the human fetal MZ, there are two transient populations of pleiomorphic cells: the CR neurons and the neurons of the subpial granule cell layer (SGL). The CR cells appear first in the preplate, and, notably, their numbers continue to increase even after the CP has formed. They are most numerous laterally, in the human temporal lobe, where they are arranged in rows just beneath the pia (Meyer and Gonzalez-Hernandez, 1993). The SGL neurons appear after both the first CR cells and the early CP neurons have arrived. Although the SGL has thus far only been definitively described in humans, it bears a striking resemblance to the dense subpial band of neurons produced in the rat after in vivo administration of NT4 (compare Figure 3 in Gadisseux et al., 1992, to our Figure 2E). The SGL contains immature (MAP-2 negative) neurons that appear to migrate tangentially (Gadisseux et al., 1992) and mature (MAP-2 positive) neurons.

In humans, SGL neurons are thought to originate in the lateral aspect of the olfactory SVZ in the frontal horn and to migrate along two major strands to the subpial layer, then migrate tangentially in the MZ across the entire cortex (Gadisseux et al., 1992). Observations in the developing rodent brain have also provided evidence for a tangential migration of neurons from the olfactory SVZ into the forming MZ. In the embryonic rat, a large cohort of early generated (E12) cells is concentrated adjacent to the olfactory ventricle; it later extends subpially along the rostrocaudal axis in the MZ of olfactory (pyriform) cortex and encroaches on the MZ of neocortex (Valverde and Santacana, 1994; De Carlos et al., 1996). Additionally, recent work in the rat describes neurons that emerge from the olfactory SVZ to enter the neocortical MZ in a similar anterior and lateral distribution (Meyer et al., 1996, Soc. Neurosci. abstract). The strong homology between these observations about early MZ cells in normal mammals and the concentration of excess neurons in anterior and lateral regions of the MZ after NT4 application in vivo suggests that the olfactory SVZ may be a source for these excess neurons.

The olfactory SVZ cannot provide the sole source of these excess neurons, however, since it is absent in our cortical slices, yet NT4 continues to produce accumulations of neurons in the MZ that are more prominent laterally. Neurons that have already reached the ventrolateral MZ at the time of our cultures (E14 mouse) could be triggered by NT4 to continue migrating tangentially into the MZ of neocortex. Alternatively, the lateral ganglionic eminence (LGE), which expresses trkB (Fryer et al., 1996), may be an additional source for neurons of the primary olfactory cortex as well as for neocortical MZ neurons. Early-born cells in the LGE of the rat are subsequently present in the olfactory cortex, including the MZ (De Carlos et al., 1996), and dye-labeled cells from the LGE migrate into the lateral aspect of the telencephalic vesicle, then turn to enter the neocortical MZ (De Carlos et al., 1996). Further support for the LGE as a source of MZ neurons is derived from preliminary observations in mice lacking the homeobox genes *Dlx1* and *Dlx2*. Mice with deletions of both genes have an abnormal LGE and a lack of GABA-reactive neurons that arise in the LGE and are present in the normal MZ (Anderson et al., 1996, Soc. Neurosci. abstract). In contrast, our preliminary studies indicate that in vivo application of NT4 increases GABA immunoreactivity in the MZ (Brunstrom and Pearlman, unpublished data).

Long tangential migrations of neurons occur during the normal development of the olfactory bulb (Zigova et al., 1996) and the cerebellum (Miale and Sidman, 1961). Substantial evidence supports the notion that a tangential migration of neurons into the MZ occurs during normal cortical development as well. The abundant extracellular matrix of the MZ (Chun and Shatz, 1988; Sheppard et al., 1991), including the ECM-like glycoprotein reelin, might provide a substrate for this migration. Reelin is expressed by neurons as they migrate from the rhombic lip to form the cerebellar anlage and by granule cells as they migrate in the cerebellar external granular layer. Reelin is also expressed in neurons of the embryonic cortical MZ and in the developing striatum (D'Arcangelo et al., 1995; Miyata et al., 1996; Schiffmann et al., 1997).

We propose that NT4 induces the tangential migration of excess neurons into the MZ of the embryonic neocortex and suggest the olfactory SVZ and the LGE as sources for these neurons. Our observations indicate a role for NT4 during normal cortical development and in the pathogenesis of certain human cortical malformations. Excess neurons are in Layer 1 in patients with primary generalized epilepsy (Meencke and Veith, 1992), and zonal heterotopias within Layer 1 in the temporal lobe have been reported in syndromes associated with severe mental retardation, tetraplegia, and intractable epilepsy (Yamaguchi et al., 1996). We suggest that human cortical dysplasias may result from increased availability of NT4 or increased sensitivity to its effects during early cortical development.

Experimental Procedures

Animals

Timed-pregnant C3H mice and Sprague–Dawley rats were obtained from Harlan Sprague–Dawley (Indianapolis; conception = E0).

Timed pregnancies resulting from matings of animals heterozygous for a deletion in *trkB* (*trkB*$^{+/-}$) (Klein et al., 1993) were provided by Dr. William Snider; the genotype of each embryo was determined by polymerase chain reaction analysis.

Organotypic Slice Preparation

Coronal cortical slices (250 μm; E13.5–E14.5) were prepared as described previously (Stoppini et al., 1991; Sheppard et al., 1995), placed individually on membrane inserts (1 μm pore size; Falcon), covered with a drop of diluted agarose, placed in sterile organ culture dishes (Falcon) over 1.6 ml of defined medium (Annis et al., 1990; lacking ethanolamine), and incubated in humidified 5% CO_2 at 37°C. Slice cultures from adjacent cortical areas were randomized to separate conditions. After 1–2 hr, half of the medium was replaced with neurotrophin- or control-containing medium; this exchange was repeated daily. Final concentrations of neurotrophins were: NT4, 20 or 100 ng/ml; NT3 and BDNF, 100 or 200 ng/ml, 1 or 2 mg/ml; and NGF, 100 ng/ml. Controls received 1 μl/ml of diluent (PBS or NT3 diluent composed of 4.5% mannitol, 0.5% sucrose, and 10 mM histidine). All solutions included 0.1% BSA. Neurotrophins were generously provided by Regeneron Pharmaceuticals (NT4, NT3, and BDNF) and Dr. E. M. Johnson, Jr (NGF). All neurotrophins were handled sterilely and stored according to the manufacturers' instructions. Several lots of each neurotrophin were tested and yielded identical results. Slices were cultured for 6–72 hr, then fixed (in 2% paraformaldehyde for 15 min at 21°C) and processed for immunohistochemistry (Sheppard et al., 1995). Each hemisphere in a slice culture (s.h.) was considered independently.

TrkB Knockout Studies

Cortical slices (E14; N = 82 s.h.) from 2 *trk B*$^{-/-}$ animals and 10 littermates (heterozygous and wild type) were prepared as described above and maintained in NT4 (100 ng/ml) or control conditions for 48 hr. Sections from fixed slices were examined after hematoxylin staining.

Immunohistochemistry

Fixed slices and embryonic brains were embedded in M1 (Lipshaw) and frozen in liquid nitrogen. Cryostat sections were collected on Vectabond-treated slides (Vector laboratories), postfixed in 1%–2% paraformaldehyde (for 5 min at 21°C), permeabilized with Triton X-100 (Sigma; 0.2% for 60 min at 21°C), and blocked in 2% fish gelatin or 0.1% BSA in PBS (Sigma; 60 min at 21°C or 18 hr at 4°C). Primary antibodies (applied for 18 hr at 4°C or 1–2 hr at 21°C) included monoclonal anti-BrdU (BU1, 1:4–1:8, provided by Dr. J. A. Katzman; Gonchoroff et al., 1985), monoclonal anti-β-tubulin (TuJ1; 1:1500, provided by Dr. A. Frankfurter; Lee et al., 1990), and polyclonal anti-cyclin A (1:1000, provided by Dr. J. Roberts; Guadagno et al., 1993), polyclonal goat anti-BrdU IgG (1:10,000, provided by Dr. S. M. Cohn; Cohn and Lieberman, 1984), and polyclonal anti-calretinin (Chemicon; 1:2000). Secondary antibodies were applied for 1 hr at 21°C. FITC-goat or donkey anti-mouse IgG (1:200), Cy3-goat anti-rabbit IgG (1:1800), Cy3-donkey anti-mouse IgG (1:1800), and Cy3-donkey anti-goat IgG (1:1800) were purchased from Jackson ImmunoResearch. Biotinylated goat or horse anti-mouse IgGs (1:400) were purchased from Vector laboratories. All antibodies were diluted in 2% fish gelatin (Sigma) unless otherwise specified. Immunolabeled sections were counterstained with bisbenzimide (Sigma; 5 min at 21°C), then coverslipped in Vectashield (Vector laboratories).

BrdU-Labeling in Slices and In Vivo

Cells were labeled in S phase for cell birth-dating studies by maternal intraperitoneal injections of BrdU (Sigma). In an initial series of injections (50 mg/kg BrdU), fluorodeoxyuridine (FldU, .075%; Sigma) was added to the solution to optimize labeling (Ellwart and Dormer, 1985). Subsequent experiments with BrdU alone (70 mg/kg) yielded the same results. Early experiments in which BrdU was added directly to the media to identify proliferating cells in slice cultures also included FldU. Although we saw no evidence of FldU toxicity, we eliminated FldU from subsequent experiments and found identical patterns of BrdU labeling. To further minimize possible toxicity from prolonged exposure to BrdU, we included equimolar amounts of deoxycytidine in some of the 24 hr pulse experiments (Meuth and Green, 1974) and found no effect on the number of BrdU-labeled cells.

To assess cell proliferation after NT4 application in vitro, cortical slices were prepared as described above and cultured with NT4 (100 ng/ml) or control (C) solutions for 6–7 hr (NT4 = 8 s.h.; C = 6 s.h.), 20 hr (NT4 = 6 s.h.; C = 6 s.h.), or 24 hr (NT4 = 10 s.h.; C = 7 s.h.). BrdU (2–3 μg/ml) was added to the dish for an additional 2–4 hr before fixation. Other slices were exposed simultaneously to BrdU and NT4 or control solutions from the outset for 10 hr (NT4 = 5 s.h.; C = 7 s.h.) or 24 hr (NT4 = 11 s.h., C = 11 s.h.; in a subset of these slices, the BrdU was replenished after 10–12 hr in culture).

Cell birth dating of early-born MZ cells in the slice was achieved by maternal injection of BrdU at E11–E11.5 (mouse). Slices from these embryos were prepared at E14 and cultured for 48 hr in NT4- (n = 13 s.h) or control- (n = 11 s.h) containing media. Brains from littermates harvested at the time of slice preparation (E14) or from separate litters harvested at E14 or E17 were immunolabeled with goat anti-BrdU (see below) to verify the birth dating.

To follow the migration of early CP cells after NT4, cortical slices (E14 mouse) were prepared after maternal BrdU injection (Takahashi et al., 1992) at E13, then cultured for 24–72 hr in NT4 (100 ng/ml; n = 28 s.h.), BDNF (100 ng/ml; n = 9 s.h.), or control (n = 24 s.h.) conditions.

Sections from each slice were immunolabeled with BU1 after Triton permeabilization and DNA denaturation with NaOH (.03 N for 20 s at 21°C). BU1 recognition was achieved with either a Cy3-conjugated secondary antibody or a biotinylated secondary antibody (1:400 at 21°C for 1 hr), followed by reaction with Vectastain (ABC Elite kit, Vector laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (20 s at 21°C). BU1 was diluted in either 2% fish gelatin or 0.1% BSA in PBS. In some experiments, a blocking step (2% horse serum for 18 hr at 4°C) was performed before addition of the secondary antibody.

To identify BrdU-labeled cells in vivo, sections from embryonic brains were denatured (in 2 N HCl for 1 hr at 21°C), permeabilized with Triton, then double immunolabeled with a primary antibody mixture of goat anti-BrdU and either TuJ1 (18 hr at 4°C) or anti-MAP-2, followed by a mixture of Cy3-labeled anti-goat and FITC-labeled anti-mouse secondary antibodies (1 hr at 21°C).

Intraventricular Injections

Injections of rat embryos were carried out under sterile conditions using previously described methods (Henderson et al., 1994). Briefly, pregnant dams (n = 13) were anesthetized with a subcutaneous injection (0.7 ml/kg) of a solution containing three parts each of ketamine (100 mg/ml) and xylazine (20 mg/ml) and one part acepromazine (10 mg/ml). Uterine horns were carefully lifted through a midline abdominal incision; embryos were visualized by transillumination with a fiberoptic light source and injected intraventricularly through a 30 gauge needle attached to a Hamilton syringe. At E16 and again at E18, 5 μl (580 μg/ml) of NT4 (N = 27 brains), NT3 (n = 7), or BDNF (n = 17) were administered. Controls received PBS (n = 14), placebo (diluent for NT3, n = 4), or were uninjected (n = 4). All solutions contained 0.1% BSA and 0.05% fast green (Sigma). Brains were used for analysis only if green dye filled both of the lateral ventricles at the time of each injection. At E19, pregnant dams were reanesthetized, and embryos were removed by Caesarian section; the brains were immersion fixed (in 2% paraformaldehyde for 4 hr at 4°C) and processed for cryostat sectioning. Coronal sections (18 μm) were examined (at 80 μm intervals) after hematoxylin staining (a total of 32–40 sections per hemisphere). A few brains (NT4 = 3; PBS = 1) were sectioned in the sagittal plane and analyzed in a similar fashion.

Cell Counts and Data Analysis

Stereological methods were used for all cell counts (Sterio, 1983). Each hemisphere within a slice (s.h.) or within an injected embryonic brain was considered independently. Statistical analyses were performed using ANOVA (Statview).

Organotypic Slices

Hematoxylin-stained sections (18 μm) of organotypic slices were viewed at 54 μm intervals over the thickness of each slice. Of 378 s.h., 255 (67%) were accepted for analysis when two sections from

the central 180 μm contained intact cortex and pia. Heterotopias were identified by their distance from the lateral angle of the lateral ventricle; the number of cells per heterotopia was counted on the hematoxylin-stained section containing the widest diameter of each collection. To determine the percentage of cells in the NT4-induced heterotopias or in the normal MZ that are neurons, slices were cultured for 48 hr in NT4 (100 ng/ml; n = 8 s.h.) or control solutions (n = 7 s.h.), then processed for TuJ1 and MAP-2 immunohistochemistry and counterstained with bisbenzimide. Heterotopias and areas of control MZ were selected from lateral, dorsal, and medial regions of each slice. Counts reflect all cells within 14 heterotopias and 14 samples of control MZ. Neurons were identified with epifluoresence microscopy (40× oil immersion lens), and the ratio of immunolabeled cells to bisbenzimide-stained nuclei was calculated for each neuronal marker.

Embryonic Brains

Comparison between neurotrophin and control conditions was carried out in embryos from the same litter. Cell birth dating was achieved by maternal injection of BrdU at E13 or E15 (70 mg/kg body weight intraperitoneal). Embryos received intraventricular injections of NT4 or PBS at E16 and E18. E13 birth-dating experiments also included BDNF-treated embryos. To account for a possible effect of the injection, each embryo received both injections in a single hemisphere. Sections were selected for analysis only if the pia and underlying neocortex were intact. BrdU-labeled cells were counted (immunofluorescence; 40× oil immersion) over the entire extent of the MZ and SP between lines perpendicular to the cortical surface that intersected the lateral and medial angles of the lateral ventricle. The neuronal identity (TuJ1 or MAP-2 positivity) of BrdU-labeled cells in the MZ was confirmed on two sections per hemisphere at the level of the hippocampus and habenulae.

In each hemisphere (injected and noninjected), counts of E13 BrdU-labeled MZ and SP cells were performed (NT4 = 7 hem; control = 6; and BDNF = 6) in two nonadjacent sections at each of three anteroposterior levels (anterior commissure, foramen of Monroe, and posteriorly at the level of the hippocampus and habenulae). The MZ was defined (on bisbenzimide counterstain) as the area between the top of the dense CP and the inner surface of the pia. For the purpose of these counts, the SP was defined as all cells in the SP just below the dense CP and the few cells that were in the upper intermediate zone. Counts of E15 BrdU-labeled cells were performed (NT4 = 7 hem; control = 6 hem) at anterior and posterior levels. Statistical analysis showed no difference in BrdU labeling between injected and noninjected hemispheres for any region or birth date. Results are displayed as the total number of BrdU-labeled cells in the MZ or SP for each hemisphere.

Cell Death

Two end-labeling methods with different enzymes, Klenow (NeuroTACS; Trevigen) and terminal deoxynucleotidyltransferase (ApopTag; OnCor), were used to examine embryonic brains (E14 mouse). Klenow, the large fragment of DNA polymerase I, generates a complementary strand of DNA (biotinylated nucleotides) for each 5′ overhang of double-stranded DNA; terminal deoxynucleotidyltransferase attaches nucleotides (tagged with digoxigenin) to free 3′ hydroxyl groups. Thymus and intestine served as positive controls, and adult brain as a negative control. Tissues were embedded in O.C.T. (Tissue Tek, VWR) and frozen in liquid nitrogen. Sections (10 or 18 μm) were collected on SuperFrost Plus slides (Fisher Scientific) and postfixed in paraformaldehyde (for 5 min at 21°C; 2% paraformaldehyde for NeuroTACS; 4% paraformaldehyde for ApopTag). Labeling was carried out according to the manufacturer's directions, except that in the NeuroTACS assay, CY3-streptavidin (1:8000; Jackson ImmunoResearch) was substituted for streptavidin HRP. These protocols consistently yielded positive staining in the thymus and intestine and negative staining (very rare positive cells) in the adult neocortex. At E14, 7–8 sections per brain (n = 3 brains) were labeled with each kit; for each brain, no difference in the number of labeled cells was evident between the two methods.

Slice cultures were prepared as described above and cultured for 24 or 48 hr in the presence of BAF (100 μM; n = 10 s.h; Enzyme Systems Products, Dublin, CA), NT4 (100 ng/ml; n = 14 s.h.), NT4+BAF (n = 9 s.h.), or control (n = 14 s.h.) conditions. Additional slices were fixed after only 1–2 hr in culture (n = 12 s.h.) to assess the amount of cell death at the start of the perturbation experiment. Slices were processed as for immunohistochemistry, except that O.C.T was used for the embedding compound. Sections (18 μm) were collected on SuperFrost Plus slides and processed with the NeuroTACS kit as described above.

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