Growth of pyramidal, but not non-pyramidal, dendrites in long-term organotypic explants of neonatal rat neocortex chronically exposed to neurotrophin-3

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Abstract

The present study was undertaken to determine the effects of neurotrophin-3 (NT3) and spontaneous bioelectric activity (SBA) on dendritic elongation and branching in long-term isolated organotypic explants of rat neocortex. Viral vector-directed expression of NT3 was used as an effective means to ensure a continuous, local production of the neurotrophic factor. Quantitative light microscopic measurement of dendritic branching patterns was carried out on Golgi-stained materials. Explants were exposed to an adenoviral vector encoding the genetic sequence for neurotrophin-3 (Ad-NT3), or to exogenous additions of the neuropeptide NT3. In order to test for activity-dependent growth effects under control and experimental conditions, explants were exposed to glutamatergic blockade using a cocktail of APV and DNQX. Both Ad-NT3 and NT3 peptide potently promoted apical and basal dendritic growth (elongation and branching) in pyramidal neurons. This growth was observed to be significant in layers II–IV and V. These growth effects were also not activity dependent, inasmuch as they were elicited from explants in which spontaneous bioelectric activity had been suppressed. Non-pyramidal neurons, throughout the neocortical slice, showed no significant dendritic responses to the prolonged presence of NT3. These findings show that pyramidal dendritic growth in long-term neocortical explants responds to at least one neurotrophic growth factor, NT3, and is independent of intrinsic bioelectric activity. The use of viral vectors in delivering a continuous high level of neurotrophic factor within developing neural tissues demonstrates its potential application to in vivo tissues during development, or in the stimulation of neuritogenesis and neuroregeneration following injuries.

Introduction

Emerging spontaneous bioelectric activity (SBA) has profound consequences on the development of neural networks through its effects on dendritic and axonal (neuritic) growth. How and when activity exerts its influence over axonal elongation and branching has been the subject of numerous studies (see Goodman & Shatz, 1993; Bailey et al., 1997). No data were given regarding the long-term effects of any given neurotrophin on in vivo slices, making the subject of numerous studies (see Goodman & Shatz, 1993; Bailey et al., 1997). Moreover, endogenous neurotrophins may also function as inhibitors of layer-specific neuritic growth, suggesting an intrinsic growth-regulating capacity during network formation (McAllister et al., 1997). No data were given regarding the long-term effects of any given neurotrophin on in vitro slices, making comparisons to the in vivo situation impossible. The reported growth effects were restricted to basal and/or apical dendrites of pyramidal neurons within a given neocortical layer. In the case of brain-derived neurotrophic factor (BDNF), these effects appear to be activity dependent (McAllister et al., 1996). In vivo studies have also shown that growth effects are elicited in neocortical neurons by a variety of

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neurotrophins and that these effects may be regulated by bioelectric activity (Castren et al., 1992; Goodman & Shatz, 1993; Ghosh et al., 1994; Schous et al., 1995; Thoenen, 1995; McAllister et al., 1996).

The present study was undertaken to determine whether a specific neurotrophin, NT3 (known to exert short-term growth-promoting effects on the basal and apical dendrites of layer IV and VI pyramidal neurons in isolated neonatal ferret neocortex in vitro; McAllister et al., 1995) could induce dendritic elongation and branching in long-term isolated rat neocortex in vitro. The delivery of NT3 was accomplished using exogenous peptide additions or by a gene transfer technique which ensured a continuous local production of the factor within the explant. Also, non-pyramidal neurons were examined to determine whether they were similarly affected by exposure to increased levels of NT3.

In addition, chronic blockade of SBA was carried out using a cocktail of glutamate antagonists in order to determine whether any changes in dendritic growth were activity dependent, or solely dependent on the presence of NT3. We have now shown that elevating the level of NT3 in these cultures, via adenoviral infection techniques or by exogenously added NT3 peptide, resulted in a significant increase in pyramidal neuron apical and basal dendritic length and number of branches. Non-pyramidal dendrites, in contrast, showed no significant growth in the presence of this neurotrophin. Moreover, the absence of SBA failed to counteract the growth effects of NT3 on pyramidal apical and basal dendrites. These results also demonstrate the usefulness of viral vector-directed delivery of neurotrophic factors as a means of promoting neuritogenesis/regeneration within intact, organotypic neural tissues.

Materials and methods

Cultures

Preparation of the organotypic slices used in these studies has been reported previously (Romijn et al., 1988). Briefly, coronal slices taken from parietal neocortex of 6-day-old rat pups were cut on a McIlwain chopper at 360 μm. Pieces ~1 mm wide, extending from pial surface to white matter, were placed on a polyamide grid assembly in warmed serum-free growth medium (Fig. 1; Romijn et al., 1984).

After attachment (~5 h or overnight), the cultures were rocked (maximum angle of 30 °C, 4 min cycle) to ensure maximal exposure to nutrients and elimination of wastes. At 2 days in vitro (DIV), the experimental groups were exposed to NT3 and/or a cocktail of APV (a NMDA antagonist) and DNQX (a non-NMDA antagonist) at 20 and 50 μm, respectively, which effectively silenced all SBA within the explants between refreshings. The culture medium was exchanged 3 × per week (~1.2 mL/refreshing), and maintained at 35 °C in 95% air/5% CO2. All explants were harvested at 10 DIV.

NT3 treatment

At 2 DIV, selected cultures were exposed to an adenoviral vector (Ad-NT3) encoding the genetic sequence for the neurotrophin NT3. The generation and characterization of Ad-NT3 have been previously documented (Dijkhuizen et al., 1997; Hermens et al., 1997).

An adenoviral vector encoding LacZ (Ad-LacZ) served as a control. Infection of neocortical explants was accomplished through a single exposure of the slices to Ad-NT3 using a viral vector dosage of 2 × 105 plaque forming units (p.f.u.). The growth medium was removed from the explants, and the grids containing the explants inverted onto 200 μL of Ad-NT3-containing medium. The cultures were allowed to sit in the culturing ovens undisturbed for 6 h before the grids were reintroduced and flooded with control growth medium, or medium containing APV/DNQX. All explants were then grown for a further 8 days in control or APV/DNQX-containing medium. Alternatively, explants were grown in the presence of 100 ng/mL purified NT3 peptide (Alamone Labs, Israel) which was added to the refreshing medium on DIV 2, 6 and 8.

Golgi staining

At 10 DIV, the explants were briefly rinsed in 0.9% NaCl to remove excess growth medium and fixed for 48 h in 3.5% potassium dichromate–5% glutaraldehyde solution at 4 °C (Caeser & Aertsen, 1991; Caeser & Schuz, 1992). The explants were then rinsed with distilled water. Excess water was withdrawn from the explant and a small crystal of AgNO3 placed at one corner of the slice at the pial/side edge. The cultures were then returned to the refrigerator for a further 24–48 h. Explants were controlled under the microscope to determine whether a successful filling had occurred. Those which were deemed successfully filled were then dehydrated in ethanol, cleared in Histoclear and mounted in Histomount (National Diagnostics). Neurons were deemed to be successfully stained when unambiguous identification of the finest endings of both dendrites and axons could be made (as shown in Baker & Van Pelt, 1997). The reliability of the rapid Golgi method as a means of quantifying dendritic/axonal development was made obvious in earlier studies which showed all dendritic segment length parameters, as well as the timing of the dendritic growth phase, were similar to those reported for age-matched in situ tissues (Uylings et al., 1994; Baker & Van Pelt, 1997).

Microscopic measurements

Successfully stained neurons were measured on a semi-automated measuring microscope developed at the Netherlands Institute for Brain Research (Overydijk et al., 1978; Uylings et al., 1986). Intact cultured slices were examined as whole mounts; only those neurons which could be traced in their entirety were selected for study. The overall cellular organization of the slices was maintained throughout the culturing period, with presumptive layers II–IV and V–VI being easily recognizable in thionine, silver and Golgi-stained preparations (see Fig. 1A,B). Pyramidal apical dendrites were orientated towards the pial surface frequently terminating within the superficial pial layers from even the deeper located cells in presumptive layers V–VI (Fig. 1A). No attempt was made to specifically select either pyramidal or non-pyramidal neurons, or to concentrate on a given cell layer within the neocortex. The following parameters of morpholo-
gically reconstructed neurons were quantitatively measured: (i) total dendritic length per neuron; (ii) total apical dendritic length (for pyramidal neurons); (iii) total (basal) dendritic length; and (iv) total number of segments.

In situ hybridization

For whole mount in situ hybridization, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and washed twice for 15 min with PBS/0.3% Triton X100. The explants were then acetylated in 1% triethanolamine with 0.25% acetic anhydride, washed twice in PBS, once in 2 × standard saline citrate (SSC) (1 × SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and prehybridized in 50% formamide, 5 × SSC, 0.3% Triton X100, 5 × Denhardt’s reagents, 250 µL baker’s yeast tRNA and 500 µg/mL heat-denatured herring sperm DNA at room temperature for 4 h. Hybridization was performed in the same solution at 55 °C with a digoxigenin-labelled cRNA probe (200 ng/mL). The cultures were washed for 5 min with 5 × SSC, 5 min with 2 × SSC, twice for 30 min with 0.2 × SSC/50% formamide (all at 55 °C), and for 5 min with 0.2 × SSC at room temperature. Cultures were then washed with B1 (100 mM Tris pH 7.5, 150 mM NaCl), blocked for 30 min in B1 with 1% blocking reagent (Boehringer Mannheim), incubated for 1 h with alkaline phosphatase conjugated with antidigoxigenin Fab fragments diluted 1 : 2000 in B1, washed twice for 15 min in B1 and washed once with B2 (100 mM Tris pH 9.5, 100 mM NaCl, 10 mM MgCl₂). Cultures were then stained with 0.34 mg/mL nitro blue tetrazolium (NBT) and 0.18 mg/mL bromo-chloro-indolyl phosphate (BCIP) in B2, washed in TE (10 mM Tris pH 8.0, 1 mM EDTA) and embedded in Kaizer’s glycerol gelatin (Merck).

Electrophysiology

The entire grid assembly, containing explants grown for 10 days in vitro, was positioned in a recording chamber mounted over an inverted microscope with recording glass microelectrodes visually positioned above the isolated neocortical slices. The explants were superfused with carbogen-equilibrated Dulbecco’s minimal essential medium (DMEM) at 35 °C with a flow rate of ≈ 3 mL/min. The area chosen for recording was a cell-rich region easily identified in each slice, corresponding to presumptive neocortical layers II–IV. Control and experimental explants were examined for the presence or absence of SBA at a number of points across the slices, the experimental slices being recorded first in medium containing APV/DNQX in order to determine whether treatment had silenced any measurable SBA. After such determinations, the APV/DNQX medium was replaced with normal DMEM in order to determine whether SBA would return to the explants.

Statistics

Culture series were examined statistically for treatment effects using the non-parametric Mann–Whitney U-test (Conover, 1990; significance taken as P < 0.05). Because of the variation between culture series, for each experiment the outcomes of the treatment were compared with control values for that experiment. When the outcome of an analysis was at the border of the P < 0.05 significance level, further analysis using the Student’s t-test was carried out to decide whether the difference was significant.

Results

Slice morphology

The neocortical slices in all groups retained their overall cytoarchitecture and three dimensionality throughout the culturing period (Fig. 1A, Romijn et al., 1984; De Jong et al., 1988; Ruiter et al., 1991). The explants assumed a somewhat fan shape with a definite, smooth pial surface and a sometimes less well defined ventricular surface, and showed no discernable degeneration, spreading or thinning of the explant with time in vitro. Cell layering within the explant reflected that reported for in vivo material, with recognizable pyramidal (Fig. 1B–D) and non-pyramidal neurons throughout. Apical dendrites from the pyramidal neurons projected towards the pial surface. Basal dendrites were easily identifiable for pyramidal neurons. Cell location within a given neocortical layer was noted for each of the neurons measured. No attempt was made to categorize the various types of pyramidal or non-pyramidal neurons used in this study.

Effect of adenoviral infection of the neocortex with NT3

Exposure of the neocortical explants to Ad-LacZ resulted in the appearance of many β-galactosidase filled cells located at the surface of the explant (data not shown). The distribution of Ad-NT3 infected cells within the explant was similar to that observed for B-galactosidase filled cells, as shown by whole mount in situ hybridization (Fig. 1F). Ad-LacZ infected cultures displayed no hybridization signal, which indicates that the signal observed in the Ad-NT3 infected cultures is due to the detection of transgene mRNA (Fig. 1E). The Ad-LacZ group showed no significant dendritic differences from their respective control group (Fig. 2). Both the total dendritic length (Fig. 2) and number of dendritic segments (data not shown) of pyramidal neurons were significantly elevated following exposure to Ad-NT3. These differences were reflected in both apical and basal dendritic lengths vis-a-vis their respective controls (Fig. 2). Non-pyramidal neurons showed no significant dendritic increases in length (Fig. 2) or number of segments (data not shown) following Ad-NT3 exposure.

Effect of adenoviral infection versus peptide supplementation of the neocortex with NT3

Both exposure to Ad-NT3 and 100 ng/mL recombinant NT3 peptide produced significant increases in pyramidal total dendritic length (Fig. 3) and number of segments (data not shown), in apical dendrites, and in basal dendrites. Non-pyramidal neurons showed no significant dendritic growth following Ad-NT3 infection or exogenous peptide treatment.

Effect of SBA and Ad-NT3 on dendritic growth

Electrophysiological recordings from APV/DNQX-treated cultures confirmed that SBA (slow waves and action potentials; see Baker et al., 1989, 1995) had been totally suppressed (data not shown). Replacing the APV/DNQX-containing medium with control DMEM resulted in the emergence of SBA within 5–20 min. Such suppression of SBA had no significant effect on total dendritic length in control pyramidal neurons (Fig. 4), as had been shown in previous studies (Baker et al., 1997). Both the Ad-NT3 and Ad-NT3 + APV/DNQX groups, however, showed significant growth effects in total dendritic length over their respective controls. This significant growth effect occurred for basal dendritic length in both these latter groups (Fig. 4). The increase in number of segments upon Ad-NT3 treatment was significant for pyramidal basal dendrites, but fell short of significance for the Ad-NT3 + APV/DNQX group (data not shown). Only the Ad-NT3 + APV/DNQX groups showed a significant apical dendritic elongation compared with controls (Fig. 4). Silenced non-pyramidal neurons continued to show no significant growth effects between the neurotrophin group compared with their respective controls (Fig. 4).
Effect of NT3 on neonatal rat neocortex

**Fig. 2.** Changes in total dendritic length for pyramidal and non-pyramidal neurons grown under control, lacZ and Ad-NT3 conditions [left histograms; mean (SEM), as in all subsequent figures]. Only Ad-NT3-grown explants showed a significant growth effect in pyramidal neurons \((n = 20); P < 0.01\) compared with LacZ \((n = 9)\) and control groups \((n = 18)\). Non-pyramidal neurons showed no significant differences between any of the groups \((n = 18\) for controls, 4 for lacZ and 15 for Ad-NT3). The significant differences observed in pyramidal neurons were seen for both the basal \((P < 0.03)\) and apical dendrites \((P < 0.01)\).

**Fig. 3.** Changes in total dendritic length for pyramidal neurons under control conditions \((n = 43)\) or exposed to NT3 peptide \((n = 10)\) or infected with Ad-NT3 \((n = 26)\), and for non-pyramidal neurons under control conditions \((n = 16)\) or exposed to NT3 peptide \((n = 17)\) or Ad-NT3 \((n = 13)\;\) (left histograms). Both neurotrophin groups showed significant growth effects compared with controls \((P < 0.02)\). There was no growth effect in non-pyramidal neurons. These growth effects were expressed in both pyramidal basal \((P < 0.02)\) and apical dendrites \((P < 0.02)\).

**Effect of NT3 on selected neocortical cell layers**
Pyramidal neurons within neocortical layers II–IV, V and VI were examined in an effort to determine whether the NT3 effect was layer specific. Since both Ad-NT3 and exogenous peptide exposure elicited significant growth effects on apical and basal dendrites, experimental groups exposed to these treatments were combined and compared with their respective controls. In all three cortical layer groupings examined, both basal and apical dendrites showed elevated levels in length and number of segments in comparison with their respective controls following NT3 exposure via either Ad-NT3 infection or exogenous peptide addition (Fig. 5). The elevation in total dendritic length (data not shown) and number of segments was found to be significant in the II–III–IV and V groups. In order to differentiate just where any significant growth occurred, total length was subsequently examined as total basal and total apical dendritic length (Fig. 5) and segment number (data not shown). Basal dendrites showed significant elevation in their length in the II–III–IV group, and in the number of segments in both the II–III–IV and V groups. Apical dendrites showed significant elevation in lengths in layer V, and in the number of segments in the II–III–IV group.

**Discussion**
The present study has shown that NT3 potently promotes apical and basal dendritic growth (elongation and branching) in pyramidal neurons in isolated rat neocortical explants. Increased values for
Fig. 4. Effect of spontaneous bioelectric activity and NT3 on total dendritic length in pyramidal and non-pyramidal neurons (left histograms). Pyramidal neurons showed a significant dendritic growth effect \((P < 0.01)\) in the NT3 exposed groups, under active \((n = 24)\) and silenced \((n = 22)\) conditions, in comparison with active controls \((n = 20)\). Non-pyramidal neurons showed no growth effect in the NT3-exposed groups under active \((n = 12)\) or silenced \((n = 15)\) conditions in comparison with active \((n = 8)\) and silenced \((n = 15)\) control conditions. There was no significant difference between active and silenced cultures \((P > 0.1)\).

Pyramidal basal dendrites (right hand histograms) showed the same overall growth effects as were seen for total dendritic growth \((P < 0.01\) for neurotrophin groups compared with controls). Apical dendrites showed a significant growth effect only in the silenced Ad-NT3 group \((P < 0.04)\).

dendritic length and branching (i.e. numbers of segments) were observed in all cortical layers, although they reached significance only in layers II–III–IV and V. The significant NT3-mediated dendritic elongation observed in pyramidal neurons was not activity dependent, but also occurred when SBA had been chronically blocked with a cocktail of glutamatergic antagonists. Non-pyramidal neurons within the NT3-treated groups showed no significant dendritic growth effect (elongation and branching) within any given layer of the neocortex.

This study is the first to be concerned with the long-term effects of a given neurotrophin (NT3) on the overall morphological development of the neocortex, and emergent spontaneous bioelectric activity (SBA). The current study shows that pyramidal neurons respond to NT3 with significantly increased growth of apical and basal dendrites. Such growth would be anticipated inasmuch as Ringstedt et al. (1993) and Altar et al. (1994) have shown that \(trkC\) mRNA (encoding the primary receptor for NT3) is expressed in all neocortical layers throughout the first 4 weeks of postnatal life in the rat, suggesting that neurons throughout the neocortex should be responsive to NT3.

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It is unknown whether the significant growth observed for dendrites in layer II–III–IV and layer V neurons is due to a differential expression of the trkC receptors in these layers at the age examined (10 DIV, roughly equivalent to PN16). Ringstedt et al. (1993) show a developmental switch in rat trkC mRNA levels from deeper neocortical layers to more superficial layers with age, suggesting that the selected, significant dendritic growth we observed in layers II–IV and V might somehow be associated with this developmental window. Ringstedt et al. (1993) were unable to differentiate between pyramidal and non-pyramidal neurons, so it is unknown whether trkC receptors were selectively present in either (or both) cell type. Our data would suggest that only pyramidal neurons express trkC receptors and respond accordingly to the presence of NT3.

Developing ferret neocortex, on the other hand, showed significant apical and basal dendritic growth responses only within layer IV and VI pyramids following 36 h exposure to exogenously presented NT3 peptide (McAllister et al., 1995). The differences between these studies may be due to differences in the degree of morphological maturation within the developing neocortex (PN14 + 36 h in ferret versus PN6 + 10 days in rat), in trkC receptor localization within a given cortical layer at the time of NT3 exposure (see Ringstedt et al., 1993), to differences in age- and/or activity-dependent molecular stages of development within ferret and rat cortex (see Zafra et al., 1991; Castren et al., 1992; Marty et al., 1996, Marty 1997), and/or to the growth characteristics of the in vitro environment used in the above studies (serum-free/antibiotic free versus serum supplemented/antibiotic supplemented growth medium; grid assembly versus milli-pore filter substratum, etc.).

Morphological plasticity within developing neural networks has been shown to be activity dependent, where the level and pattern of emerging SBA form the basis for synaptic competition (see Goodman & Shatz, 1993). In the neocortex, neurotrophins can potentiate both neuronal activity (Kim et al., 1994) and dendritic/axonal morphologies (see Cabelli et al., 1995). Recent work has implicated the neurotrophin BDNF in activity-dependent growth of neocortical (McAllister et al., 1996) and hippocampal (Marty et al., 1997) pyramidal and non-pyramidal neurons. McAllister et al. (1996) have shown that dendritic growth in pyramidal neurons within isolated slices of developing ferret neocortex is strongly, and differentially, effected by BDNF. BDNF-associated growth in apical dendrites appeared to be strongly inhibited by the addition of glutamate and L-channel calcium antagonists (see Cabelli et al., 1995). In contrast to the above reported BDNF effects, the present study has shown that apical, as well as basal, dendrites evince significant increases in NT3-promoted growth, following total SBA blockade by combined APV/DNQX application. These findings suggest that NT3 and BDNF may differ in their abilities to modulate dendritic arborization under in vitro conditions. In agreement with this view are the recent findings showing opposing roles of exogenously added BDNF and NT3 in ferret neocortex (McAllister et al., 1997). In this model, NT3 inhibited BDNF stimulated growth in layer IV pyramids, while BDNF inhibited NT3 stimulated growth in layer VI pyramids. It is unknown whether these opposing roles are activity dependent. Whether the production of neurotrophins within in vitro slices is activity dependent has yet to be determined. Increased production of neurotrophins (and in particular NT3) following cross-innervation in control co-cultured neocortical explants may underscore sustained dendritic growth in this in vitro model (Baker & Van Pelt, 1997).

NT3-mediated growth on pyramidal neurons of the neocortex could be expected to exert growth-enhancing effects on non-pyramidal neurons. It has been hypothesized that pyramidal neurons synthesize and secrete neurotrophins under activity-dependent constraints, and that these target-derived molecules then become trophic factors for innervating interneurons. Recent work has shown that NT3 and BDNF act in an activity-dependent manner to modulate GABAergic neuron synaptic transmission (Kim et al., 1997). Moreover, any increase in pyramidal neuron size which would allow for increased numbers of synaptic contacts and possible neupeptide production might also be expected to influence the size of interneurons. For the first time, we show no significant increases in non-pyramidal dendritic length or branching following prolonged exposure to NT3. This suggests that neither NT3 nor increased pyramidal size exert a significant influence over non-pyramidal dendritic growth under the current in vitro conditions.

Finally, the present study has demonstrated the reliability of inducing long-term neuritic growth and complexity using a viral vector encoding a neurotrophin. Previous studies with viral vectors encoding neurotrophins have shown that such vectors can induce radial neurite outgrowth from cochleovestibular ganglia (Geschwind et al., 1996) and dorsal root ganglia without disrupting the overall health of the infected tissues (Dijkhuizen et al., 1996; also see Durham et al., 1996). Furthermore, cultured astrocytes transduced with an adenoviral vector for NT3 secreted sufficient biologically active NT3 to support the survival of co-cultured embryonic neurons (Smith et al., 1996). The present study differs from the previous reports in that we have now shown that dendritic growth intrinsic to and occurring in the natural cellular microenvironment of a postnatal cortical explant, can be promoted with the use of a viral vector encoding a neurotrophin. This study, together with the results of the studies on cultured ganglia, suggest that viral vectors can be applied in vivo in future studies, to direct local and high-level expression of neurotrophins to examine their effects on the patterning of neuritic growth during development, or on neuritogenesis and neuroregeneration following injury.

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Abbreviations

Ad-NT3 adenoviral vector encoding the genetic sequence for NT3
APV NMDA antagonist
BCIP bromo-chloro-indolyl phosphate
DIV days in vitro
DMEM Dulbecco’s minimal essential medium
DNQX non-NMDA antagonist

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