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Research report

Cocultured, but not isolated, cortical explants display normal dendritic development: a long-term quantitative study

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Abstract

Dendritic growth has been studied in long-term organotypic neonatal rat occipital neocortex explants grown either apart as isolated explants or in tandem as cocultures. Quantitative light microscopic measurement of dendritic and axonal branching patterns within the cortical slice was accomplished using rapid Golgi stained materials. In both isolates and cocultures the overall cellular organization of the slice was maintained over 4 weeks in vitro with morphologically distinguishable pyramidal and nonpyramidal neurons located within the same layers and with the same orientations as observed in situ. Long-term increases in the total length of basal dendrites, apical dendrite and axons were observed only in cocultures and were similar to growth patterns reported for in situ materials. Dendritic growth was mainly due to elongation of terminal dendritic segments. Surprisingly, isolated explants showed no long-term increases in total (basal) dendrites, apical dendrites or axons with time in vitro. A transient decrease in the number of basal dendritic segments and increase in terminal segment lengths at the end of the first week in vitro, however, was observed in nonpyramidal neurons. It is hypothesized that (i) afferent inputs and/or efferent targets develop only in cocultures and provide a crucial condition for the continued growth of dendritic/axonal arborization for neocortical neurons in vitro, (ii) intrinsic interconnectivity within isolated explants is not sufficient to maintain long-term growth of neuritic arbors, and (iii) remodelling of dendritic arbors within isolated explants occurs at the same time as these explants are showing noticeable increases in the level of spontaneous bioelectric activity, which suggests that dendritic growth and network formation may be function dependent.

Keywords: Neocortex; Organotypic explant; Coculture; Rapid Golgi; Quantitative morphology; Dendrite/axon

1. Introduction

During development neurites grow and make synaptic connections in the formation of functional networks. Critical stages occur during embryogenesis and in the immediate postnatal period during which a neuron finds its place within the network and, in synergy with its emerging bioelectric properties, finalizes its detailed morphological characteristics (e.g., [11,37,45–48]). While the genetic program of the neuron is capable of allowing for the development of distinctive neural phenotypes (see [23]), epigenetic factors exert powerful influences on the ultimate morphology attained. Of these, emerging spontaneous bioelectric activity (SBA) and trophic factors appear to be most influential [3,11,12,16,20,22,33,34,38,47].

In studying the role of SBA on morphological development and synapse formation in intact animals, one is faced with experimental difficulties: surgery is often complicated, limited accessibility to tissues of interest, and, more importantly, compensatory anatomical and/or functional changes occur making interpretation of the results difficult and tenuous (see [39]). A variety of long-term in vitro model systems have now been developed and provide reliable systems for studying mechanisms underlying the morphological development of neurons within an emerging neural network. Detailed morphological studies are feasible because the anatomical organization of long-term rodent brain slices grown under a variety of culturing conditions retains its cytoarchitectural characteristics over 3–4 weeks in vitro [1,6–8,13–15,17,21,24,25,32,35,36]. Additionally, neocortical slices exhibit lamina-specific connectivity when two such explants are grown together as cocultures [50], or when innervated by various thalamic nuclei [5,9,26,49,50].

Abbreviations: SBA, spontaneous bioelectric activity; DIV, days in vitro

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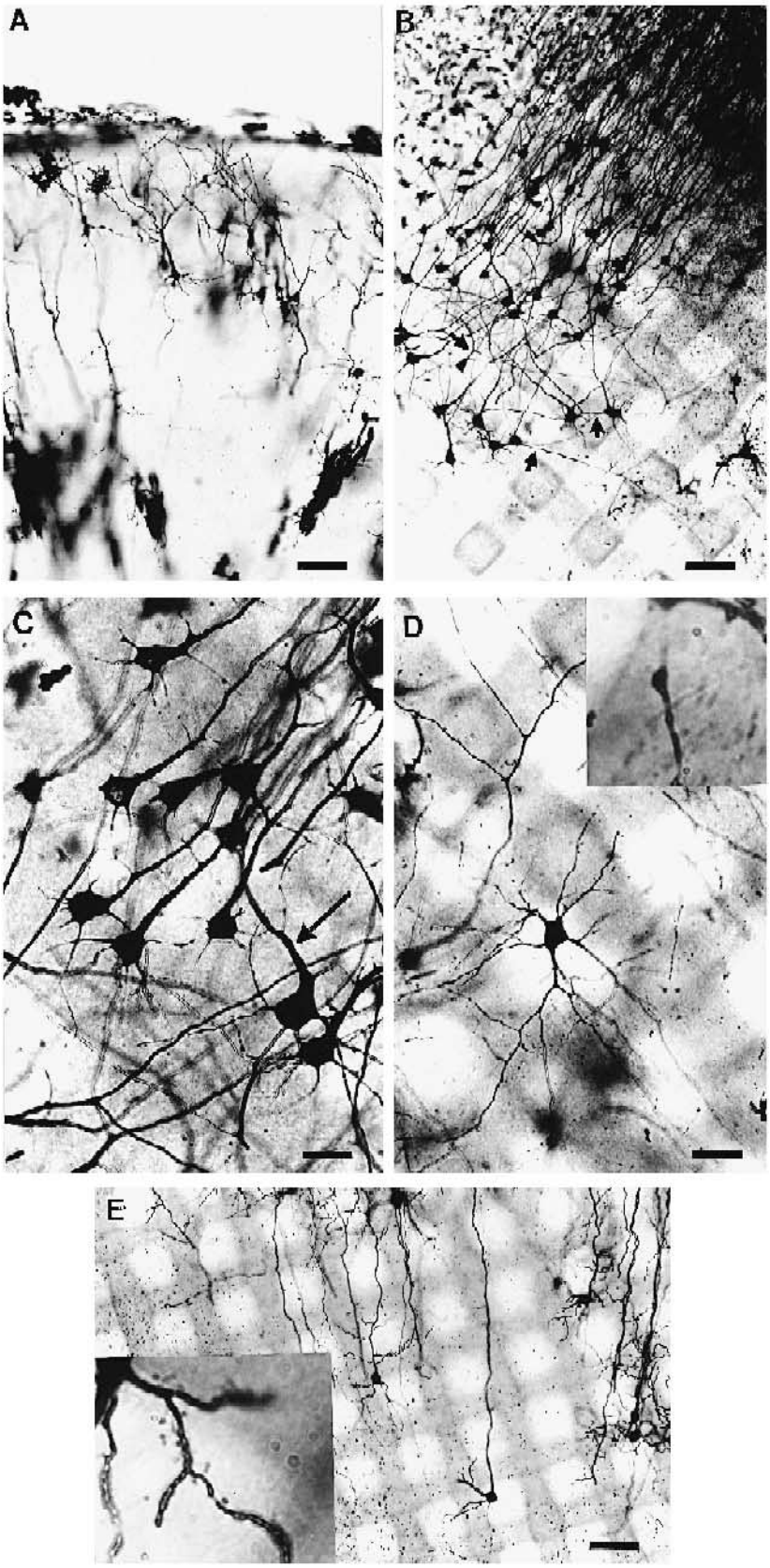
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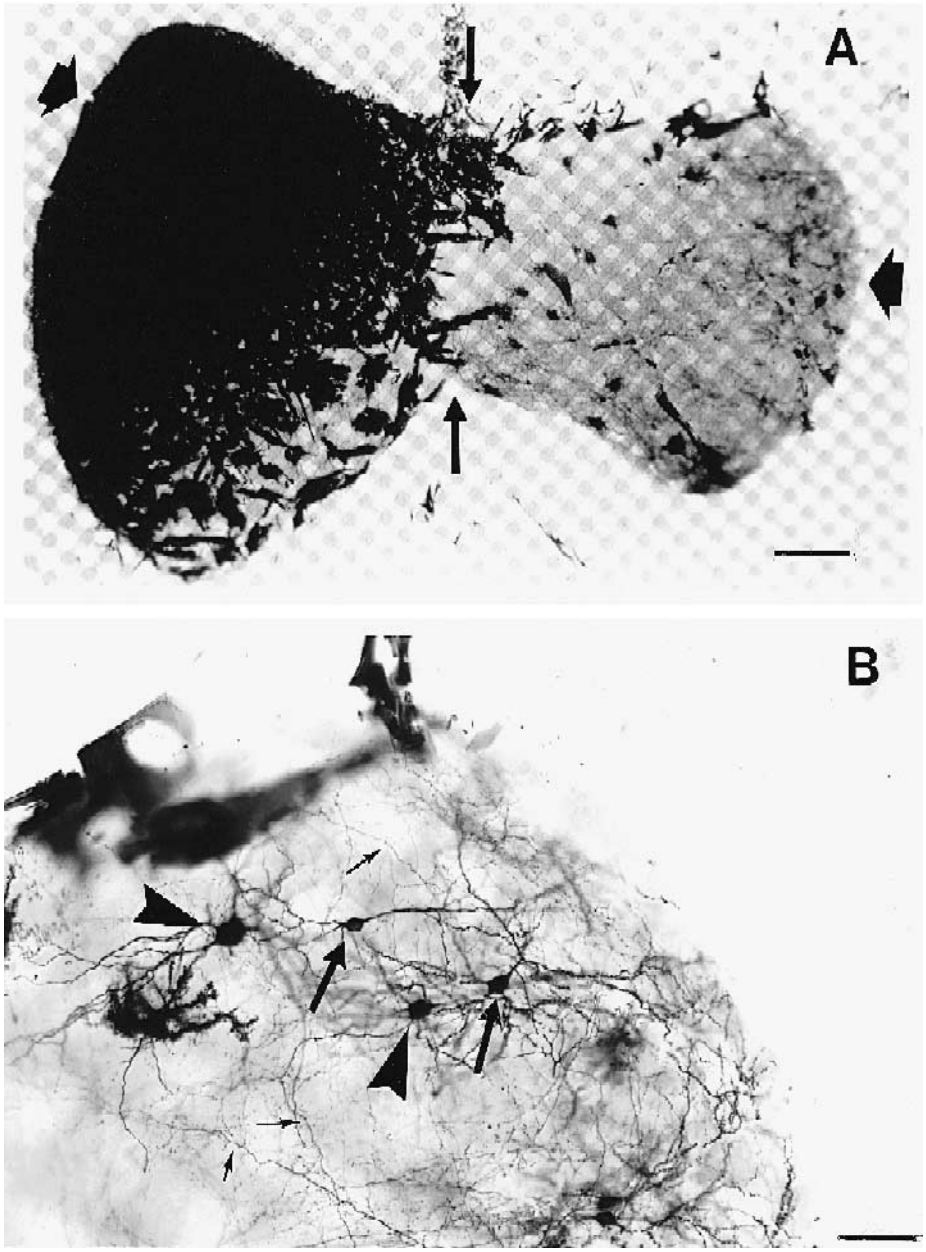

Fig. 2. Example of Golgi impregnated corticocortical coculture grown for 28 DIV. A: The dark precipitate at the left resulted from placement of a crystal of silver nitrate at the pial surface. Only the cocultured neocortical slice has been successfully stained. Pial surfaces (heavy arrows), ventricular abutment zone (thin arrows). Bar = 0.2 mm. B: Enlargement of the upper pial surface of the coculture shown in A, showing pyramidal (arrows) and nonpyramidal (arrowheads) neurons located within the presumptive layers II–III–IV. A heavy network of axons is also seen within this whole mount (thin arrows). Bar = 100 μm.

In spite of the increased interest in the development of in vitro models for studying neuronal development, surprisingly little is known about the quantitative nature of morphological changes that occur under a variety of culturing conditions. Quantitative measurement of dendritic/axonal arbors has been reported in isolated

Fig. 1. Examples of Golgi impregnated fresh (A) and cultured (B–E) neocortical tissues. A: In situ tissue stained from PN6 occipital cortex. Pial surface facing upwards. Bar = 100 μm. B: Overview of neocortical explant stained at 2 DIV. Pial surface located towards upper right-hand corner of photograph. Bar = 50 μm. C: Enlargement of 2 DIV layer V pyramidal cells showing short spike-like basal dendrites, smooth apical dendrites and a misdirected apical dendrite (arrow, also see B at arrows) which has 'righted' itself and projects with its neighbors towards the pial surface. Bar = 16 μm. D: Enlargement of 2 DIV nonpyramidal neuron from layer IV/V interface. Inset shows a growth cone typical of many neurons at this age. Bar = 25 μm. E: Overview of 22 DIV neocortical explant showing layer V pyramidal neurons with their apical dendrites projecting towards the pial surface. Inset shows dendritic spines which are common on many neurons at this age. Bar = 100 μm.

organotypic neonatal mouse neocortex (at 7 DIV) [1]. Intrinsic connections within the slices are sufficient to allow for the early differentiation of both morphological and functional characteristics in presumptive layers V and VI. This maturation shows correlation with emerging SBA on neuronal structure during early stages of network formation. Recent work has also shown that proper Purkinje cell dendritic differentiation relies on receiving innervation from granule cells [3]. Thus, selective afferent input to a given cell type may be essential to the regulation of neuritic growth, in effect, requiring the presence of such input for in vivo levels of maturation to occur under in vitro growth conditions.

The objective of the present study is to establish baseline morphological criteria for studies involved with activity-dependent neurite outgrowth, overgrowth and retraction during the formation of neural networks. The present study is the first to quantitatively examine the development of dendritic and axonal arborization within isolated and cocultured organotypic neocortex explants for up to 4 weeks in vitro. We expected that cellular differentiation and morphological maturation in both types of explants would be similar to that reported for in vivo tissues at similar stages of development [4,19,24,25,29,42], much as has been reported for the functional maturation of these explants within similar time frames in vitro [2]. Unexpectedly we discovered that only in cocultured cortical explants were dendritic outgrowth similar to that reported in situ. This suggested that some facet of coculturing was able to compensate for whatever factor was missing within the isolated explant. Based on these findings in vitro models will allow us to differentiate between the roles connectivity, function and/or trophic factors play in the proper maturation of neural networks, studies which are now in progress.

2. Materials and methods

2.1. PN6 material

Pieces of occipital cortex (caudal to the splenium) were removed from 6 day old rat pups (PN6), fixed in a freshly made solution of 2% glutaraldehyde and kept in the dark overnight at room temperature. The tissue blocks were then briefly rinsed in distilled water and placed in 2% potassium dichromate–1% osmium tetroxide (4 parts to 1 part, respectively) for 3–6 days in the dark at room temperature. The pieces were then washed with 0.75% silver nitrate until the red precipitate could no longer be seen in the wash and then transferred to fresh silver nitrate and kept for 3–6 days in the dark at room temperature. The pieces were then transferred to 70% ethanol and cut on a vibratome at 160 μm. The sections were transferred to subbed slides, dehydrated, cleared in Histoclear and mounted in Histomount.

2.2. Explants

Slices taken from 6 day old Wistar rat pup occipital cortex (caudal to the splenium) were cultured according to the procedure described by Romijn et al. [31]. Briefly, 360 μm thick, coronal sections were cut from the occipital cortex from which three pieces of cortex, approximately 1 mm wide, extending from the dorsal midline were cut and grown on a polyamide carrier grid. Explants (two) were grown apart from one another (isolates) or positioned so that two explants made contact with one another at their ventricular edges (cocultures, Figs. 1 and 2). The growth medium consisted of a serum-free nutrient medium (see [31]). The explants were continuously rocked to insure an optimal exchange of nutrients and wastes and were maintained at 35°C in an atmosphere of 95% air/5% CO_2. The nutrient medium was refreshed three times a week. Isolates were selected for study at 2, 4, 6, 8, 10, 14, 18, 23 and 28 days in vitro (DIV), while cocultures were selected at 4, 8, 14, 17 and 28 DIV. All explants were taken from culture series which were set in from animals taken from the same litter.

2.3. Staining techniques

The explants were stained as whole mounts using a variation of the Colonnier method of rapid Golgi impregnation according to the protocol described by Caeser and coworkers [6,8]. The cultures were fixed in 2–3.5% potassium dichromate and 5% glutaraldehyde for 2–4 days at 4°C. They were then washed briefly in distilled water and a small grain of silver nitrate placed (under microscopic control) onto the edge of the explant at the pial surface using a broken glass micropipette. The cultures were then maintained for 24–48 h in the dark at 4°C before being dehydrated in ethanol, transferred to Histoclear and mounted with Histomount.

2.4. Measuring techniques

Stained neurons were morphologically reconstructed using a semi-automated dendrite measuring system [28,40]. We have quantitatively measured the following parameters: (i) total dendritic length per neuron; (ii) total apical dendritic length; and (iii) total axonal length. Total dendritic length measurements were further analyzed as follows: (a) path length (length of dendritic path from soma to a terminal tip); (b) intermediate segment length (segment length between two consecutive branch points); (c) terminal segment length (segment length between a final branch point and terminal tip); (d) number of dendritic segments per neuron; and (e) number of tips per dendrite. All measurements on cultured explants were accomplished from intact cultures as whole mounts. Occipital neocortex from postnatal day 6 animals (PN6) was taken as a quantitative starting point for the various measurements carried out on cultured tissues.

2.5. Statistics

The developmental curve of each morphological parameter was statistically tested for possible age effects using the nonparametric Kruskal-Wallis test [10]. Only when a significant age effect was found (at a 0.05 significance level) was a multiple comparison procedure applied in which pairs of age groups were tested to elucidate more precisely age related differences. The outcomes of the multiple comparison procedure were used to identify certain trends which occurred in more than one variable and/or were present over several age points in succession.

3. Results

3.1. Organization of the organotypic explant

The cytoarchitecture of both isolates and cocultures used in the current study did not differ from those described previously [14,30,32]. Unlike serum-grown explants, tissues grown under our serum-free culturing conditions maintained their original organotypic shape and three-dimensionality throughout the in vitro period with no discernable cellular migration away from the explant borders. Only an initial thinning of the explant from the original 360 μm thick slice occurred over the first several days in vitro, probably associated with the loss of those cells mortally damaged at the time of sectioning. Final explant thickness generally exceeded 150 μm (to over 200 μm); 6–10 cells deep from surface to grid.

The overall cellular organization of the neocortical slice was maintained throughout the periods of cultivation. Pyramidal cells occupied positions in presumptive layers III–IV and V–VI; their apical dendrites oriented towards the pia (Fig. 1B,C,EFig. 2B). Most apical dendrites from the deeper (V–VI) layers appeared to end well below the pial surface (in presumptive layers III–IV) in the isolated explants. In cocultures, apical dendrites from pyramidal neurons in layers V and VI frequently projected to the pial surface. Apical dendrites from pyramidal neurons located in layers III–IV in both types of explants terminated at or near the pial surface. Occasional inverted pyramidal cells or misdirected apical dendrites were observed (Fig. 1C). Basal dendrites were easily distinguished as was the axon which usually projected towards the ventral surface of the explant. Dendritic spine densities appeared much denser in cocultures compared with the isolates, and the number of identifiable growth cones appeared to decrease with age (Fig. 1D,E). No attempt was made to quantify either structure.

3.2. Neuritic development

The anatomy of PN6 tissue is presented in Fig. 1A and included in Fig. 3 as 0 DIV in the isolate explant panels. Location of each cell within the explant was noted. No attempt was made to classify large or small pyramidal cells, or to identify a particular type of nonpyramidal neuron.

Those cell parameters showing a significant developmental age effect according to the Kruskal-Wallis test were followed by multiple comparisons. Tissues taken at PN6 were compared with in vitro isolated explants plated from the same brains and maintained for 2–4 DIV as a means of determining whether surgical effects might have occurred following plating of the tissues in vitro. There was no difference between PN6 tissue measurements and early culture values, the only exception being that a significant difference occurred in the branching of pyramidal cell dendrites (Fig. 3K). The same age effects observed in the 2–28 DIV culture series continued to be seen in the PN6-early in vitro series. We therefore felt confident that the two culture groups could be combined in this study and that no serious surgical effects on the overall morphology of the tissues were occurring.

3.2.1. Total (basal) dendritic length

Total (basal) dendritic length of both pyramidal and nonpyramidal cells showed a remarkable constancy during the entire culturing period. However, pyramidal neurons did show a transient, but significant, increase at 10 DIV. Cocultures, on the other hand, showed a significant increase in total (basal) dendritic length during the first 3 weeks in vitro for both cell types (Fig. 3B) which plateaued over the subsequent 2 week growth period. The amount of tissue explanted had no effect on total basal dendritic length as measured in the isolates. Wide, narrow (half the size of wide explants) and thick (ca. 550 μm) explants showed no significant differences in this parameter (data not shown).

(a) Path length. Nonpyramidal neurons within the isolates showed alternating patterns in mean path length, with an increase in the first 10 DIV, a decrease in the third and again an increase in the fourth week in vitro (Fig. 3C). Pyramidal neurons, however, showed a significant increase in mean path length during the second week in vitro followed by a rapid decrease and stable level thereafter. In the cocultures, mean path length significantly increased for the pyramidal neurons to about 14 DIV and levelled off, while for nonpyramidal cells this increase continued to about 17 DIV before levelling off (Fig. 3D).

(b) Intermediate segment length. Pyramidal neurons within isolated cultures showed a short-lived significant increase in intermediate segment length at the end of the first week in vitro (Fig. 3E). A similar increase was observed for pyramidal neurons in cocultures, except that the increase began shortly after plating and peaked somewhat later (ca. 10 DIV; Fig. 3F). Transient, fluctuating changes were observed for intermediate segment lengths for nonpyramidal neurons in isolated explants.

(c) Terminal segment length. Pyramidal neurons in iso-

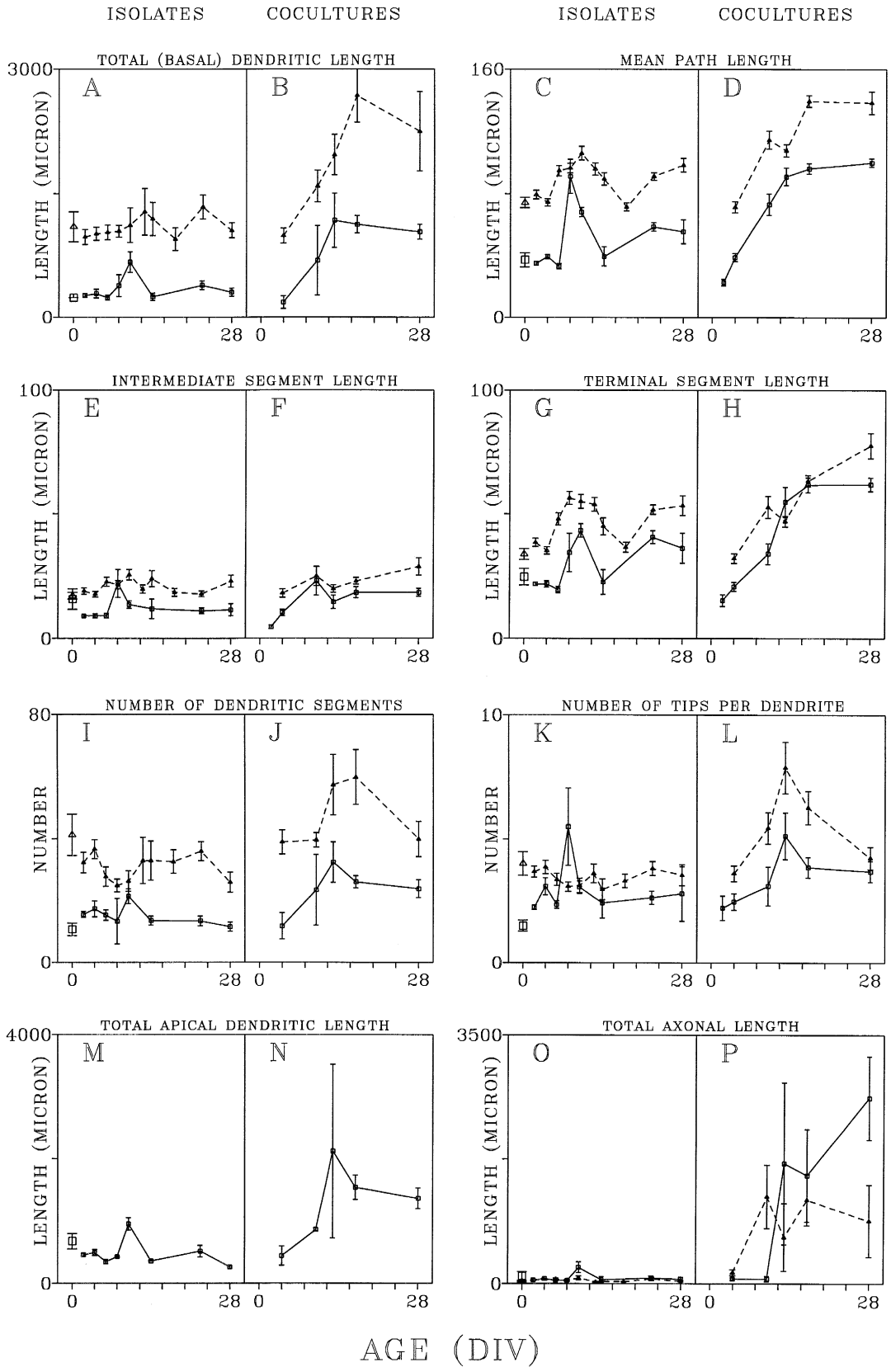

Fig. 3. Age curves for occipital neocortical explants grown for up to 28 days in vitro. Each graph couplet represents similar measurements for both isolated (left) and cocultured (right) explants. Mean (S.E.M.) values are presented for pyramidal (continuous lines) and nonpyramidal (broken lines) neurons. PN6 tissue is represented at 0 DIV in the isolates panels. See text for description of each parameter.

lated explants showed variable increases and decreases in this parameter throughout the culturing period (Fig. 3G). Nonpyramidal neurons showed a similar, somewhat earlier occurring, growth pattern. In cocultures, both pyramidal and nonpyramidal neurons showed a significant increase in terminal segment lengths with time in vitro (Fig. 3H). This increase appeared to plateau for the pyramidals at about 17 DIV, but continued up to 28 DIV for the nonpyramidal neurons.

(d) Dendritic segments. No significant age effects were observed for either pyramidal or nonpyramidal neurons throughout the culturing period (Fig. 3I). Cocultured explants also showed no significant age effects in dendritic segments over the four weeks in vitro (Fig. 3J).

(e) Number of tips per dendrite. A significant increase in branching of dendrites in pyramidals occurred during the first week in vitro (Fig. 3K). Nonpyramidal neurons did not show any significant age effect throughout the culturing period. A significant branching in both cell types occurred in cocultures during the first 2 weeks in vitro followed by significant decreases in the third and fourth weeks for nonpyramidal cells (Fig. 3L). Pyramidal neurons plateaued in the last 2 weeks.

3.2.2. Total apical dendritic length

A significant, short-lived increase was observed to occur in isolated apical dendritic length during the second week in vitro (Fig. 3M). Apical length decreased by the end of the second week and remained unchanged for the remainder of the growth period. In cocultures, an apparent, but not significant, apical length increase occurred at the end of the second week in vitro and then remained stable during the last 2 weeks (Fig. 3N).

3.2.3. Total axonal length

Throughout the full culturing period pyramidal and nonpyramidal neurons showed no significant growth in their axons in isolated cultures (Fig. 3O). In cocultures, however, pyramidal neurons showed a strong and continued increase in axon length between the second and fourth weeks in culture (Fig. 3P). Nonpyramidal neurons showed a substantial increase in axonal length between 4 and 10 DIV and a plateau for the remainder of the growth period.

4. Discussion

The present study is the first to quantitatively document the development of neuritic growth in long-term neocortical explants maintained in vitro as isolated explants or grown as cocultures. The major findings of our investigations are that (i) the overall organization of the explant (recognizable major cell types with layering and orientation of major neuritic processes as observed in vivo) remained stable throughout at least 4 weeks in vitro; (ii) the growth patterns of total (basal) dendritic lengths within the cocultures were similar to those described in situ for both pyramidal and nonpyramidal neurons, and were concerned most prominently with elongation of the terminal segments; (iii) in contrast to the cocultures, isolated neocortical explants displayed markedly constant total basal segment lengths in both cell types, demonstrating a lack of growth with age in these explants. However, a transient increase in dendritic segment length was observed at the end of the first week in vitro in these explants.

Comparing dendrite length at day 6 (i.e., 0 DIV) with data reported for in situ tissue [41,42] we found equal values for pyramidal neurons, but higher values for nonpyramidal neurons. This difference may be due to the strain of rat examined (Sprague-Dawley vs. Wistar) but is most likely related to our measuring a variety of nonpyramidal cells throughout the depth of the neocortex, while those reported by Uylings et al. [41,42] came from only multipolar cells within layer IV. Also, during subsequent culturing nonpyramidal dendritic length exceeded the in situ data for both pyramidal and nonpyramidal cells. However, peak values for total (basal) dendritic and terminal segment lengths as well as number of dendritic segments of small layer V and layer II–III pyramidals were similar to those reported in situ [42]. These data demonstrate that the growth conditions used in the current study have not provoked an undue effect on our explants with regard to their morphological development.

Based on our present results we conclude that neocortical dendritic/axonal development for both pyramidal and nonpyramidal neurons shows a rapid growth phase that ends shortly after the second week in vitro within cocultured explants. While the growth phase resembles that observed in situ, the onset of the growth phase occurs ca. 4 days later than reported for intact tissues (see [42]), which undoubtedly reflects a lag time in growth for tissues brought into culture. The important observation here is that the growth phase we report for cocultured tissues is similar to that reported in situ in both duration (ca. 12 days) and scope (length of neurites) ([42]; see Fig. 3B).

It was highly surprising that cocultures of **like** tissues exhibited network maturation at in situ levels, whereas two isolated explants within the same dish showed no long-term neuritic growth. Since growth conditions were identical for both explant types several possible conditions (or combinations) may underscore the observed retardation of neuritic growth: size of explant, isolation, configuration of coculturing, growth factors or SBA.

4.1. Size

We observed that increasing the size (width or thickness) of the isolated explant did not result in an overall increase in dendritic lengths. Thus, we must conclude that it is not merely the amount of tissue present which effects outgrowth in these explants.

4.2. Isolation

Isolation denies developing networks their natural afferent inputs and efferent targets suggesting that cues for proper dendritic/axonal differentiation are not inherent in individual neocortical neurons or available from the intact local neocortical environment. Reduced afferent input has a pronounced effect on dendritic outgrowth (e.g., [20,38]), which may be more pronounced in an in vitro preparation since the majority of the afferent inputs to the explant have been severed. Notwithstanding the absence of maintained changes in a number of cell and segmental parameters reported here for isolated cortical explants, significant age effects nonetheless did occur. One such effect was a significant increase in total basal dendritic length in pyramidal neurons (and to a lesser degree in nonpyramidal cells) between 6 and 14 DIV (Fig. 3A). Similar increases in dendritic lengths were also observed by Annis et al. [1] at these same ages. Moreover, there appeared to be an alternating pattern in terminal segment length, peaking at about 8 DIV and in the fourth week, and the counteracting pattern in the number of dendritic segments (see Fig. 3C,I).

4.3. Configuration

The data presented in the present study are taken from cocultures which have been grown ventricle-to-ventricle. It is unknown, therefore, whether other possible configurations might also result in the same growth responses as are reported here (e.g., side-by-side; pia-to-pia; side-to-ventricle). However, it has been reported that, at least for thalamic-neocortical cocultures, position of the thalamic tissues posed no obstacle for target location by outgrowing neocortical efferents [5,9,26,27,49,50].

4.4. Growth factors

Cross-innervation might contribute to dendritic development by release or stimulation of production of growth factors which mediate dendritic maturation. Neurotrophins appear to mediate survival and developmental effects on Purkinje and granule cells (see [3]). Cortical growth factors appear to mediate elongation of murine and kitten neocortical neurons and may foster selective innervation of the visual neocortex [18,22]. Maturational differences within the neocortex as reported in the present study suggest that either isolated neocortical explants may not produce growth factor(s) which would allow intrinsic neuritic growth to occur, or that such production is insufficient to sustain growth. Moreover, it must be concluded that no diffusible factor operates between isolated explants grown on a common grid, and that the presence of afferent inputs may stimulate growth factor/receptor production, perhaps under the influence of SBA (see [3,18,22]).

4.5. Spontaneous bioelectric activity

An obvious mechanism by which afferent input could operate on neuritic differentiation would be that of bioelectric activity. SBA has been shown to promote growth, branching and stabilization of dendritic form (e.g., see [3,23,43,47]). The onset of dendritic/axonal growth seen here coincides with the emergence of SBA within isolated explants [2]. Preliminary data from our laboratory have shown that SBA may be operating in our model systems: there is a strong increase in the frequency of bioelectric discharges in cocultured explants compared with that registered from isolated slices (manuscript in preparation). Since electrical activity, depolarizing media and neurotransmitters all may affect neurite outgrowth in many cell types, including pyramidal neurons [2,23,43–45], we suggest that the segmental responses are associated with emerging bioelectric activity.

Experiments are now in progress to determine the roles coculture configuration, growth factors and their relationship with SBA as well as SBA itself play within both isolated and cocultured neocortical slices.

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