Research report

Chronic blockade of glutamate-mediated bioelectric activity in long-term organotypic neocortical explants differentially effects pyramidal/non-pyramidal dendritic morphology

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Abstract

Dendritic/axonal growth has been examined in long-term organotypic neocortical explants taken from neonatal rat pups and grown either as isolated slices or as co-cultures. The quantitative light microscopic measurement of dendritic and axonal branching patterns within both types of explants was carried out on Golgi-stained materials. Spontaneous bioelectric activity (SBA) was blocked within both types of explants using a combination of APV and DNQX, NMDA and non-NMDA receptor antagonists, respectively. No extracellularly measurable SBA was observed to occur in the silenced explants in the presence of both antagonists but reappeared following wash-out with control medium. In both control and silenced explants, the overall cellular organization of the slice was maintained throughout the culturing period, with distinguishable pyramidal and non-pyramidal neurons located within the same layers and with the same orientations as observed in situ. The major findings of the present study show the following. (i) *Pyramidal* neurones chronically exposed to APV/DNQX exhibited no basal dendritic growth in *co-cultured* explants, while growth of apical dendritic lengths was similar to control values in the absence of SBA. (ii) *Pyramidal* neurones, nonetheless, exhibited significant terminal segment growth under SBA blockade which was correlated with a concomitant decrease in number of basal dendrites. (iii) Axonal growth in co-cultures was not sustained in silenced pyramidal neurones. (iv) *Non-pyramidal* neurones showed significant total dendritic and axonal growth in co-cultures following APV/DNQX treatment. (v) *Non-pyramidal* cells in co-cultures experienced an increase in terminal segment length at 2 weeks which declined in the third week. This increase–decrease was correlated with a decrease–increase in the total number of dendritic segments during the second and third weeks, respectively. (vi) In *isolated* explants the only departure from control growth curves was a significant increase in terminal segment length which was offset by a similar decrease in number of dendritic segments under APV/DNQX growth conditions. Thus the chronic loss of glutamate-mediated SBA differentially effected pyramidal and non-pyramidal neurones in isolated and co-cultured explants, with pyramidal neurones experiencing the more pronounced effects. We conclude that SBA effects the dynamics of neuritic elongation and branching and that these changes are most dramatically seen in co-cultures which cross-innervate one another, presumably via pyramidal axons. We hypothesize that the activity-dependent changes associated with reduction in pyramidal dendritic and axonal growth may be associated with neurotrophin receptor production/maturation. © 1997 Elsevier Science B.V.

Keywords: Tissue culture; Neocortex; Organotypic slice; Dendritic morphology; APV/DNQX; Bioelectric activity

1. Introduction

During development, dendrites and axons grow out from neurones to form interconnected neural networks which ultimately determine behaviour and function of the organism. Spontaneous bioelectric activity (SBA) and trophic/growth factors play a crucial role in the development of emerging neural networks [6,16–18,23,24,34–37,42]. Emerging SBA has been positively correlated with the elongation and branching of dendritic and axonal arbours in early neonatal mouse neocortex [1]. While both SBA and trophic/growth factors have been shown to influence neuritic growth (trophic/growth factors also appear to be under the influence of SBA), it is unknown what interplay occurs between the two mechanisms to promote morphological maturation.

Previous studies have shown that neonatal rat neocortex can be successfully maintained in long-term cultures as organotypic explants [19,20,32]. The cytoarchitectural or-

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ganization of the slice remains virtually unchanged throughout cultivation, with pyramidal and non-pyramidal neurones occurring within recognizable layers throughout the dorsal–ventral extent of the explant. Further studies have demonstrated that when neocortical tissue was grown as an isolated slice no sustained growth (i.e., elongation and/or branching) occurred in either dendrites or axons throughout 4 weeks of culturing [5]. Lack of growth was not dependent on the *amount* of tissue cultured, since doubling the width or thickness of such explants failed to enhance dendritic/axonal growth characteristics. When two such explants were placed ventricle-to-ventricle and grown as a co-culture, on the other hand, sustained dendritic/axonal growth occurred and was similar to that reported for the same tissues and ages in situ [39]. It was unknown, however, whether the configuration of the co-cultures played a major role in efferent fibre ingrowth. Others have reported that positioning of presented slices around neocortical explants does not appear to inhibit the location of and invasion by outgrowing pyramidal axons [8,29,38,44]. It is unknown whether the location of target neurones by presented explants would have pronounced effects on neuronal maturation in either slice. We hypothesize that afferent inputs from contralateral neocortical explants underscored the sustained growth observed in co-cultures and that this growth occurred in association with emerging SBA.

The objective of the present study was to determine whether *bioelectrical activity* per se promotes sustained dendritic/axonal growth in our co-culture model. Total glutamatergic blockade can be achieved with the use of the NMDA and non-NMDA antagonists (APV and DNQX, respectively) rendering the explants silent between refreshings. We expected that cytoarchitectural details of both isolated and co-cultured explants, as well as cellular differentiation, would be unaffected under control and blockade conditions, as had been shown in previous studies using TTX or elevated magnesium to block bioelectric activity [2,33]. It was unknown how the dendritic/axonal development of neurones within silenced explants (both isolated and co-cultured) would be effected, since both elongation and suppression of dendritic outgrowth has been reported in neocortical and hippocampal cultures experiencing TTX- or NMDA receptor-induced blockade [9,30,39,40]. We hypothesized, however, that if SBA was responsible for sustained dendritic/axonal growth in co-cultures (either on its own or through an interplay with growth factors) that SBA blockade would block such growth, and that if SBA were not involved growth would continue. Finally, we expected that positioning of the two slices would not significantly affect cytoarchitecture, interconnectivity or growth within the model.

We now report that elongation and branching of dendritic/axonal arbours in long-term isolated and co-cultured organotypic neocortical slices was differentially effected in pyramidal and non-pyramidal neurones following chronic blockade of glutamate-mediated neurotransmission. We hypothesize that these activity-dependent differential effects are due to afferent target induced changes associated with neurotransmitter and/or neurotrophin receptor production/maturation.

2. Materials and methods

2.1. Cultures

Isolated and co-cultured neocortical slices (360 μm thick, from presumptive visual cortex) were taken from 6-day-old Wistar rat pups and grown on a polyamide grid assembly in a serum-free growth medium for 4–28 days in vitro (DIV; see [32]). For isolated explants, two similarly sized slices were grown apart from one another on the grid assembly. No physical contact developed between these slices over the entire growth period. For co-cultured explants, two similar sized slices were placed in physical contact with one another, varying the orientation of the slices at the point of contact (see below). The cultures were housed in stainless steel boxes which were continuously rocked in order to assure maximal exchange of nutrients and wastes. The explants were maintained in 5% CO_2–95% air at 35°C and refreshed 3 times per week. At 2 DIV, the experimental groups were exposed to 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.

2.2. Histology

Explants were fixed for a rapid Golgi impregnation as described by Caeser and co-workers [11,12]. Briefly, cultures were fixed in 3.5% potassium dichromate–5% glutaraldehyde for 3 days at 4°C. The explants were then briefly rinsed with distilled water. Excess water was then removed and a small crystal of silver nitrate was placed at the pial surface near one edge of the explant. The cultures were returned to the refrigerator for a further 2 days. Successfully stained explants were then dehydrated and mounted with Histoclear and Histomount.

A number of co-cultured explants were also examined with the fluorescent dyes, DiI and DiA. The cultures were lightly fixed with 4% paraformaldehyde ($\approx$ 2 h) and then a small crystal of either DiI or DiA was inserted into one of the two explants. The explants were then housed in Eppendorf tubes in paraformaldehyde at room temperature for 3–6 weeks. Explants were examined under fluorescent microscopy.

2.3. Dendritic measurements

Successfully Golgi-stained pyramidal and non-pyramidal neurones were examined under a semi-automated

measuring microscope [31]. From 3D reconstructed neurones the following morphological features were extracted: (i) (basal) dendritic length, (ii) axonal length; (iii) terminal segment length, (iv) apical dendritic length; (v) number of basal dendrites, (vi) degree (number of terminal tips per dendrite).

2.4. Electrophysiology

The entire grid assembly containing the cultured explant was positioned in a recording chamber mounted over an inverted microscope with recording and stimulating glass microelectrodes visually positioned above the explants. The explants were superfused with a carbogen-equilibrated Dulbecco's minimal essential medium (DMEM) at 35°C with a flow rate of approximately 3 ml/min. The area chosen for stimulation and recording of evoked potentials was a cell-rich region easily identified in each slice, corresponding to presumptive layers II–IV. Control explants were examined for the presence or absence of SBA at a number of points across the slices with a recording electrode (filled with 300 mM NaCl; tip diameter: 2–5 μm; tip resistance: 0.6–3.0 MΩ). Explants grown in the APV/DNQX cocktail were examined for the presence of SBA in DMEM 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 explant. Explants were stimulated with a single, monopolar cathodal pulse of 0.1 ms duration and 30–60 nA amplitude as a means of evoking responses within and between explants (electrode tip diameter: 3–5 μm). A site was scored as positive when consistent responses to the applied stimulus were observed.

2.5. Statistics

The developmental curve of each morphological parameter was statistically tested for possible *age* effects using the non-parametric Kruskal-Wallis test [15]. When a significant age effect was found (at $P = 0.05$) a multiple comparison procedure was applied in which pairs of age groups were tested to elucidate more precisely where the age-related differences occurred. In the case of an immediate comparison between two age groups, the Mann-Whitney U-test was applied. When borderline significance was obtained, a further inspection with Student's t-test was applied to decide whether the outcome of the comparison was considered to be significant.

3. Results

3.1. Explant configuration

A number of possible co-culture configurations were examined in order to determine whether the manner in which explants were joined in vitro would influence growth characteristics. As such, two similar sized neocortical explants were placed in physical contact with one another at their side edges (side-by-side), pial surfaces (pia-to-pia), ventricular surfaces (ventricle-to-ventricle) and with ventricle-to-side connections. Pia-to-pia, ventricle-to-side and ventricle-to-ventricle co-cultures showed significant total (basal) dendritic growth as compared with their respective isolate controls. Only the side-by-side configuration failed to show the significant growth characteristics in total segment length, total basal dendritic length and total apical dendritic length (for pyramidal neurones) as compared with respective isolated slices.

3.2. Explant and cellular morphology

Both isolated and co-cultured explants retained their overall cytoarchitecture throughout the culturing period (Fig. 1A,B). No appreciable loss of cells occurred during the first 2 weeks in vitro as measured by DNA levels (data not shown). Recognizable Golgi- and fluorescent-stained pyramidal and non-pyramidal neurones (Fig. 1C,D,F) were observed throughout the explant(s), as well as neocortical cell layering. Pyramidal neurones had well-defined apical dendrites projecting towards the pial surface. Apicals from layer III–IV pyramidals inevitably terminated at the pial surface. Apicals from layer V and VI pyramidals did terminate within layer I, but it was not unusual to observe them terminating well below the surface, in layers II–IV (see [39]). Occasionally, misdirected apical dendrites were seen.

Axonal fields were often seen in the Golgi-stained co-cultures and crossing of the abutment zone between co-cultures was common (Fig. 1E). The presence and density of such cross-innervation between co-cultures was most evident when fluorescent dyes were used. Both retro- and anterograde transport of DiI and DiA revealed large numbers of filled fibres and cells (Fig. 1F).

3.3. Dendritic / axonal measurements

There was no measurable age-related growth in basal dendritic lengths in *pyramidal* neurones following glutamatergic blockade in co-cultures ($P > 0.25$; Fig. 2). Nevertheless there was a significant increase in terminal segment length ($P > 0.0001$; Fig. 3) without an accompanying loss in the number of dendritic segments (Fig. 4). Length of apical dendrites in the experimental explants showed a similar age effect as that observed in their respective control group. Pyramidal axons did not show a growth effect into the third week, as was reported for their respective controls (Fig. 2).

Dendritic growth in *non-pyramidal* neurones in co-cultures showed significant increases in total dendritic length following glutamatergic blockade ($P > 0.04$; Fig. 2). During the second week growth was due to significant increases in terminal ($P < 0.0001$; Fig. 3), and intermediate

(measurements not shown) segments lengths, whereas in the third week the increases come about from increases in the total number of dendritic segments ($P < 0.05$; Fig. 4). Axonal growth in non-pyramidal neurones in co-cultures was unaffected by SBA blockade (Fig. 2).

Pyramidal neurones within *isolated* explants showed no basal, apical dendritic/axonal growth upon SBA blockade (Figs. 2–4). Nor was there any significant growth observed in non-pyramidal dendrites and axons. There was, nonetheless, a significant increase in terminal length values for both pyramidal ($P < 0.05$) and non-pyramidal ($P < 0.001$) neurones which was offset by decreases in the total number of dendritic segments ($P < 0.01$ for non-pyramidals; Figs. 3 and 4).
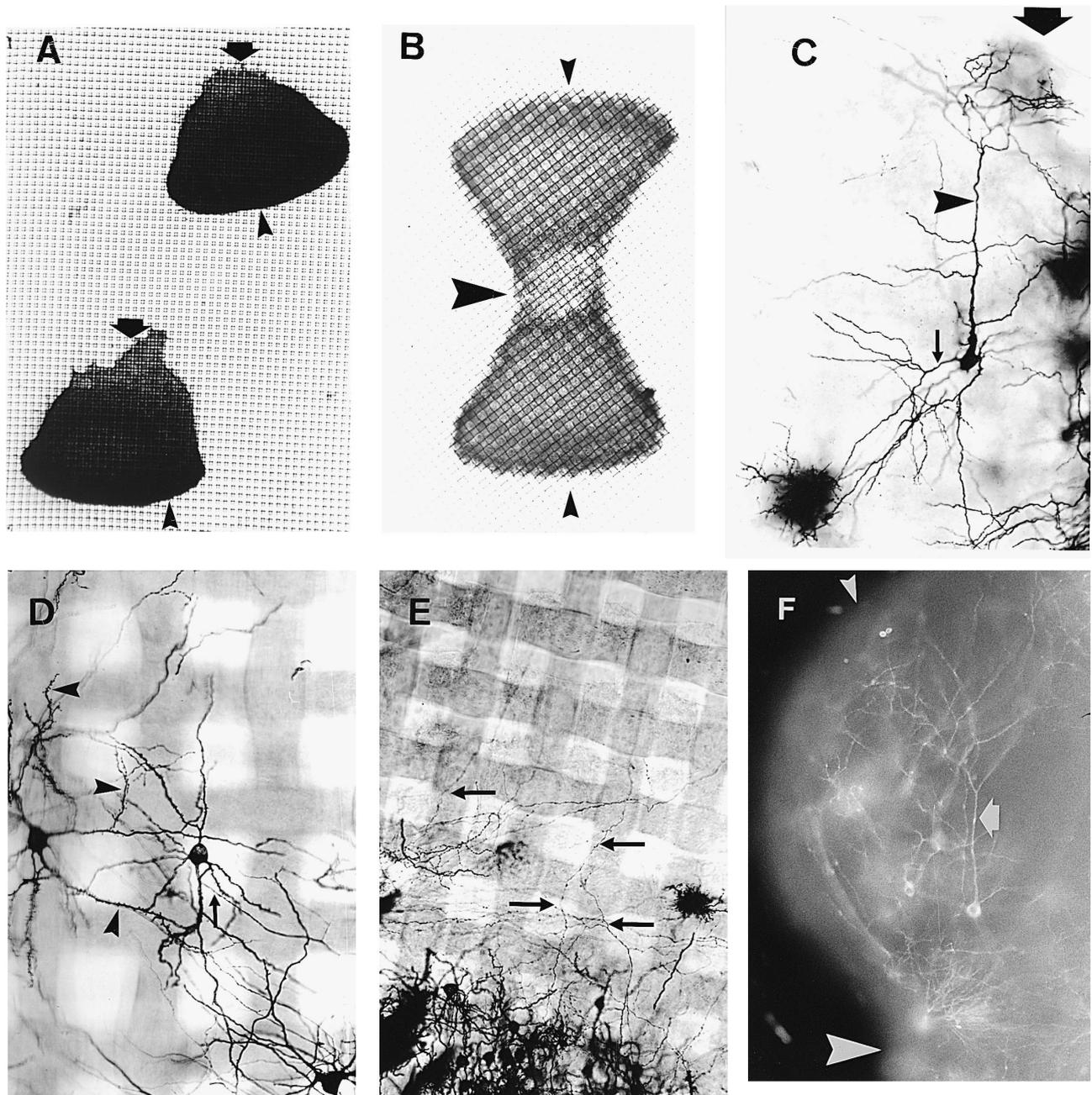

Fig. 1. A: overview of thionine-stained isolated neocortical slices. A well-defined pial surface (arrowheads) and ventricular surface (larger arrows) can be readily recognized. Explants are 6 DIV. B: thionine-stained co-cultured control neocortical explants (6 DIV). Pial surfaces are well defined (arrowheads) with an abutment occurring at the ventricular surfaces (large arrowhead). C: pyramidal neurone (10 DIV) in layer IV is shown with a recognizable apical dendrite (arrowhead) extending to the pial surface (large arrow) with basal dendrites (arrow). D: non-pyramidal neurones (10 DIV) in layer V with spine-covered dendrites (arrowheads) and axons (arrow). E: Golgi-stained axons seen at the abutment zone between two opposing neocortical slices (28 DIV). Several axons can be seen to cross between the two explants (arrows). F: DiA-stained co-culture (13 DIV) showing retrogradely filled pyramidal neurone in layer VI with its apical dendrite (arrow) extending towards pial surface (small arrowhead). Abutment zone shown at large arrowhead.
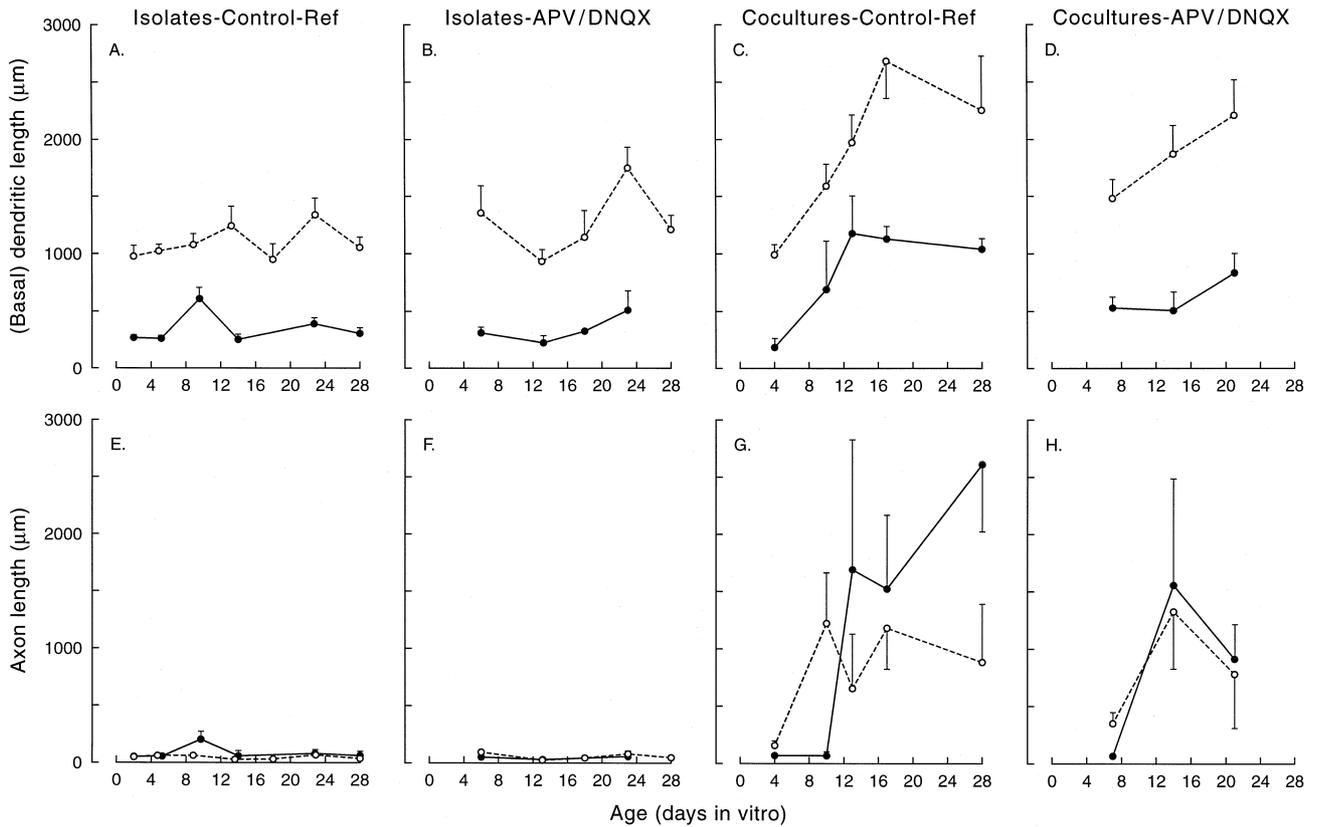
Fig. 2. Curves showing total (basal) dendritic and axonal growth in control and chronically silenced isolated and co-cultured neocortical explants. Reference curves for Fig. 3, Fig. 4, and Fig. 5 have been taken from Baker and Van Pelt, 1997. Mean and S.E.M. values are displayed for this and the next two figures. ●, pyramidal neurones; ○, non-pyramidal neurones here and in following figures.
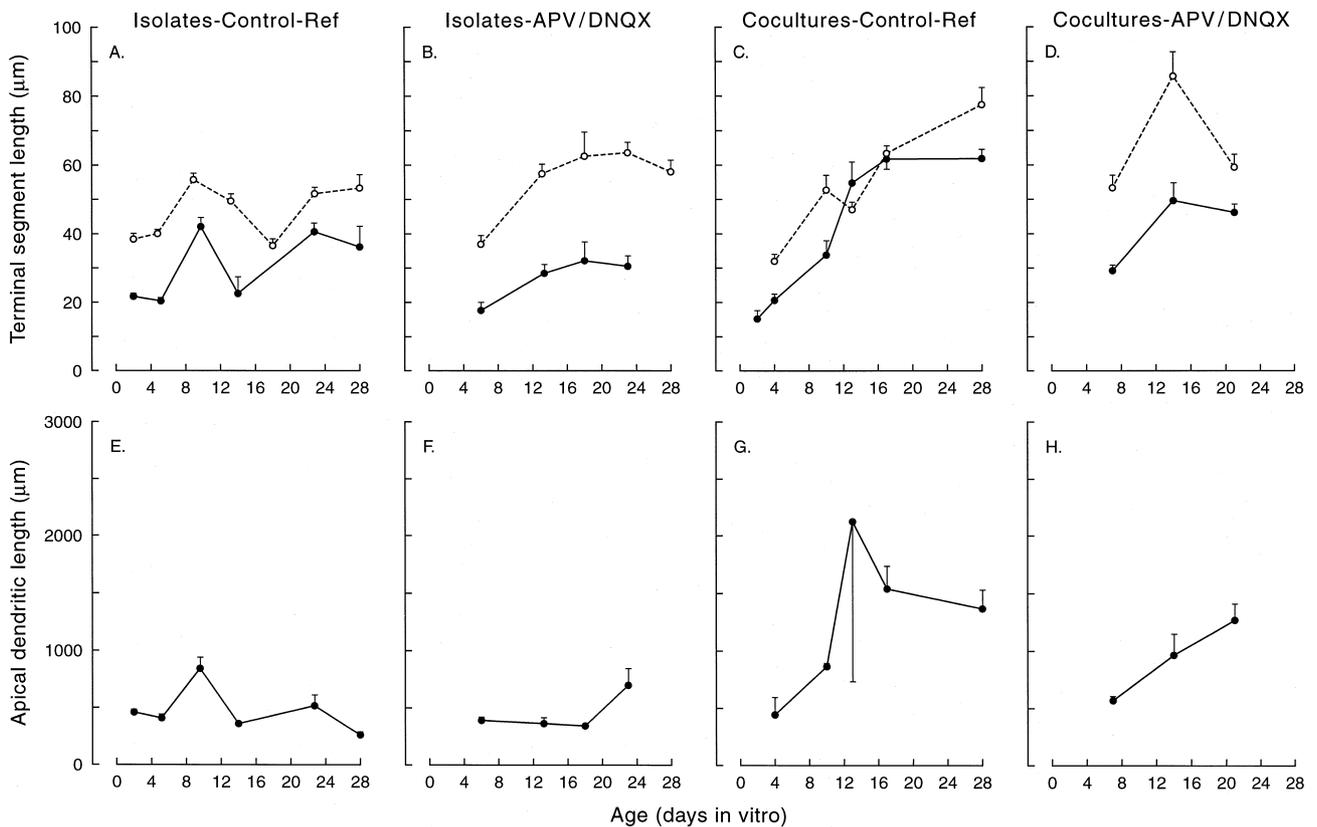
Fig. 3. Curves showing terminal dendritic segment and apical dendritic growth in control and experimental isolate and co-cultured explants.
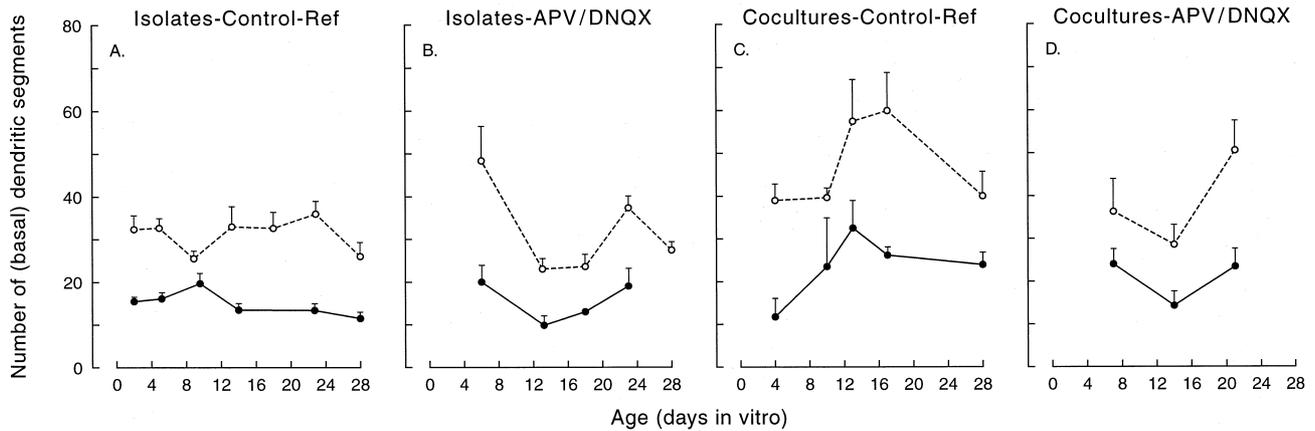
Fig. 4. Curves showing total number of (basal) dendritic segments in control and experimental isolate and cocultured explants.

3.4. Electrophysiological measurements

Explants were examined under control and experimental conditions to determine whether SBA was present. Cultures grown and recorded in the presence of APV/DNQX showed no measurable spontaneous activity in the presence of both antagonists, but became active when the growth medium was replaced by control recording medium (within $\approx$ 2–3 min). Several changes back and forth between APV/DNQX recording medium and control medium verified the suppressive effect of the cocktail on SBA. Stimulation of one ventricle-to-ventricle explant invariably resulted in an evoked response in the same explant at some distance from the stimulation site, *and* in the contralateral
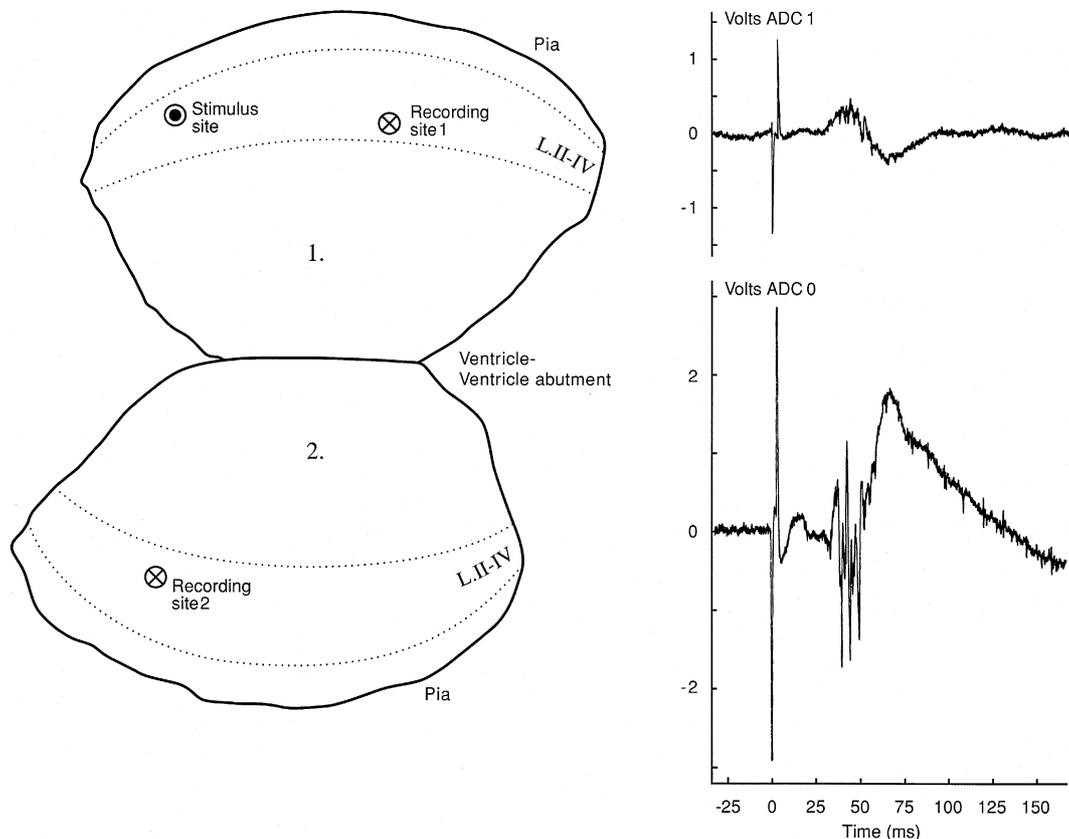

Fig. 5. Electrophysiological traces showing the functional connectivity between two explants in a coculture. The upper explant was stimulated at site 'S' and an evoked reponse recorded in the the the same explant at 'x' (upper trace) as well as in the co-culture at 'x' (lower trace).

explant (Fig. 5). This demonstrated the functional interconnectivity of the two slices. No attempt was made to make a detailed map of electrophysiologically interconnected sites between the two explants.

4. Discussion

The present study shows that chronic blockade of glutamate-mediated spontaneous bioelectric activity (SBA) exerts differential growth effects on the formation of pyramidal and non-pyramidal dendritic or axonal arbours within neocortical explants. Since morphological maturation within the CNS is due to elongation and branching of neuronal dendrites and axons, alterations in either or both of these processes would be expected to change the fundamental characteristics of pyramidal/non-pyramidal connectivity. The results of the present study are the first to show that basal dendrites and axons of pyramidal neurones within co-cultured explants of neonatal rat neocortex experience a significant reduction in total neuritic elongation and branching following blockade of glutamate-mediated SBA. Growth of non-pyramidal neurones, in contrast, show little dendritic or axonal response to the loss of SBA. In *isolated* neocortical explants the only significant growth effects associated with SBA blockade were the increases in pyramidal and non-pyramidal terminal segment length and accompanying decreases in total number of dendritic segments. These responses appear to be a common response in pyramidal and non-pyramidal neurones in both isolates and co-cultures following glutamatergic blockade of bioelectric activity.

Only neocortical co-cultures have been shown to contain conditions for sustained dendritic/axonal growth in vitro [5]. How and why neuritic growth in neocortical neurones is effected by SBA blockade is unknown, but may be associated with a variety of activity-dependent, target-induced effects. Wise et al. [43] suggested that appropriate afferent innervation of neocortical pyramidal neurones effected subsequent dendritic elongation, branching and spine formation. These suggestions receive support from studies reported for Purkinje granule cell co-cultures, where only an appropriate afferent input was sufficient to trigger dendritic elongation, branching and spine formation [6,34]. Recent work suggests that one role selected afferent innervation might play in neuronal development would be that of inducing neurotransmitter receptor production and/or maturation. Production of GABAa and NMDA receptors in isolated neocortical explants fail to reach control levels following chronic SBA blockade, suggesting that at least the production of these receptors is activity dependent [3,4]. It is unknown whether either GABAa or NMDA receptor levels would be increased in co-cultures. Developmental maturation of subunit composition within NMDA and GABAa receptors, from immature to adult forms, also appears to be under afferent or activity dependent control [10,21,41]. Maturational switch of GABAa receptor subunits occurs in bioelectrically active, interconnected co-cultures, but not within active isolated explants (Brussaard and Baker, unpublished observations).

Such neurotransmitter receptor changes may have profound consequences for activity-dependent neuronal plasticity as mediated via neurotrophins. Neurotrophin levels and function have been shown to be activity-dependent [14,22,35], and the balance between glutamatergic and GABAergic neurotransmission may significantly alter a variety of neurotrophin expressions [7,27,28,45]. It is also known that neuronal survival and differential dendritic/axonal growth effects occur in response to a variety of neurotrophins [45]. Indeed, McAllister et al. [25,26] have recently shown that the developing ferret visual cortex shows differential growth effects on pyramidal neurones to a variety of exogenously presented neurotrophins. Pyramidal neurones within cortical layers IV–VI responded to specific neurotrophins which exerted selective effects on basal and apical dendrites. This seminal study did not report on whether non-pyramidal neurones were similarly effected by neurotrophins, or whether the explants evinced SBA, or what role SBA might play in their results. Recent work by the above group suggests that one growth factor, BDNF, is activity dependent, though physiological details of successful SBA blockade are unknown [26]. The question now arises as to whether production of a given neurotrophin is activity dependent, and if so, whether there would be a correlation between SBA blockade and a given neurotrophin on dendritic/axonal development in both pyramidal *and* non-pyramidal neurones.

Finally, physical contact of explants with an abutment at some angle other than side-by-side appears essential for sustained dendritic growth in control co-cultured explants. Side-by-side co-cultures, which show little cross-innervation between explants, showed no sustained dendritic growth. This demonstrates the crucial role afferent innervation plays in the development of dendritic/axonal morphology in this system. The other co-culture configurations examined here confirm reports regarding directed outgrowth between presented explants, no matter where the explants might be located in relation to one another [8,13,29,38,44]. In addition, we have shown with both fluorescent labelling and electrophysiological recordings that abundant axonal crossover occurs between co-cultures and that bioelectrically functional connections form between these explants.

The present work has shown that SBA blockade exerts significant, and differential, effects over pyramidal and non-pyramidal dendritic/axonal morphology within co-cultured, organotypic neocortical explants in vitro. The effect of SBA on dendritic development appears to be mediated by afferent inputs from apposing neocortical explants possibly under the influence of a variety of neurotrophin receptors. Studies are now underway which

should allow us to determine what role SBA plays in the maturation of neurotrophin receptors in this model system.

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