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Aspects of Early Postnatal Development of Cortical Neurons that Proceed Independently of Normally Present Extrinsic Influences

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SUMMARY

To examine the contribution of local versus extrinsic influences on postnatal development of cortical neurons, we compared the maturation of deep (infragranular) layer neurons in isolated slices of neocortex grown in organotypic culture to a similar population of neurons developing *in vivo*. All slice cultures were prepared from sensorimotor cortices of newborn mice (P0) and neurons in these cultures were examined at daily intervals during the first 9 days *in vitro* (DIV). The maturational state of neurons developing *in vivo* over this same time period was assessed in acute slices prepared from animals of equivalent postnatal age, P1–P9. Electrophysiological recordings were obtained from neurons in both cultured and acute slices, using Lucifer yellow filled whole-cell recording electrodes, enabling subsequent morphometric analysis of the labeled cells. We report significant changes in both cellular morphology and electrical membrane properties of these deep layer cortical neurons during the first week in culture. Morphological maturation over this time period was characterized by a two- to three-fold increase in cell body size and total process length, and an increase in dendritic complexity. In this same population of cells a three-fold decrease in input

resistance and changes in the action potential waveform, including a two-fold decrease in the AP duration, also occur. The degree of morphological and electrophysiological differentiation of individual neurons was highly correlated across developmental ages, suggesting that the maturational state of a cell is reflected in both cellular morphology and intrinsic membrane properties. A remarkably similar pattern of neuronal maturation was observed in neurons in layers V, VI/SP examined in acute slices prepared from animals between P1–P9. Because our culture system preserves many aspects of the local cortical environment while eliminating normal extrinsic influences (including thalamic, brainstem, and callosal connections), our findings argue that this early phase of neuronal differentiation, including the rate and extent of dendritic growth and development of AP waveform, results from instructive and/or permissive local influences, and appears to proceed independently of the many normally present extrinsic factors. © 1993 John Wiley & Sons, Inc.

Keywords: organotypic, slice cultures, somatosensory cortex.

INTRODUCTION

An issue of broad interest in developmental neurobiology is the understanding of the interplay be-

tween intrinsic, local, and systemic influences on differentiation of individual cells. At one extreme, expression of a number of early phenotypes of amphibian neurons and muscle cells has been demonstrated in single cells raised in complete isolation (Henderson and Spitzer, 1986). Such autonomous differentiation may be limited, however, and although a cell's genetic program clearly plays an important role in expression of specific cellular properties, considerable evidence indicates that expression of mature morphological and physiological

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phenotypes of central nervous system neurons is influenced by factors present in the local environment (e.g., cell-cell, synaptic, and trophic interactions) (Rakic, 1979; Hillman, 1988). In addition to local influences, extrinsic factors originating from other regions of the nervous system, including afferent/efferent connections with distant loci and blood-born factors, have been shown to affect normal development (Rakic, 1979; Hillman, 1988; Mattson, 1988; Baier and Bonhoffer, 1992).

The cerebral cortex is an attractive system in which to examine the relative roles played by local and extrinsic factors in the development of CNS neurons. In newborn rodents the cortex is relatively immature, consisting of the subplate (SP) region, layers V and VI, the undifferentiated cortical plate, and the marginal zone (Berry and Rogers, 1965; Rice and Van der Loos, 1977; Bayer et al., 1991). While the deep layers V, VI, and SP can be identified, previous studies have demonstrated that neurons in these laminae are immature at birth, both in terms of their morphological differentiation and their electrical membrane properties (Miller, 1988; McCormick and Prince, 1987; Miller, 1981). Early postnatal development of cortical neurons is characterized by a sequence of changes in a number of properties including cell body size, dendritic length, and action potential waveform (Valverde, 1968; Wise et al., 1979; Miller, 1981, 1988a; McCormick and Prince, 1987; Petit et al., 1988; Kreigstein et al., 1987).

Comparison of the maturation of neurons in cell culture with those developing *in vivo* is a useful strategy for determining which aspects of neuronal differentiation are intrinsic to the cell, and which are influenced by either local environmental factors or by extrinsic influences such as the presence of afferent connections. Previous studies have shown that embryonic cortical cells grown in dissociated cell culture differentiate into distinct morphological classes, including pyramidal, bipolar, and multipolar neurons, suggesting that expression of these neuronal phenotypes is controlled by the genetic program of individual cells (Dichter, 1978; Kreigstein and Dichter, 1983). However, the observation that dendritic arborization of pyramidal cells in these cultures is more elaborate than those developing *in vivo* suggests that the environment of the dissociated cell cultures lacks cues that normally limit the extent of dendritic branching. These signals could arise from either the local cortical environment or from outside the immediate cortical region. To distinguish between aspects of the maturation of cortical neurons controlled by

intrinsic and local influences from those regulated by extrinsic factors, the differentiation of cortical neurons can be examined in isolated sections of cortical tissue grown in organotypic culture. In these cultures both afferent fibers and subcortical efferent targets are absent, but the local neuronal environment seems to closely resemble the environment *in vivo* (Gahwiler, 1981; Robertson et al., 1991; Annis et al., 1990). Several recent studies have noted that cortical neurons grown in organotypic slice culture appear more mature after several weeks *in vitro* than they were at the time the cultures were prepared (Caesar et al., 1989; Wolfson et al., 1989; Wolburg and Bolz, 1991). All of these studies, however, have been qualitative and none have examined the question of whether development of cortical neurons in culture parallels that *in vivo*; such information is essential to identifying factors that regulate development of cortical neurons.

The aim of the present study was to determine which aspects of early postnatal differentiation of cortical neurons are supported by intrinsic/local cues and which properties appear to be influenced by factors arising extracortically. To address these issues we have directly compared the pattern of morphological and physiological development of layer V, VI/subplate neurons growing in slice cultures with those developing *in vivo*. In order to examine the maturation of these neurons from a relatively immature state of differentiation, all of our cultures were prepared from slices of sensorimotor cortex obtained from mice on the day of birth (P0). Our analysis demonstrates that many aspects of early development of infragranular cortical neurons results from intrinsic and/or local influences.

METHODS

Acute Slice Preparation

Neonatal C57 BL/6 mice between the day of birth (P0) and P9 were used in these studies. Coronal slices (400 μ m) from sensorimotor cortex were prepared using a McIlwain tissue chopper. Slices were transferred to the recording chamber and perfused with oxygenated artificial cerebral spinal fluid (ACSF) at room temperature. The ACSF solution consisted of (in mM): NaCl, 126; KCl, 3; NaH_2PO_4 , 1.25; MgSO_4 , 1.3; NaHCO_3 , 26; D-glucose, 10; CaCl_2 , 2.5.

Preparation of Organotypic Slice Cultures

Coronal slices of sensorimotor cortex were prepared from P0 mice as described above. Freshly cut slices were

bathed in a dish of cold EOL 1 medium (Annis et al., 1990) for 30 min and then mounted onto coverslips coated with poly-D-lysine and dialyzed rat tail collagen (Bornstein, 1958). Coverslips with attached slices were placed into 16-mm culture tubes with 500 μ l of EOL 1 defined medium and sealed with 18-mm gas permeable caps (Biomedical Polymers). The tubes were placed into a 5.0% CO₂ incubator at 36.0°C and left stationary for 24 h. After the initial 24-h attachment period, cultures were rotated in the incubator at 10 rev/h. The medium was subsequently changed every other day and fresh medium was prepared once a week.

Histology of Acute and Organotypic Slice Cultures

Acute and cultured slices processed for Nissl staining were fixed in 4% paraformaldehyde, immersed overnight in 20% sucrose in sodium-phosphate buffer and embedded in mounting medium (Tissue Tek). Cultures were sectioned on a Reichert cryostat at 20–30 μ m in a plane parallel to the principle plane of the culture (i.e., parallel to the coronal plane). Acute slices were resectioned in the coronal plane. Sections were stained with cresyl violet for Nissl substance, dehydrated, cleared, and coverslipped. Thickness of cultures was estimated by determining the number of sections obtained from each culture. Cultures typically were somewhat thicker in the middle than at the edges, so that the cross-sectional areas of sections diminished in the sections from the top of the cultures; the smallest section included in calculating culture thickness was no smaller than $\frac{1}{3}$ the area of the largest section.

Neuronal cross-sectional areas were determined by analyzing Nissl stained sections with a BioQuant 3 image analysis system. The cortical regions examined included layers V and VI in the acute slices and the lower half of cultured slices. Sections of acute and cultured slices were examined on a video monitor at magnification of 1000 $\times$. Every neuron (identified by the presence of a pale nucleus) within a given field was measured; the areas of 50 or 100 neuronal somata per section were measured.

Imaging of Single Cells and Morphological Analysis

Inclusion of 0.1% Lucifer yellow in the recording solution resulted in fluorescent labeling of recorded neurons. The number of cells filled in each slice was limited to an average of three to enable unambiguous comparison of physiological and morphological properties of individual cells. Upon completion of physiological experiments, slices were fixed in 4% paraformaldehyde for 24 h, dehydrated through graded ethanols, then cleared and mounted in methyl salicylate. Cleared slices were examined at low power and the outline of the slice and posi-

tion of each filled cell within the slice was recorded using a camera lucida. Individual cells were imaged using a laser scanning confocal microscope (BioRad MRC 600) with a 60 $\times$ oil immersion lens. A series of optical sections was obtained for each cell and digitally projected into a single 2-D representation. A montage of these 2-D images was used to reconstruct each cell from which measurements were taken. A Zeiss Zidas digitizing tablet was used to determine soma size, dendritic branch length, and total process length. Total process length for each cell was calculated by summing the lengths of all individual dendrites. A distribution of dendritic branch order was constructed by counting the number of dendrites of a given branch order for each cell. Dendrites emerging from the cell body were considered primary with each successive daughter branch designated the next higher order (apical dendrites of pyramidal neurons were considered primary for their entire length). The lengths of individual branches were also measured and averaged for each cell to determine the average length of each process order. Finally, Sholl distributions were produced as previously described (Sholl, 1953) by placing concentric rings (the first at a 10- μ m radius, the second at 25 μ m, with all subsequent rings spaced at 25- μ m increments) centered over the cell soma and counting the numbers of dendrite intersections at given distances from the cell body.

Intracellular Recording

Electrophysiological recordings were obtained from neurons in cortical slices maintained in culture for 1–9 days and from acute slices between P1 and P9. Both whole cell voltage-clamp and current-clamp techniques were used (Hamill et al., 1981; Blanton et al., 1989). Uncoated patch pipettes of 5–10 M Ohm resistance were used to establish GOhm pipet-membrane seals. The residual capacitance associated with the electrode and patch was subtracted electronically prior to rupture of the patch. To facilitate measurement of rapid physiological events, all recordings were performed at room temperature (22–24°C). The internal recording solution consisted of (in mM): KF, 70; KCl, 70; Hepes, 10; CaCl₂, 1; MgCl₂, 2; EGTA, 11, with 0.1% Lucifer yellow. The bathing solution consisted of oxygenated ACSF.

Electrophysiological Data Acquisition and Analysis

Data were collected and analyzed using a List EPC-7 patch-clamp amplifier, a Dell 386 computer, and P-Clamp (Axon Instruments) software. The voltage-clamp records were sampled between 5 and 20 kHz and filtered at 2.5 kHz through an 8-pole Bessel filter. Input resis-

Figure 1 Histological organization of cerebral cortex in acute and cultured slices. In P0 and P1 acute slices, layers V and VI were located below the cell dense compact cortical plate (CP). A similar laminar organization is seen after 1 DIV. At P7, all six layers of the cortex could be clearly identified in acute slices. After 7 DIV there was some distortion of radial organization but laminae corresponding to layer V, VI, and subplate (SP) could be identified. Note the increase in cortical width of both acute and cultured slices. Acute and cultured slices were resectioned at 20 μm and stained for Nissl substance. Scale bar = 100 μm .

tance was measured as the plateau current level during a 10-mV depolarizing voltage step from a holding potential of -70 mV. A series of depolarizing voltage steps from a holding potential of -100 mV was used to elicit voltage-activated currents. Following initial examination of the currents in voltage-clamp mode, the amplifier was switched to current clamp and the resting potential of each cell noted. Holding current was applied to the cells to maintain a consistent holding potential of -75 to -80 mV. Action potentials (AP) were elicited by depolarizing current steps. All of the current clamp measurements were obtained from records sampled at 10–20 kHz, were taken from the first action potential in each spike train, and represent the average value from at least three AP's recorded from each cell. The AP amplitude was measured as the height (in mV) from threshold to peak. AP duration was measured as the time interval (ms) between the rising and falling phases of the action potential at half amplitude. The rate of rise was calculated as the AP amplitude divided by the time interval from threshold to peak. The rate of fall was calculated as the change in amplitude from peak to 50% of peak, divided by the time interval from peak to 50% of peak. All data are expressed as mean $\pm$ SEM and significant differ-

ences between data groups were evaluated with the Student's *t* test.

RESULTS

Cellular Organization of Slice Cultures

The cytoarchitecture of cortical slices in culture was assessed using Nissl stains and was compared to acutely prepared slices from animals of equivalent age (Fig. 1). At the time cultures were prepared (day of birth, P0), parietal cortex consisted of the marginal zone, the compact cortical plate, cortical layers V and VI, and the subplate. The laminar organization evident at P0 is maintained in cultured slices after 1 day *in vitro* (DIV) and appeared similar to the organization seen in acute slices at P1 (Fig. 1). Layers I–VI could be identified in acute slices prepared at P7 (Fig. 1). Although laminar borders were less clear in cultured slices after 6–9 DIV than in acutely prepared tissue

slices at corresponding ages, layers V, VI, and SP could be identified on the basis of cell size, cell shape, and packing density (Fig. 1: P7, 7 DIV). Layers II, III, and IV could not be distinguished in Nissl stained preparations of slice cultures.

An increase in the size of neuronal somata throughout the cortical tissue occurred over the first 9 days *in vivo* and in culture. This change is illustrated by comparison of the relative sizes of neuronal somata in layers V and VI between 1 and 7 days both in cultures and in acute slices [Fig. 2(A)]. Quantitative analysis revealed a significant increase in the mean cross-sectional area of neurons in layers V and VI of the acute slices. At PO, the average cell area was $45.1 \pm 10.5 \mu\text{m}^2$. Between P1–2 and P6–9 the mean cross-sectional area increased from 53.4 ± 12.1 to $135.9 \pm 27.4 \mu\text{m}^2$ [Fig. 2(B)]. Examination of neurons in the lower half of the cortical slice cultures revealed a similar developmental increase. The average cell area at 1–2 DIV was $53.8 \pm 10.6 \mu\text{m}^2$ compared with $126.9 \pm 28.2 \mu\text{m}^2$ at 6–9 DIV [Fig. 2(B)].

In addition to changes in the cross-sectional area of individual cells in the cultures, the width of the cortex, measured from the inner border of the subplate to the pial surface, increased markedly with time in culture (Fig. 3). When first placed in culture, slices had an average cortical width of $450 \mu\text{m}$, similar to that previously reported for sensorimotor cortex of newborn mice (Rice and Van der Loos, 1977). By 1 DIV the average cortical width was approximately $650 \mu\text{m}$, about 50% greater than the P0 slices used to make the cultures [Fig. 3(A)]. By 6–9 DIV the average cortical width increased 50% over that seen at 1 DIV. A similar increase in cortical width over comparable time periods was noted in acute slices [Fig. 3(A)].

Previous studies have shown that slices from a variety of brain regions maintained in cell culture are subject to thinning and spreading (Gähwiler, 1981, 1988). To determine the contribution that this process might make to the apparent growth of cortical slices in our cultures, the average thickness in each age group was examined. The average slice thickness decreased from the original $400 \mu\text{m}$ to approximately $150 \mu\text{m}$ over the first day in culture [Fig. 3(B)] suggesting that much of the increase in cortical width in the first 24 h may be due to spreading of the tissue. However, no further change was seen in the slice thickness between 1–2 and 6–9 DIV, the same time period over which an additional 50% increase in cortical width occurred [Fig. 3(B)]. These data indicate that the increase in cortical width that occurs after 1 DIV cannot be

accounted for simply by further thinning and spreading of the tissue.

Morphological Cell Types

Histological analyses presented above suggest that the lower half of the cultures correspond to layers V/VI and SP throughout the first 9 DIV. We examined the cellular morphology of 27 Lucifer yellow filled neurons located in this region from 11 slice cultures. The cultured neurons were compared to 21 cortical neurons in the deep layers (V, VI, SP) developing *in vivo* and examined in 8 acute slice preparations. In young acute (P1–2) and cultured (1–2 DIV) slices, all neurons had small cell bodies, few dendritic processes, and many were not identifiable as belonging to a distinct morphological class. However, by 3 days, both in acute and cultured slices, the cells could be classified as either nonpyramidal or pyramidal. Nonpyramidal cells included bipolar [Fig. 4(A)] and multipolar cells [Fig. 4(B)]. Cells with triangular-shaped cell bodies, an apical dendrite, and several basal dendrites were classified as pyramidal [Fig. 4(C)]. The apical dendrites of all pyramidal cells in acute slices were oriented perpendicularly to the pial surface [Fig. 5(E,F)]. It was noted that the apical dendrites of some of the cultured pyramidal cells were not perpendicularly oriented [Figure 4, 5(C)].

Morphological Development of Individual Lucifer Yellow Filled Cells

Qualitative changes in morphological differentiation of individual cells between 1 and 9 days are illustrated by the photomicrographs in Figure 5. Cell body size and dendritic complexity of the neurons increased as a function of age both *in vivo* and in culture. Lucifer yellow filled neurons examined in acute slices at 1–2 days had small somata with an average cross-sectional area of $61.8 \pm 9.7 \mu\text{m}^2$ which increased to $102.0 \pm 8.4 \mu\text{m}^2$ by P6–9. The cultured neurons sampled had an average cross-sectional area of $47.0 \pm 6.0 \mu\text{m}^2$ at 1–2 DIV, which increased to $149 \pm 11 \mu\text{m}^2$ by 6–9 DIV. To quantify developmental changes in dendritic arborization, photographic montages of confocal images of cells in acute and cultured slices were constructed. Examples of filled pyramidal neurons in the acute [Fig. 6(B)] and cultured [Fig. 6(A)] slice, at 9 and 8 days, respectively, demonstrate labeling throughout the cell body and fine dendritic processes. Also in evidence are dendritic spines which were first

Figure 2 Increase in cell size both *in vivo* and *in vitro*. (A) Closer examination of the layer V–VI region shown in Figure 1, at 1 and 7 days, revealed an increase in the size of individual somata in both the acute and cultured slice. Note typical pyramidal shaped cell bodies with apical processes both at P7 and at 7 DIV. Pial surface is at the top in all of the photographs. Scale bar = 50 μm . (B) Average cross-sectional area of neurons in layers V, VI of acute slices and in the deep half of slice cultures. Neuronal cross-sectional areas were measured from Nissl-stained sections using a BioQuant 3 image analysis system. Numbers of neurons measured for each condition are indicated. Numbers of slices examined: one each for P0 and 1–2 day histograms; two each for 3–5 day histograms; five for 6–9 DIV; four for P9.

detectable at 6–9 days in both the cultured and acute slices.

Intracellularly filled cells were grouped into three age categories (1–2, 3–5, and 6–9) either reflecting days *in vitro* or postnatal age. In the two

older age groups, data for pyramidal cells were examined separately and also were compared to pooled data from all morphological classes. Comparisons were made for cells between age groups and for cells between the acute and cultured slices.

Figure 3 Width of cortical tissue and cultured slice thickness. (A) A significant increase in cortical width ($p < .01$, Student's t test) between 1–2 and 6–9 days was seen both in slices developing in culture and in acute slices (mean $\pm$ SEM, n indicated for each age group). Width measurements were made from the inner border of the subplate to the pial surface. (B) All cultures were prepared from 400 μ m thick slices made at P0. The thickness of the slices after varying times in culture was estimated by determining the number of 20 or 30 μ m frozen sections obtained from a number of cultures upon resectioning. As not all of the cultures were of uniform thickness, the smallest section included in calculating culture thickness was no smaller than one-third the area of the largest section. Slices thinned significantly over the first 24 h in culture but did not change over the next 9 days *in vitro* (mean $\pm$ SEM, n indicated for each age group).

Total Dendritic Length. The total dendritic length for each cell was determined by summing the lengths of all the individual dendrites of each branch order (Fig. 7). No statistically significant increase in the dendritic length was detected between the 1–2 and 3–5 age categories either in acute or cultured slices [Fig. 7(A)]. A large (3–4 fold) significant increase (Student's t test, $p < .01$) in total length occurred between 3–5 and 6–9 days, both in the acute and cultured slice. The subset of neurons classified as pyramidal displayed changes in dendritic length that were similar to the den-

dratic changes of neurons of all morphological classes [Fig. 7(A,B)].

Sholl Analysis. Data gathered from Sholl analyses indicate a significant increase not only in process length but also in branching during the developmental period examined, for neurons from both acute and cultured slices. The average Sholl distribution for neurons in acute slices at 1–2 days is roughly linear, with an average of three intersections at 10 μ m from the cell body and a smaller number of intersections at increasing distances from the soma [Fig. 7(C,D)]. There was no significant change in the Sholl distribution of acute slice neurons between P1–2 and P3–5. A marked change was seen between P3–5 and P6–9, with significant increases in the number of intersections at 25, 50, 75, and 100 μ m from the cell body [Fig. 7(C,D)]. The average Sholl distributions for neurons developing in cultured slices were similar to those obtained from cells examined in the acute slices, with a roughly linear profile at 1–2 DIV. At 3–5 DIV, the average number of intersections increased from 2.6 to 5.2, accompanied by a shift (from 10 μ m to 25 μ m) in the distance from the cell body at which the maximal number of intersections was seen [Fig. 7(C)]. Similar to the pattern observed in the acute slice, the major developmental increase occurred between 3–5 and 6–9 DIV, with significant increases (Student's t test, $p < .01$) in the number of intersections at distances between 25 and 100 μ m. The peak number of intersections in this older age group (6–9) both in cultured and in acute slices, occurred at 25 μ m from the cell body.

Branch Order. The number of processes of each branch order were counted for individual cells and an average distribution constructed [Fig. 7(E,F)] for each age category. Neurons in acute slices from the first two age groups, P1–2 and P3–5, had predominantly first- and second-order processes with few or no higher order branches. At P6–9, the number of first-order processes was unchanged; however a significant increase in the number of second-, third-, and fourth-order processes was observed in addition to the appearance of higher order branches. The number of first- and second-order processes at 1–2 DIV was slightly lower than that seen in cells from P1–2 acute slices. At 3–5 DIV there was no significant change in the number of first-order processes, an approximately two-fold increase in second-order dendrites, and the appearance of a few higher order branches [Fig. 7(E)].

Figure 4 Three morphological classes of neurons in slice cultures. Lucifer yellow filled cells in organotypic slice cultures revealed (A) bipolar, (B) multipolar, and (C) pyramidal neurons. Fluorescent images were obtained from a confocal microscope. These images were constructed from 25–40 optical sections obtained at 1 μm increments.

The numbers of second-, third-, and fourth-order branches increased markedly by 6–9 DIV and the number of higher order processes also increased. At the later ages (3–5 and 6–9), the distribution of branch orders did not differ significantly between cells in cultured and acute slices.

Process Order Length. The average length of each dendritic branch order was determined for neurons from cultured and acute slices, and these data are presented in Figure 8. Cortical neurons from acute slices had an average first-order dendrite length of approximately 45 μm , with second- and third-order lengths each of approximately 20 μm (Fig. 8). In acute slices prepared from older animals, neurons had a larger number of higher order processes but there was no significant change in the

length of existing process orders. Dendritic length measured in the cultured slice neurons showed a very similar developmental profile, in which young cells at 1–2 DIV exhibited average lengths of first-, second-, and third-order processes of 45, 30, and 15 μm , respectively. Similar to results from acute slices, the lengths of existing processes did not appear to change, although the number of higher order processes increased as a function of time in culture. No statistically significant difference was found between neurons from acute and cultured slice in the two older groups.

Electrophysiological Development of Individual Cells

To determine whether developmental changes in intrinsic membrane properties of cortical neurons

occur in organotypic slice cultures, we used the whole-cell patch technique to examine the responses of individual cells. We report the results of physiological recordings from 55 neurons in layers V and VI from 24 acute slice preparations and 98 neurons located in the lower half of 32 slice cultures.

Developmental Change in Input Resistance and Resting Potential. Neuronal input resistance was estimated from the plateau current level in response to a small depolarizing (+10 mV) voltage step from a holding potential of -70 mV, voltages chosen to minimize the contribution of voltage-activated currents. A three-fold decrease in input resistance of cells in acute slices occurred between P1-2 and P6-9 from 1.7 ± 0.2 to 0.56 ± 0.06 GOhms. A similar decrease in input resistance from 2.1 ± 0.56 to 0.87 ± 0.08 GOhms was observed in the cultured neurons between 1-2 DIV and 6-9 DIV (Table 1). This developmental decrease in input resistance is consistent with the increase in membrane surface area, as indicated by soma size and dendritic length measurements above, occurring over this same time period. The resting potential measured upon breaking into cells in current clamp mode was relatively depolarized (-40 to -50 mV) when using our standard intracellular solution (70 mM chloride/70 mM fluoride). In light of recent evidence that the resting potential of immature neurons is sensitive to intracellular concentration of Cl^- (Spigelman et al., 1992), all cells in the present study were held at hyperpolarizing potentials between -75 and -80 mV to ensure that voltage-sensitive channels important in spike generation were available for activation to an equal extent.

Developmental Changes in Action Potential. Developmental changes in the properties of neuronal action potentials both in acute and cultured slices

were assessed in current clamp mode. Though spontaneous action potentials were recorded in only a small minority of cells, injection of depolarizing current elicited regenerative responses in the majority of cells at all ages examined. Figure 9 illustrates the qualitative changes in the AP's and underlying currents in neurons from both acute and cultured slices. Neurons examined in young cultured slices exhibited action potentials that were of smaller peak amplitude and longer duration than those recorded from cells after longer periods of time in culture [Fig. 9(A)]. In addition, the peak amplitude of the inward sodium current elicited in voltage-clamp mode increased over this same time period. This developmental change was similar to that seen for the action potential waveform and sodium current amplitude in neurons examined in acute slice preparations [Fig. 9(B)].

Changes in specific aspects of the AP waveform were quantified and expressed as a function of postnatal age or days *in vitro* (Fig. 10). Action potential amplitude was measured from threshold rather than from resting potential because all cells examined were held near -75 mV by hyperpolarizing current injection. A significant increase in AP amplitude (Student's *t* test, $p < .01$) was seen between 1-2 and 6-9 days, in neurons from both acute and cultured slices, from slightly less than 40 mV to just over 50 mV [Fig. 10(A)]. The AP duration, measured at half spike amplitude, showed a decrease from 5.7 ± 0.38 ms to 3.9 ± 0.37 ms in acute slices between P1-2 and P6-9. A similar change in duration occurred over the first 9 DIV from 6.8 ± 0.58 ms to 3.2 ± 0.17 ms [Fig. 10(B)]. To determine whether changes in the rising and or falling phases of the action potential were responsible for the decrease in AP duration, the average rate of rise (AP amplitude divided by the time interval from threshold to peak) and fall (change in amplitude from peak to 50% of peak, divided by the time interval from peak to 50% of peak) of the spikes were examined. The average rate of rise increased from 20.5 ± 1.6 mV/ms to 30.5 ± 3.6 mV/ms in acute slices and from 18.4 ± 2.1 mV/ms to 33.9 ± 2.6 mV/ms in cultured slices [Fig. 10(C)]. Also contributing to the decrease in AP duration was a two-fold increase in the average rate of fall in both acute and cultured slices [Fig. 10(D)].

In addition to examining the voltage response of individual cells to current injection, voltage-activated currents were recorded in response to a series of depolarizing voltage steps from a holding potential of -100 mV. As illustrated in Figure 9, an in-

Table 1 Input Resistance (Mean $\pm$ Standard Error, *n* Indicated) for Neurons from Cultured and Acute Slice Preparations for Each Age Group

	Input Resistance (mOhm)	
	Cultured Slice	Acute Slice
1-2 d	2172.8 $\pm$ 565 (<i>n</i> = 23)	1722.5 $\pm$ 249 (<i>n</i> = 21)
3-5 d	1188.7 $\pm$ 97 (<i>n</i> = 21)	1887.8 $\pm$ 496 (<i>n</i> = 16)
6-9 d	871.3 $\pm$ 82 (<i>n</i> = 35)	560.4 $\pm$ 65 (<i>n</i> = 18)

Figure 5 Examples of morphological development of neurons in cultured and acute slices. Confocal images of Lucifer yellow filled neurons in cultured (A–C) slices and in acute (D–F) slices. At 1–2 days, cells in cultured (A) and acute (D) slices both had small somata and few, relatively unbranched dendrites. By 6–9 days in culture (C) and in acute slices (F) there was a large increase in cell body size, number, and branching of dendritic processes. At 3–5 days, the degree of morphological differentiation was intermediate both in culture (B) and in acute (E) slices. Direction of the pial surface indicated by the top edge of each photomicrograph except in (C), where the arrowhead points toward the pial surface. Scale bar = 25 μ m.

Figure 6 Reconstruction of the labeled dendritic fields of pyramidal neurons *in vitro* and *in vivo*. (A) A montage of seven confocal images, each of which was constructed from a maximum of 30 optical sections taken at 1.5 μm increments, of an 8 DIV pyramidal neuron. (B) A montage of 3 confocal images, each of which was constructed from a maximum of 41 optical sections taken at 2.0 μm increments of a P9 pyramidal neuron. Scale bar = 25 μm .

crease in the amplitude of the peak inward sodium currents was observed as a function of developmental age. However, with the exception of a few cells in the youngest age category, the voltage control was not sufficient to clamp the transient sodium currents recorded from cells in either acute or cultured slices and therefore this change was not examined quantitatively.

Table 2 Correlation Coefficients from Linear Regression Analyses between Electrophysiological and Morphological Measures for 25 Neurons. All Figures Represent Values of $p < .01$, except where indicated as $p < .05$

	Cell Area	Total Dendritic Length	Morph. Index
Amplitude	.64	.38 ($p < .05$)	.53
Duration	-.84	-.61	-.76
Input Resistance	-.64	-.55	-.63
Phys. Index	.82	.58	.73

Developmental Change in Multiple Spiking Activity

Depolarizing current injection evoked trains of action potentials in some cells, at all ages examined, both in acute and cultured slices [Fig. 11(B)]. A long duration (150 ms), just suprathreshold, stimulus elicited a single-action potential while injection of larger currents evoked the first spike at shorter latency and also evoked a varying number of subsequent spikes. There did not appear to be substantial frequency accommodation during the repetitive discharge nor did we see evidence of the various classes of spiking neurons that have been previously described in adult cortical neurons (Connors and Gutnick, 1990). In addition to repetitively firing cells, there were some cells in each of the age categories, both in acute and cultured slices, in which only a single-action potential could be elicited [Fig. 11(A)]. For cells in which injection of large suprathreshold currents elicited only one spike, it was possible to continue to elicit the single spike without significant decrease in ampli-

Figure 7 Developmental changes in dendritic structure. (A) The average total process length (mean $\pm$ SEM, n indicated for each age group) for cultured and acute neurons of all morphological classes increased significantly between 3–5 and 6–9 days ($p < .01$, Student's t test). A small increase was noted in the process length in cultured cells between 1–2 and 3–5 DIV while there was no change over that same time period seen in the cells from acute slices. (B) The subset of pyramidal neurons showed the same developmental increase in total process length. Average Sholl distribution (mean $\pm$ SEM) determined for cells of all morphological classes demonstrated a significant increase in complexity of the dendritic arbor between 3–5 and 6–9 days both in cultured (C) and in acute (D) slices. The average number (mean $\pm$ SEM) of dendritic branches of each branching order for all morphological classes of cells increased as a function of developmental age in cultured (E) and acute (F) slices.

tude or duration for up to 30 min after the onset of recording (data not illustrated). In addition, the average input resistance of the single spiking cells

was not statistically different from the input resistance of the multiple spiking cells (input resistance was 1270 ± 126 MOhm for multiple spiking and

Figure 8 Dendritic branch length for each branch order during development. The average lengths (mean $\pm$ SEM) of the different process orders of neurons developing in cultured slices were similar to those from acute slices, over the age range examined.

1560 $\pm$ 232 MOhm for single spiking cells at P1–2, mean $\pm$ SEM). Similar results were obtained from cultured slice neurons). It is, therefore, unlikely that the inability of these cells to generate multiple spikes is due simply to excessive damage to the cell during the formation of the whole-cell patch, which would be reflected in a decrease in input resistance.

Although there were both single and multiple spiking cells in each age category, there was a developmental change in the percentage of cells in

which multiple spikes could be elicited. In the younger age category (P1–2) in acute slices, approximately 40% ($n = 20$) of the cells examined were able to generate two or more spikes in response to a 150-ms depolarizing current pulse [Fig. 11(B)]. In the P3–5 age group, the multiple spiking cells comprised 55% of the total population while at P6–9 75% of the cells could generate multiple spikes [Fig. 11(B)]. A similar developmental trend was observed in culture where 55% ($n = 27$) of the cells at 1–2 DIV were able to generate two or more spikes. The percentage of cells capable of generating multiple spikes increased to 86% by 3–5 DIV, and by 6–9 DIV, 97% of all cells were able to fire repetitively [Fig. 11(B)].

Correlation of Morphological and Physiological Development

Both electrophysiological and morphological data were obtained for 25 cells in 11 cultured slices. For each of these cells, specific electrophysiological parameters (input resistance, AP amplitude, AP duration or AP rate of rise) were compared to specific morphological parameters (total process length, cell area). Data from each of these comparisons were examined and fit with simple linear regressions to determine the degree of correlation in development between any two of these properties. Correlation coefficients with significance levels of $p < .05$ indicate statistically significant correlations in the development of a number of specific properties (Table 2) including input resistance and cell area [Fig. 12(A)].

An index of morphological development was created by expressing the total process length and the somal area of each cell as a percentage of the mean values in the 6–9 day age category and then combining those two figures to obtain a single value. This value was used to represent the degree of morphological differentiation of each individual cell. A similar index was created for the degree of physiological maturation using AP amplitude and duration data. The indices of physiological development and of morphological development were plotted for each cell [Fig. 12(B)]. The three different symbols on this graph indicate the age category from which each of the points was obtained and show that the young cells tend to be less mature both physiologically and morphologically than the older cells. These data were well fit with a simple linear regression, demonstrating a statistically significant correlation between morphological and

Figure 9 Representative action potentials and ionic currents in cortical neurons in acute and cultured slices. (A) Action potentials (upper traces) and ionic currents (lower traces) recorded from neurons in organotypic slice cultures at 1–2, 3–5, and 6–9 days *in vitro*. Note the progressive increase in the action potential amplitude, overshoot, and decrease in duration. (B) A similar progressive change in the action potentials and ionic currents was seen in neurons in acute slices at corresponding ages. Action potentials were elicited by injection of a 60 ms depolarizing current of 70–100 pA from a holding potential of -75 mV. Total ionic currents were recorded during a 60-ms voltage step from -100 to -10 mV.

physiological development in individual neurons ($r = .73$, $p < .01$).

neurons or present in the local cortical environment.

DISCUSSION

During early postnatal development, cortical neurons undergo a period of rapid differentiation characterized by changes in both cellular morphology and electrical membrane properties. In order to distinguish aspects of neuronal development governed by cortically derived factors from those influenced by factors extrinsic to the cortex, we quantitatively compared various features of neurons from P0 animals developing for up to 9 days in organotypic slice cultures of sensorimotor cortex with those of neurons maturing over the same time period *in vivo*. The results of the present study demonstrate that early differentiation of a number of morphological and electrophysiological properties of cortical neurons proceed along a normal time course, independently of extrinsic influences, and thus must be governed by factors intrinsic to the

Development of Cerebral Cortical Laminae *in vitro*

Histological analyses indicated that although there was an initial increase in cortical width (distance from "pial" surface to the inner border of the subplate) in the first 24 h, the laminar organization present at the time of culturing was maintained in all of the cultures examined at 1 DIV. This initial change in width was probably due to thinning and spreading of the culture, as judged by the corresponding decrease in slice thickness over this time. Several studies have documented that roller tube slice cultures prepared with the plasma clot technique spread out and reduce in thickness, in some cases to monolayers (Gähwiler, 1981; Gähwiler, 1988; DeJong et al., 1988; Caeser et al., 1989). However, use of collagen as a substrate in the present culture system resulted in no further thinning

Figure 10 Developmental changes in electrophysiological properties. Data (mean $\pm$ standard error, n indicated) for cultured neurons and acute slice neurons at 1–2, 3–5, and 6–9 days for (A) Action potential amplitude, measured from threshold to peak, (B) Duration, measured at $\frac{1}{2}$ amplitude, (C) Rate of rise and (D) Rate of fall. Note changes in these electrical membrane properties in cultured neurons exhibited a similar developmental profile to the changes seen in acute slice neurons recorded from under similar conditions. All recordings were obtained at room temperature.

of the slices after 1 DIV, even though cortical width increased an additional 50% between 1 and 9 DIV. This increase in cortical width may result, in part, from the observed three- to four-fold increase in the average size of neuronal somata and the marked increase in dendritic fields. The finding that the increase in volume of the total slice is of lesser magnitude than the increase in average size of individual neurons, in conjunction with the observation of small darkly stained nuclei that become more prominent over time in culture, suggests that regressive events such as cell death are probably also occurring in culture.

Despite the increase in cortical width, accompanied by variable amounts of distortion in the normal radial organization of the tissue, layers V, VI, and subplate could be identified as occupying the lower half of the cortical slice in the majority of cultures examined, throughout the 9-day culture period. Unlike the laminae already formed by the time of culture preparation, layers II–IV were not

clearly distinguishable in these cultures. These findings are consistent with other reports suggesting that cortical cytoarchitectonic features formed by the time of slice preparation are maintained in culture (De Jong et al., 1988; Behan et al., 1991; Distler and Robertson, 1992). Neurons that would normally give rise to layers II, III, and IV are generated later and are either contained within the undifferentiated compact cortical plate or are still in the process of migration to the cortical plate at P0 (Angevine and Sidman, 1961; Berry and Rogers, 1965; Walsh and Cepko, 1992). Studies of neuronal migration in slice cultures indicate that migration, begun *in utero*, continues in culture (Bolz et al., 1992; Yamamoto et al., 1992; Roberts et al., 1993). Thus, these previous studies suggest that neurons migrating through layers V and VI at the time the cultures were prepared likely would continue their migration, supporting the notion that the neurons examined in this study were, indeed, layer V and VI/SP neurons.

Figure 11 The ability to generate multiple spikes develops over the first postnatal week. (A) Single action potentials in response to a 150-ms suprathreshold depolarizing current pulse in a cultured and an acute slice neuron at 1–2 days. Multiple spikes in response to a suprathreshold depolarizing current pulse in a cultured and an acute neuron at 6–9 days. (B) The percentage of cells capable of generating multiple spikes, in the cultured and acute slices, increased during this developmental period.

Development of Distinct Morphological Classes in Culture

Early in culture (1–2 DIV) all neurons appeared immature, with small cell bodies and short unbranched processes, and many could not yet be unambiguously identified as pyramidal or nonpyramidal. Neurons at this stage often displayed a thick process oriented toward the pial surface, probably a remnant of the leading process evident during migration. During development *in vivo*, this neurite is believed to develop into an apical dendrite in pyramidal cells or is partially retracted in stellate cells (Katz and Callaway, 1992; Marín-Padilla, 1992). Cells examined at later times could be classified as pyramidal or nonpyramidal, based on cell body shape and dendritic arbor, suggesting that development of basic morphological class, in this sys-

tem, is not dependent on extrinsic influences. Previous studies have demonstrated that both pyramidal and nonpyramidal cells differentiated in dissociated cortical cultures (Kreigstein and Dichter, 1983), suggesting that differentiation of distinct morphological classes may be under intrinsic control.

Somal and Dendritic Differentiation

An increase in size of neuronal somata is a well-documented characteristic of developing neurons throughout the nervous system (La Velle, 1956) and within the cerebral cortex (Miller, 1981, 1988a,b). Results reported here document a remarkably close parallel increase in average cell body area for cells developing in organotypic slice cultures and those developing in the animal. It is

Figure 12 Correlated morphological and physiological development *in vitro*. (A) Correlation of input resistance and cell area. Each point represents data from an individual cell. These data were fit with a simple linear regression line with $r = -.64$ and $p < .01$, indicating a significant correlation between increase in cell area and decrease in membrane resistance. (B) An index of physiological development was created for each cell (based on AP amplitude and duration) and was graphed vs an index of the morphological development (based on cell area and total dendritic length) of the same cell. These data were fit with a simple linear regression line with $r = .73$ and $p < .01$, indicating a significant correlation in the relative rates of physiological and morphological maturation. Symbols represent cells taken from the three age groups (Open symbols indicate nonpyramidal neurons and closed symbols indicate pyramidal neurons).

clear that these data represent a true increase in neuronal cell size over time in culture and are not simply a result of selective survival of large cells in culture as the average size of neurons between 6–9 DIV was substantially greater than even the largest cells at P0. Similar increases have been documented in neurons from a variety of CNS structures developing in culture (Gähwiler, 1981, 1988; Zimmer and Gähwiler, 1984).

Analysis of neurons developing in dissociated

cell culture support the suggestion that basic features of cellular morphology, such as the initial orientation and number of outgrowing dendrites, are intrinsic properties of individual neurons. However, the observation of abnormally high levels of dendritic complexity in cultured pyramidal neurons developing in dissociated cell culture suggests that factors which normally limit the extent of dendritic branching are lacking in the dissociated culture environment (Kreigstein and Dichter, 1983). Changes in the patterns of dendritic arborization seen in neurons developing in slice culture reported in the present study closely parallel the maturation of dendritic morphology observed in neurons developing *in vivo* and examined in acutely prepared slices (present study) or in Golgi stained preparations (Juraska and Fifkova, 1979; Wise et al., 1979; Miller, 1981, 1988a; Petit et al., 1988). These findings demonstrate that the isolated cortical slice environment is sufficient for normal maturation of early dendritic morphology and suggests that cues important in controlling these processes are provided by the local cortical environment rather than factors intrinsic to the cell or extrinsic to the cortex.

Preparation of the slice cultures clearly eliminates brainstem, basal forebrain, thalamic, as well as callosal and many intrahemispheric cortical afferents and efferents. In light of the large number of studies describing the effects of reduced afferent activity on dendritic development (Valverde, 1967; Coleman and Riesen, 1968; Greenough and Volkmar, 1973; Diamond et al., 1987; Höhmann et al., 1991), it is perhaps surprising that the present experiments detected few differences between neurons grown in slice cultures and neurons in acutely prepared slices. It should be pointed out, however, that we have confined our analyses to a limited number of quantifiable aspects of morphological development. The determination of developmental changes in dendritic complexity based on Sholl distributions and branch order analysis performed in this study would not have revealed more subtle changes in dendritic orientation, such as that described in mouse barrel cortex after early lesions of mystacial vibrissae (Steffen and Van der Loos, 1980). In addition, we have confined our analysis to cells in layers V, VI, and SP during the first 9 DIV. Cells at this stage are still not fully mature and some of the developmental events resulting in the remarkable complexity of pyramidal cell form seen in the adult cortex undoubtedly occur after P9 (Lund and Booth 1975; Lorente de No, 1992; Marín-Padilla, 1992).

Electrical Membrane Properties

The results of this study show that in parallel with their morphological maturation, cortical neurons developing in slice cultures are also undergoing marked development of their intrinsic membrane properties. The developmental decrease in neuronal input resistance, in both acute and cultured slices, is consistent in magnitude with the change reported previously during early postnatal development of cortical neurons from rat neocortex (McCormick and Prince, 1987; Kriegstein et al., 1987). The relatively high input resistance values recorded at all ages in our study probably resulted from our use of the whole-cell recording technique instead of conventional intracellular recording (LoTurco et al., 1991; Spigleman et al., 1992). We also observed a significant increase in spike amplitude and a decrease in spike duration over the first 9 DIV. In previous studies, recordings from layer V pyramidal neurons in acute slices from rat neocortex revealed a similar two-fold decrease in spike duration during the first 10 days of postnatal development (Kriegstein et al., 1987; McCormick and Prince, 1987). However, in contrast to the previous work, all of our recordings were performed at room temperature, which resulted in slower kinetic properties of the ion channels underlying the action potential and accounts for the relatively long AP durations reported in the present study. As the time course and extent of the developmental changes in AP waveform were very similar in cells grown in the isolated cortical slice, when compared with cells in acute slices, it can be concluded that the early development of electrical membrane properties, important in generation of the electrical impulse, is not dependent on extrinsic factors. It may even be the case that expression of these early AP characteristics in cortical neurons is under intrinsic genetic control, as has been shown for other neuronal cells (O'Dowd, 1983; Henderson and Spitzer, 1986).

Our data, from both acute and cultured slices, also demonstrate an increase over time in the percentage of neurons capable of firing multiple spikes in response to a prolonged depolarizing current pulse. While it is possible that the low percentage of young cells capable of firing repetitively is due to membrane damage during seal formation, the high input resistance of these cells, and their ability to fire single-action potentials for up to 30–45 min suggests that the integrity of the membranes has not been compromised. Therefore, it seems likely that as the cortical cells mature and acquire

the ionic currents necessary for developmental changes in action potential waveform, changes in these same or additional ionic currents are also occurring to confer the ability to generate repetitive spikes.

All of the multiple spiking cells examined in the present study displayed trains of single spikes whose frequency did not accommodate during a long depolarizing current injection. Previous studies in mature neocortical neurons have demonstrated three distinct patterns of repetitive firing behavior: regular spiking, bursting, and fast spiking (Connors and Gutnick, 1990). Studies using conventional intracellular electrodes have shown that cells in neocortical explant cultures after 3–6 weeks *in vitro* have the same three patterns of repetitive firing described in the acute slice preparations (Wolfson et al., 1989; Gutnick et al., 1989). The lack of distinct physiological classes of neurons in the present study may be due to the use of the whole-cell recording technique or may be related to the relatively immature state of the neurons. Using whole-cell recording, the inside of the cell is rapidly perfused with the contents of the pipet, which includes the calcium chelator EGTA, perhaps preventing the activation of ionic currents underlying distinct patterns of repetitive firing. Alternatively, these early postnatal cells may not yet have developed the mature complement of ionic conductances that are necessary for expression of the full range of spiking patterns seen in mature cells.

Correlation of Morphological and Physiological Development

While it is usually assumed that morphological and physiological development of neurons occur in concert, this issue has received little direct attention (Gruol and Franklin, 1987). Because both electrophysiological and morphological data were obtained for 25 cultured cells over the first 9 DIV, it was possible to compare the expression of specific physiological parameters with specific morphological measurements as a step toward determining whether there may be common mechanisms controlling their development. Without assuming any changes in specific membrane resistance, one predicts that an increase in membrane surface area will result in a decrease in input resistance, analogous to adding resistors in parallel. Input resistance in the cultured neurons decreased dramatically during the first 9 DIV. Over this same time period the membrane surface area increased

due to an expansion in soma size and elaboration of dendritic processes. When input resistance was compared to a morphological index, taking into account both soma area and dendritic length of individual cells, a highly significant correlation was seen. Comparison of input resistance with soma area alone revealed a similar level of correlation. However, a slightly lower correlation was observed when input resistance was compared to dendritic length alone. These data suggest that additional membrane in the cell body may contribute more to the measured decrease in input resistance than the increase in dendritic length. A highly significant correlation was also noted between the degree of physiological maturation (as indicated by AP amplitude, duration, and rate of rise) and soma area, consistent with the hypothesis that ion channels in the soma membrane contribute to developmental changes in AP waveform. In addition, a significant correlation between the dendritic length and AP properties suggests that dendritic membrane may also contain channels that are important in generation and development of the AP waveform. Examination of the correlation between indices of relative morphological maturity and physiological maturity revealed a highly significant correlation ($R = .73, p < .01$), demonstrating that as cortical neurons develop morphologically there is a concomitant maturation in the intrinsic membrane properties. Future studies aimed at dissociating these properties will be needed to determine if these properties are simply co-developing, or whether they are in fact coupled.

In summary, we find that neurons in the mouse sensorimotor cortex, maintained in culture for up to 9 days in the absence of many of the normal extrinsic influences, develop dendritic morphologies and electrical membrane properties along a time course very similar to that of cortical cells developing *in vivo*. Further, the maturational state of individual cells was reflected in the degrees of both morphological and physiological differentiation. These quantitative data describing the pattern of growth of neurons differentiating in slice culture, when compared with similar measurements *in vivo* and in dissociated cell culture, provide important insights into the mechanisms underlying the expression of early neuronal phenotypes. The fact that many aspects of early cortical neuron differentiation, including the rate of maturation, are similar in cells developing in slice culture with those developing *in vivo*, suggests that differentiation of these features is governed by local influences (i.e., genetic regulation and/or local environmental in-

teractions). Our finding that early dendritic branching proceeds normally in slice culture, while abnormal branching had been previously noted in cells developing in dissociated cell culture (Kreigstein and Dichter, 1983), suggests that local cortical interactions and not factors intrinsic to the neuron, are involved in determining the normal pattern of dendritic branching. Though neurons in culture appear to grow and branch at rates that are not altered by the lack of extrinsic influences, our studies do not address such issues as the determination of exact location of particular branches and their orientation. It seems likely that extrinsic factors, such as the amount of available synaptic input (Steffen and van der Loos, 1980) may influence fine details of distal dendritic branch patterns.

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