Synapses on Demand Require Dendrites at the Ready: How Defining Stages of Dendritic Development In Vitro Could Inform Studies of Behaviorally Driven Information Storage in the Brain

ABSTRACT: Bill Greenough’s work provides a framework for thinking about synaptogenesis not only as a key step in the initial wiring of neural systems according to a species typical plan (i.e., experience-expectant development), but also as a mechanism for storing information based on an individual’s unique experience over its lifetime (i.e., experience-dependent plasticity). Analysis of synaptic development in vitro brings a new opportunity to test the limits of expectant-expectant development at the level of the individual neuron. We analyzed dendritic growth, synapse formation, and the development of specialized cytoplasmic microdomains during development in cultured hippocampal neurons, to determine if the timing of each of these events is correlated. Taken together, the findings reported here support the hypotheses that (1) dendritic development is rate limiting in synapse formation and (2) synaptic circuits are assembled in a step-wise fashion consistent with a stage-specific shift from genomically pre-programmed to activity-dependent mechanisms. © 2011 Wiley Periodicals, Inc. Dev Psychobiol 53: 443–455, 2011.

Keywords: dendrite; dendritic development; synapse formation; postsynaptic development; cultured hippocampal neuron; experience-expectant brain development

INTRODUCTION

The functional architecture of the human brain is built through an extended process that begins during gestation but continues for years after birth (Huttenlocher, 1990; Purpura, 1975; Shaw et al., 2008). In fact, there is reason to believe that brain development never stops during the human lifespan, given the capacity for experience-induced modification of synaptic circuits (Greenough, Black, & Wallace, 1987; Holmaat & Svoboda, 2009). Despite the daunting complexity of the neural systems created, it is possible that both development and experience-dependent plasticity operate via the same basic cellular processes associated
with building any synaptic circuit. According to this hypothesis, it will be of fundamental importance to understand the sequence of synaptic development in terms of key stages of cell autonomous maturation versus steps that depend on the history of synaptic activity.

So what are the rules that operate in development? Neural circuits are assembled, station-by-station, as neurons extend their axons to the appropriate post-synaptic target, while, in parallel, the neurons that will receive these afferents extend a dendritic arbor that presents a surface receptive to prospective synaptic contacts at the right time. By explicitly laying out the set of events that must take place in development (e.g., initial specification of axons and dendrites vs. subsequent outgrowth and maturational steps), we are able to test the extent to which each event is governed by cell autonomous, or intrinsic, programs, and which are influenced by external signals.

In principle, both axonal outgrowth and formation of a dendritic arbor could be regulated by developmental programs intrinsic to the neuron. Evidence that this is the case comes from studies of dissociated primary culture of CNS neurons, where neurons removed from their native environment still become polarized (Dotti, Sullivan, & Banker, 1988). As development in vitro proceeds, dendritic arbors grow to reach a size and geometry appropriate to the cell type, whereas axonal outgrowth is sustained, creating wandering loops of axons that are presumably searching in vain for the extracellular cues that would mark the path to their intended targets in situ (Banker & Waxman, 1988). Given the wealth of data on the role of extrinsic cues in axon pathfinding, this axonal behavior is not surprising. But, even from these simple observations, the pattern of dendritic growth contrasts starkly, and suggests different mechanisms of regulation (Kollins, Bell, Butts, & Withers, 2009).

As the primary recipients of input from other cells, the size and orientation of dendrites limit the number and pattern of connections a neuron can receive. Understanding how that growth is controlled at the cellular level is therefore critical to understanding how synaptic circuits form. For example, in vitro, the onset of synapse formation appears to be dictated by the maturational state of the postsynaptic cells (Fletcher, DeCamilli, & Banker, 1994). Slow rates of growth and an extended period of maturation are characteristic of dendrites, and so one question that arises is whether this protracted growth also reflects extensive maturation required to support the capacity for synaptogenesis. In humans, the dendritic arbor does not reach its full extent for years after birth (Huttenlocher, 1990; Purpura, 1975); in rats, it takes weeks (Eayrs & Goodhead, 1959; Juraska & Fifkova, 1979; Watson, DeSesso, Hurt, & Cappon, 2006). This extended period of dendritic development correlates well with cognitive development. Accordingly, anything that interferes with the normal developmental process might be predicted to increase the probability to learning disabilities, memory defects, and other neurological defects expressed later in life (Rodier, 1994; Wallace, Reitzenstein, & Withers, 2003). In support of this, a host of dendritic and synaptic abnormalities are associated with developmental delays and mental retardation (e.g., Benavides-Piccione et al., 2004; Comery et al., 1997; Dierssen & Ramakers, 2006; Huttenlocher, 1991; Weiler et al., 1997). Given that the state of the dendrite may limit the capacity for synapse formation, the more concretely we are able to define benchmarks of dendritic morphogenesis, the more readily we can assess the biological consequences when development goes awry.

Much of what we know about the cell biological mechanisms of dendritic growth and maturational stages comes from studies using in vitro models, like cultured hippocampal neurons prepared from embryonic rat brain (Banker & Goslin, 1998). The advantages of such a preparation are numerous—the cells are accessible and readily manipulated. Even with simple wide-field microscopy, dynamic changes in morphology can direct attention to potential changes in molecular state. Because the cells are plated onto optical-quality glass coverslips, development can be observed over time to test the hypothesis that the instructions for generating appropriate dendritic architecture are intrinsically programmed. Such experiments are not possible in vivo, where the cellular environment cannot be controlled.

This in vitro preparation was first used to describe a stereotyped sequence of neuron development that was divided into 5 stages (Dotti et al., 1988). The first 3 stages take place within the first days in culture and include the initial formation and outgrowth of undifferentiated “minor processes” from the cell body, and the subsequent development of polarity as one of these minor processes extends rapidly to become the axon. The axon forms before dendrites develop, similar to what has been observed in vivo (Barnes & Polleux, 2009; Brittis, Lemmon, Rutishauser, & Silver, 1995). The remaining two stages follow the growth of the dendrites over the next several weeks. During stage 4, the remaining undifferentiated minor processes differentiate into a dendritic arbor by adding branches, and tapering. In stage 5 the dendritic arbor continues to increase in complexity, but is most notably distinguished by the presence of dendritic spines, the primary site of excitatory synapses (Dotti et al., 1988). Synaptic contacts are forming by the time cells reach this developmental
state, but the relationship between the rate of dendritic development and rate of synaptogenesis is not known.

If the sole limitation is amount of available dendritic territory once synaptogenesis begins in vitro, then synapse addition should proceed at the same rate as dendritic outgrowth. Here, we analyze the timing of dendritic outgrowth, synapse formation, and the appearance of cytoplasmic specializations associated with local protein synthesis in dendrites to test whether they develop at the same rate. These data reveal two new findings. First, there is a plateau phase in dendrite extension that coincides with the time at which the first presynaptic contacts are made. After several days of little to no outgrowth, dendritic growth then resumes. Second, there is a period of exuberant synapse formation that begins days after dendritic outgrowth begins. Accelerated synapse formation overlaps in time with the appearance of microdomains of protein synthesis machinery within the cytoplasm. We propose that these findings show previously unrecognized, additional stages of dendritic development that represent key steps in “molecular readiness” within the cell and use these data to frame a new hypothesis about how the completion of one stage might be enable the initiation of another.

METHODS

Low density neuronal cultures prepared from embryonic day 18 rat hippocampi, and grown in a co-culture with a glial feeder layer were prepared as described previously (Banker & Goslin, 1998) with minor modifications (Kollins et al., 2009).

To analyze the dendritic arbor and presynaptic contacts, cells were fixed at time points across development in vitro and prepared for immunostaining (for detailed methods see Kollins et al., 2009; Withers & Banker, 1998). The dendritic arbor was stained with antibodies to the dendritically-localized protein MAP2 (1:3,000, Sigma Aldrich, St. Louis, MO); synapses were localized using the presynaptic vesicle protein synapsin 1 (1:3,000, from P. DeCamilli). Images of 15 neurons per developmental timepoint were acquired using MetaMorph software and a Leica IRB inverted microscope coupled with a CCD camera (Photometrics CoolSnap). The synapsin-1 and MAP2 images of the same cell were combined in MetaMorph to yield a merged image, to ensure that only synapsin-1 puncta in direct contact with dendrites were counted. Dendritic outgrowth was estimated using a Sholl concentric ring analysis (Sholl, 1956) with rings placed at 15 μm intervals, and the number of dendrite intersections at each ring were tallied (rings were made using Image J open source freeware). Counting and summing the number of branches at each order determined the total number of branches/cell.

The localization of protein synthetic machinery was studied in neurons fixed as described above, and double-stained with a fluorescent RNA-binding Nissl stain (NeuroTrace, Molecular Probes, Invitrogen, Carlsbad, CA, 1:40 in Phosphate Buffered Saline [PBS], 5′ at room temp), and fluorescently conjugated phalloidin (Molecular Probes, Invitrogen, 1:40 in PBS, for 45′ at 37°C) which binds to polymerized actin and is highly concentrated in filopodia, dendritic spines and presynaptic terminals, and thus used as an indirect marker of these structures. Digital images of eight cells per developmental timepoint were acquired, similar to methods described above. Line scans (1 pixel/diameter, corresponding to approximately 0.1 μm) were taken along a representative dendrite in MetaMorph to determine relative fluorescence intensity. Data were also analyzed with a simple pattern recognition algorithm in which the line scan data was divided into 230 pixel sections representing approximately 25 μm, from which we calculated an approximation of the derivative for all segments so that \( \frac{dx(i)}{dx(i+1)} = x(i+1) - x(i) \). Each derivative was then compared to the means of the derivatives of each age group and classified based on the Euclidean distance from each point to its closest mean.

RESULTS

Growth of Dendrites Precedes Rather Than Parallels Synapse Formation

Dendritic length is an index of the available receptive surface for synaptic contacts, and so a fundamental question is whether dendritic growth precedes or parallels an increase in the number of synaptic contacts formed. Presynaptic contacts first appear around 3 days in vitro (DIV), and previous work has shown that there is little growth of the immature dendrites/minor processes before this time, and so we chose this time point to begin quantifying dendritic growth. Qualitative analysis of neurons between 3 and 14 DIV showed both growth of the dendritic arbor, and the addition of synapses over time (Fig. 1 A–E). Quantitative analysis revealed that active outgrowth of the dendritic arbor did not begin until 5–7 DIV, but once initiated, growth followed a steady expansion as neurons matured in vitro, reflected by both the territory covered by branches (Fig. 1F), and number of branches within this territory (Fig. 1G). Once growth of the arbor began, significant additions in both length and branch number occurred in a relatively short time, and continued at a progressive rate at least through 14 DIV, the oldest time point measured.

In contrast to the relatively steady growth of the dendritic arbor, analysis of the number of presynaptic contacts indicated an apparent exuberant burst of synaptic contact formation between 10 and 14 DIV (Fig. 1H). This surge interrupted a previously gradual rise in the number of synapses from 3 to 10 DIV. This initial pattern
of steady accretion of synaptic contacts contrasts with
the significant dendritic growth occurring in the same
cells at the same time. Thus, it is clear that it is not
simply the availability of dendritic surface per se that
limits the rate at which new synaptic contacts form.

The Pattern of Synapse Addition Changes With
Development

If, as the results summarized in Figure 1 suggest, the
rate of synapse formation is significantly accelerated
when dendrites reach a certain stage of maturity, then it
is important to determine whether all branches of the
dendritic arbor mature synchronously or whether there
is a detectable proximal-distal gradient. These two hy-
potheses predict distinctly different distributions of pre-
synaptic terminals. If recently added distal regions are
less mature than the stable shaft that formed earlier in
time, then it would make cell biological sense for syn-
apses to form along a developing dendrite in proximal
to distal fashion. On the other hand, if the cell deter-
mines the maturational state and all dendrites are
equivalent, an incoming axon would likely form con-
tacts wherever the first point of contact was, indepen-
dent of location. These predictions of differential
spatial bias in synapse formation could be tested
directly using Sholl analysis to map the distribution of
synapses across the dendritic arbor (Fig. 2). If the
entire dendritic arbor is equally receptive to inner-
vation, and chance determines where along the arbor
the axon forms a contact, then the distribution of pre-
synaptic contacts should show the same shape of cure
in a Sholl should show the same shape of curve in a
Sholl analysis as does the dendritic arbor (Fig. 2, left

![Figure 1](image_url)
If however, synapses appear preferentially on parts of the dendritic arbor that have formed earlier than those that are added later, then the curves for the two measures would have different shapes (Fig. 2, right panel).

The data suggest a temporal shift in preference for location of synaptogenesis (Fig. 3). When presynaptic contacts first began to appear (3 DIV), most synapses were detected further from the cell body. By 14 DIV, however, this bias was no longer apparent, and most of the presynaptic contacts were distributed nearer to the cell body, and within the Sholl rings that had the most intersections with dendrites. The peripheral distribution of newly forming synapses at 3 DIV could simply reflect that these are the sites where initial contact is most likely to occur. Alternatively, it could reflect differential receptivity of the dendritic surface related to initial sorting of different classes of synaptic inputs (e.g., excitatory vs. inhibitory) that might arrive at different times (Christie & DeBlas, 2003; Swanwick, Murthy, Mtchedlishvili, Sieghart, & Kapur, 2006). Regardless, the distribution of presynaptic contacts later in development suggests a shift away from a distal, or unbiased distribution and that a disproportionate number of synapses are added within the proximal zone of the dendritic arbor.

**Maturation of the Cytoplasmic Machinery for Dendritic Protein Synthesis**

As molecular components of the synaptic junction are being assembled at the surface of dendrites, changes in the molecular organization of cytoplasm also become
apparent in the form of a preferential positioning of protein synthetic machinery, or polyribosomal aggregates (PRAs) near synapses, and particularly at the base of spines (Steward, 1983b). Not only does this positioning correlate with the timing of synaptogenesis during development and in regeneration in vivo (Steward, 1983a,b), but work from Bill Greenough’s lab also demonstrated that more spines had PRAs following housing in a complex environment (Greenough, Hwang, & Gorman, 1985). Together these findings suggest that the selective positioning of this machinery beneath spine synapses in vivo may be a marker for active synapse formation.

In vitro, the development of such localization might also be an important benchmark for assessing stages of dendritic maturation. For example, if the machinery for local protein synthesis were in position when dendrites become receptive to innervation, the proteins being delivered might be involved in readying locations for synaptic contact. On the other hand, if protein synthetic machinery does not take on such localization until after synapses form in the membrane above, the localization of this machinery would be implicated in later stages of synaptic maturation.

Previous work has shown that RNAs are concentrated in distinct granules in mature neurons in vitro (reviewed in Hirokawa, 2006). We sought to determine if RNA-associated machinery (i.e., material associated with ribosomes) was present in immature neurons as well by analyzing the localization of a fluorescent Nissl stain (Fig. 4). With this stain, protein synthesis machinery appeared to be distributed diffusely early in development, but as time progressed, it became increasingly organized into the discrete clusters characteristic of the cytoplasm beneath mature synapses (compare Nissl staining at 1 and

![Diagram of Quantitative analysis of the distribution of presynaptic contacts along dendrites from 3 to 14 DIV (Panels A–E) analyzed by density along Sholl rings (at 15 μm intervals). The heavy black bar represents the mean number of dendrite intersections at each ring/neuron, the hatched line shows the number of presynaptic contacts within each ring. The shape of the two curves is similar, suggesting that synapses are added proportionally along the dendrite, rather than preferentially to proximal, or to distal dendrites. The robust increase in presynaptic contacts after 10 DIV is evident in (E).]
Quantitative analysis of fluorescence using linescans taken along the dendrites showed that with increased developmental age, the size of Nissl aggregates also increased both in size, and in intensity of fluorescence (Fig. 4 C, F, I, respectively) show that the intensity and size of the fluorescent aggregates increases. In addition, there is an apparent shift in localization from the core of the dendrite, to along the edges, nearer to the location of synapses. Arrowheads point to candidate postsynaptic sites of filopodia (D,E), or spines (G,H) with Nissl-stained aggregates beneath. Arrow (G,H) points to one of several axons in the field, devoid of Nissl staining. Fluorescence intensity is measured in arbitrary units. Scale bar = 10 μm.

**DISCUSSION**

If the mechanism of brain information storage converges on the brain’s capacity to generate new synapses on behavioral demand (Greenough, Withers, & Wallace, 1990) then the capacity to learn must emerge from successful dendritic development. Our analyses suggest that during development, growth of the dendritic arbor is not constant. There are several critical phases of dendritic growth with the potential to
Table 1. A Pattern Recognition Algorithm Was Used to Predict the Developmental Age of the Cell From Which Line Scan Data Was Taken

<table>
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<th>Days in vitro</th>
<th>% Correctly identified</th>
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<tr>
<td>1</td>
<td>80.00</td>
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<td>4</td>
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<td>8</td>
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<td>11</td>
<td>68.75</td>
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<td>15</td>
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<td>18</td>
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The percentage of data that was correctly identified was high, particularly as cells became more mature. That this analysis sorted data according to developmental age supports qualitative analysis of the line scan data that the pattern of Nissl staining changes over time.

determine a readiness for synapse formation (Fig. 5). Detected as periods of active dendritic growth interspersed with at least one plateau, these stages may anticipate, or enable, contact with presynaptic partners. These stages also suggest potential points of molecular regulation that may be critical for the next stage of growth. In addition to confirming that similar regulation occurs in vivo, a key next step will be to identify what this regulation entails in vitro. With the suite of molecular imaging tools that are now available we can begin to account for the molecular assembly of structural specialization using live cell imaging.

Multiple Stages of Dendritic Growth and Synapse Formation Suggest Multiple Opportunities for Regulation

The timecourse of dendritic maturation, as revealed by morphological analysis of cultured hippocampal neurons, begins with the formation and extension of minor processes (e.g., stages 1–3, Banker & Waxman, 1988; Dotti et al., 1988; Withers, Higgins, Charette, & Banker, 2000). These “first growth” processes lack the phenotype of mature dendrites, for example, taper, length and branching, and importantly, are not receptive to innervation (Fletcher et al., 1994). Although the tips of these processes are motile, they progress little in length and appear to “loiter” (Kollins et al., 2009, Withers & Banker, unpublished observations). Prior to the analysis reported here, a common assumption was that once dendrites became receptive, both dendritic growth and synaptogenesis would proceed in parallel. We were therefore surprised when our analysis showed that dendritic growth appeared to be stalled for an additional 2 days. Therefore, in light of our quantitative analysis that showed no net dendritic growth between 3 and 5 days, well after the initial phase of minor process formation, it is tempting to hypothesize that growth is deferred until some level of commitment toward a dendritic phenotype is attained, making these dendrites synapse-ready.

Regardless of what events enable the onset of synaptogenesis, the timing of the late surge in number of presynaptic contacts onto dendrites (e.g., between 10 and 14 DIV) has implications for mechanism. This period of development suggests additional layers of regulation of synapse formation that may be downstream of, or distinct from, the first events that render dendrites receptive to innervation. The molecular regulation that enables synapse formation could be intrinsic to the postsynaptic cell, but it could also be facilitated by extrinsic factors. For example, evidence has shown that signals from astroglia can regulate the rate at which new synaptic contacts form (Christopherson et al., 2005; Elmariiah, Oh, Hughes, & Balice-Gordon, 2005; Withers, Lambruschi, Brown, & Wallace, 2008, data published in preliminary form). Further evidence of step-wise regulation comes from findings that some factors secreted by astroglia appear to regulate the probability with which structural contacts form on dendrites, while other factors may determine when they become physiologically active (Barker, Koch, Reed, Barres, & Ullian, 2008; Eroglu & Barres, 2010).

Key Events in Dendritic Maturation

Observable structural changes provide benchmarks of early dendrite maturation, for example, the appearance of taper along the dendrite shaft, growth of the arbor, and the addition of new branches and the appearance of presynaptic contacts (Craig & Banker, 1994; Dotti et al., 1988; Fletcher, Cameron, De Camilli, & Banker, 1991; Hirokawa & Takemura, 2005). Presumably, however, these morphological changes are the expression of underlying changes in the molecular state of immature minor processes that change the composition from undifferentiated to a dendritic phenotype (reviewed in Barnes & Polleux, 2009; Craig & Banker, 1994).

Appropriate development of specialized cytoplasmic compartments, like localized protein synthesis machinery, could contribute to a molecular state change in dendrites, and appears to be critical for normal dendritic development (Beckel-Mitchener & Greenough, 2004). Yet the question of how these specializations are developmentally regulated is largely unanswered. Observations reported here suggest that early in dendritic development in vitro, ribosomes and associated protein synthesis machinery are distributed diffusely throughout the dendrite, but over time become increasingly clustered in locations near the base of synapses and dendritic spines. Although the mechanism for
FIGURE 5  Notable phases of dendritic development are shown in the top panel, and the relative timetable for key events (e.g., dendritic outgrowth, branch formation, synapse formation, and postsynaptic specialization) is charted in the lower panel. Initial growth begins with the formation of immature minor processes during the development of neuron polarity (Phase A, days 1–3), but then lags (Phase B) as the first presynaptic contacts begin to form. Dendritic outgrowth resumes (Phase C) after dendrites have assumed a dendritic phenotype in dendritic. During this phase, dendrites extend, and branch, at a steady pace, and synapses continue to be added in modest numbers. Phase D is initiated with robust synapse formation, coincident with the development of specialized postsynaptic structures. It is around this time that fluorescent Nissl aggregates appear to accumulate near synapses. In the neuron depicted in the illustration, the progress of dendritic development is shown over time. The initial segment of the axon emerges from the lower right of the cell body, and incoming synaptic contacts are shown in red.
localization is not known, other organelles, such as mitochondria, are also recruited to synapses based on differential metabolic activation (Li, Okamoto, Hayashi, & Sheng, 2004). One mechanism by which synaptic activity might recruit protein synthetic machinery to synapses is the activation and localized diffusion of αCam II kinase (Rose, Jin, & Craig, 2009). In turn, protein synthesis is necessary to maintain immature synapses (Sebeo et al., 2009). Developmental disruption of local protein synthesis, as in the case of the Fragile X mutation, can lead to abnormal synapse development, and mental retardation (Irwin, Galvez, & Greenough, 2000; Weiler et al., 2004). These findings suggest a two-step process, where protein synthesis machinery comes to be localized to synapses after they form, then enabling local protein synthesis that then helps to stabilize those synapses.

The generation of dendritic spines, specialized microdomains associated with the postsynaptic side of excitatory synapses, is a key event in later maturation. In these cultured hippocampal neurons, they begin to appear typically after 2 weeks in vitro (Bartlett & Banker, 1984), shortly after the period of exuberant synapse formation that we report. Following the appearance of spines, a number of cell biological mechanisms come into play to build a mature and stable synapse. Receptors for the excitatory transmitter glutamate organize into clusters in the spine head (Craig, Blackstone, Huganir, & Banker, 1994) and a grid work of structural scaffolding, requiring the actin cytoskeleton (Zhang & Benson, 2002) and linker proteins such as PSD-95, or gephryn, contribute to the architectural stability and recruit signal transduction proteins into the postsynaptic complex (Craig, Graf, & Linhof, 2006; Lardi-Studier & Fritschy, 2007; Svitkina et al., 2010). Each of these steps provides potential opportunities for regulation by multiple signals. As a late event in dendritic maturation, and hence an apical property of healthy development, abnormal spine development can serve as a biomarker of disrupted development. Furthermore, because spine formation is clearly influenced by external factors, like synaptic activity, this late stage of development appears to represent one point of vulnerability associated with a shift from cell-autonomous development to extrinsic regulation.

How Far Can Analysis of Development In Vitro Take Us?

Thus far, comparisons of dendritic development in the intact organism and under in vitro conditions have documented a striking similarity. In both cases, the time course for maturation is slow (Dotti et al., 1988; Eayrs & Goodhead, 1959; Juraska & Fifkova, 1979; Withers et al., 2000), and the development of specialized postsynaptic structure (i.e., evidenced in the formation of dendritic spines, the assembly of clustered receptors and aggregation of postsynaptic machinery), occurs after axons have begun forming presynaptic contacts (Bourne & Harris, 2008; Craig et al., 2006; Schuman, Dynes, & Steward, 2006; Ziv & Smith, 1996).

There are notable contrasts, however, between the in vivo and in vitro environments, that are equally important to consider as they might reveal the limits of growth and plasticity in the dish. While basic aspects of dendritic development appear to occur in a normal sequence for neurons growing in vitro, primary culture cannot but fail to represent the diversity of the synaptic neighborhood available in intact tissue. The vascular system is entirely absent, and in many cases, physical contact between neurons and astrocytes eliminated. Undeniably, these considerations make the culture dish an artificial milieu for development, yet at the same time, the subtractions of these influences may illuminate all the more clearly the limits to what pre- and post-synaptic neurons can do on their own. The pause in dendritic growth prior to the onset of synapse formation, for example, could reflect a gap in signaling that physical contact with astrocytes might normally fill. Some current hypotheses about the contributions of nonneuronal brain cells to neuron function even consider astroglia as powerbrokers that mediate the energy supplied by the vasculature (Araque, Parpura, Sanzgiri, & Haydon, 1999; Bushong, Martone, & Ellisman, 2004; Stevens, 2008). Given that both types of these nonneuronal brain cells show plastic responses to environmental enrichment (Black, Sirevaag, & Greenough, 1987; Sirevaag, Black, Shafron, & Greenough, 1988; Wallace et al., this issue), timely metabolic interactions between neurons, glia, and blood vessels could be critical for support of plasticity on demand. Whether aspects of such a partnership could be modeled in vitro remains relatively unexplored.

Finally, because neurons growing in vitro develop in a random organization on a uniform field of poly-L-lysine, our findings reflect the growth of dendrites in an unpatterned environment. Neuronal activity in the dish is spontaneous and unpatterned as well. Nonetheless, polarity, the cornerstone event in establishing neuronal phenotype arises in an environment barren of external cues. The determination, and orientation of the axon, however, is most likely under external influence (Barnes & Polleux, 2009;Brittis & Silver, 1995; Esch, Lemmon, & Banker, 1999). Similarly, the orientation of the dendritic arbor is also modified by extrinsic
cues, like members of the cadherin adhesion molecule family (Arikkath, 2009). Individual molecules of the external environment can be selectively presented in vitro with techniques like microcontact printing, however, to tease out the influence of these cues on dendritic growth (Shi, Shen, & Kam, 2007; Withers, James, Kingman, Craighead, & Banker, 2006; Withers & Mumford, unpublished observations). The present data highlight how important it is to consider timing of development in designing these kinds of experiments.

While dendritic development in vivo is certainly influenced by factors outside the culture dish, we would argue that the fundamental architecture and phenotype of the dendritic arbor of principle neurons in vitro emerges nonetheless. Further, the fact that the developmental stages discussed here are observed in the stripped down environment of the culture dish supports the hypothesis that the sequence of development leading up to a robust capacity for synapse production, and postsynaptic maturation represent pre-programmed, experience-expectant phases of development independent of specific patterns of input. Now, a molecular accounting of the stages of dendritic development described here would give improved biomarkers for healthy experience-expectant brain development, as well as identify the cellular prerequisites for the expression of experience-dependent plasticity.

NOTES

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REFERENCES

developing synapses. Journal of Neuroscience, 29(31), 9778–9793.


