Ginger S. Withers
Christopher S. Wallace
Elizabeth M. Gibbs
Isaac R. Emery
Marja L. Applegate
Department of Biology
Whitman College
Walla Walla, WA 99362
E-mail: withergs@whitman.edu

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 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

Received 10 April 2011; Accepted 18 April 2011
Elizabeth M. Gibbs's present address is University of Michigan Neuroscience Graduate Program, Ann Arbor, MI 48109.
Isaac R. Emery's present address is Purdue University Ecological Science and Engineering Graduate Program, West Lafayette, IN 47907.
Correspondence to: G. S. Withers
Contract grant sponsor: NIH
Contract grant number: 1R15HD061831
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/dev.20560

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 postsynaptic 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, HM-2, Sigma Aldrich, St. Louis, MO); synapses were localized using the presynaptic vesicle protein synapsin I (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 synapsin1 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 $dxj(i) = xj(i + 1) - xj(i)$. 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 inital pattern

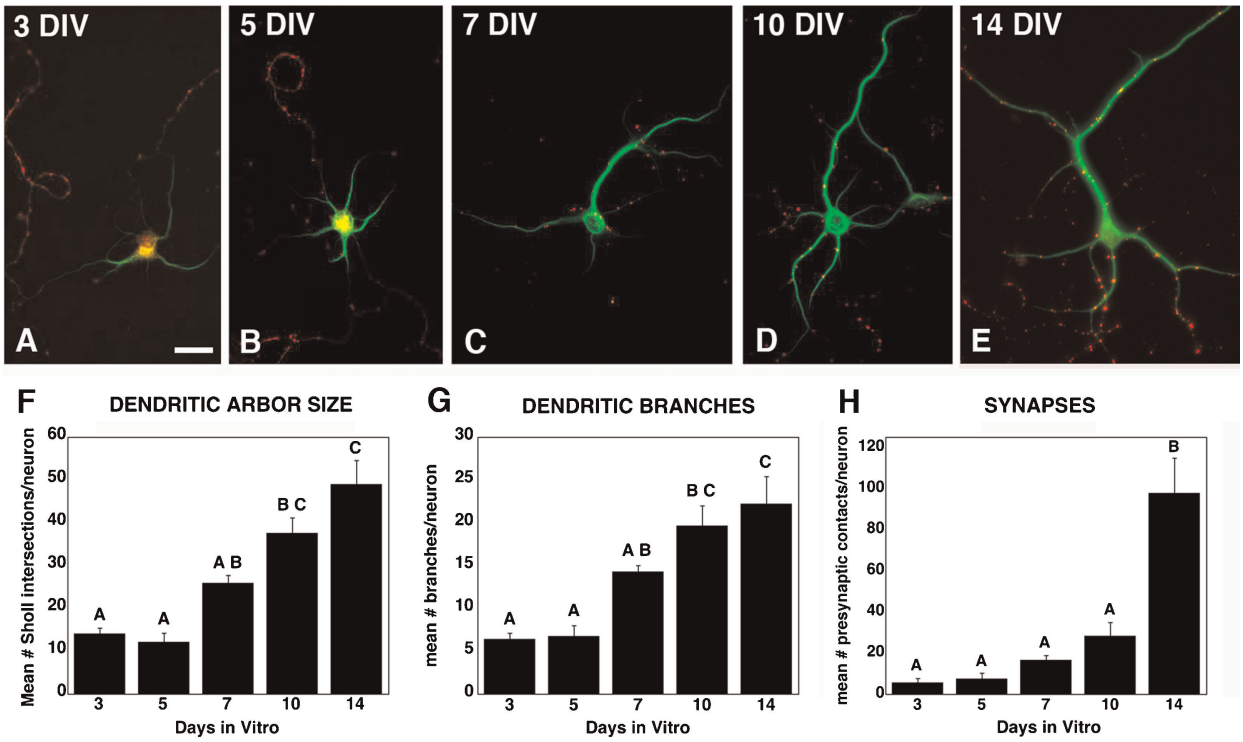

FIGURE 1 Development of the dendritic arbor and synapse formation during the first 2 weeks in culture. Panels A–E show representative images of hippocampal neurons from 3 to 14 days in vitro (DIV) immunostained with antibodies to MAP2 (green), to show the dendritic arbor, and Synapsin I (red) to show presynaptic contacts. Quantitative analyses of the extent of the dendritic arbor (F, as determined by the total number of Sholl ring intersections with rings at 15 μm intervals), total number of branches (G) and number of presynaptic contacts (H) shows that branches are added at a rate that parallels outgrowth, and net growth begins after about 5 DIV. The number of presynaptic contacts increases slowly until between 10 and 14 DIV, when there is a significant increase in the total number of contacts. Differences were determined using ANOVA, and Tukey posthoc comparisons (JMP Statistical Analysis Package). Bars with different letters are significantly different at $P < 0.05$. Scale bar = 10 μm.

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 hypotheses predict distinctly different distributions of presynaptic 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 synapses to form along a developing dendrite in proximal to distal fashion. On the other hand, if the cell determines the maturational state and all dendrites are equivalent, an incoming axon would likely form contacts wherever the first point of contact was, independent 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 innervation, and chance determines where along the arbor the axon forms a contact, then the distribution of presynaptic 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

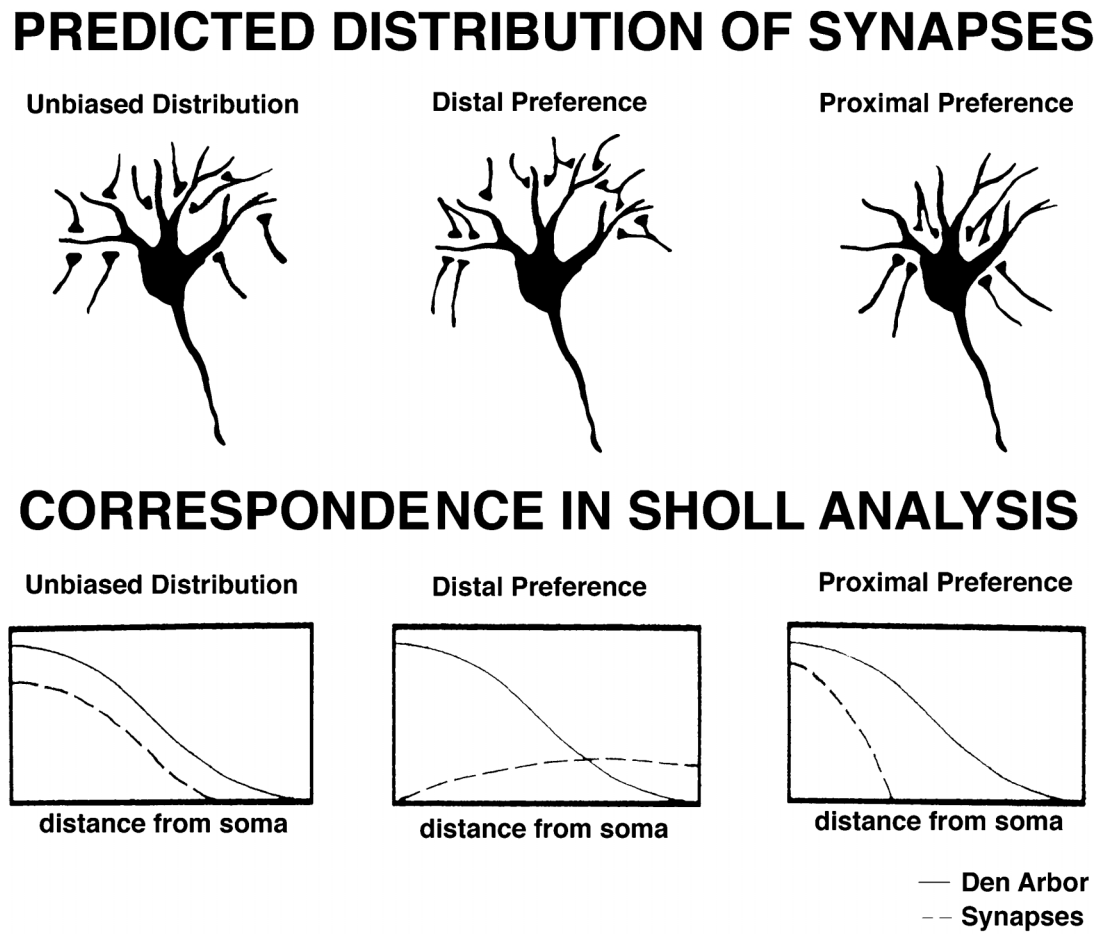

FIGURE 2 Predictions of the distribution of synapses along dendrites as determined using a Sholl Ring analysis. If presynaptic contacts have no particular preference along the dendritic arbor (e.g., proximal vs. distal to the cell body), then the distribution of presynaptic contacts should have essentially the same shape of curve as the distribution of dendrite intersections (left panels). Alternatively, if synapses form preferentially along distal dendrites (middle panels), or proximal dendrites (right panels), the curve reflecting density of synapses should shift to reflect that preference.

panel). 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

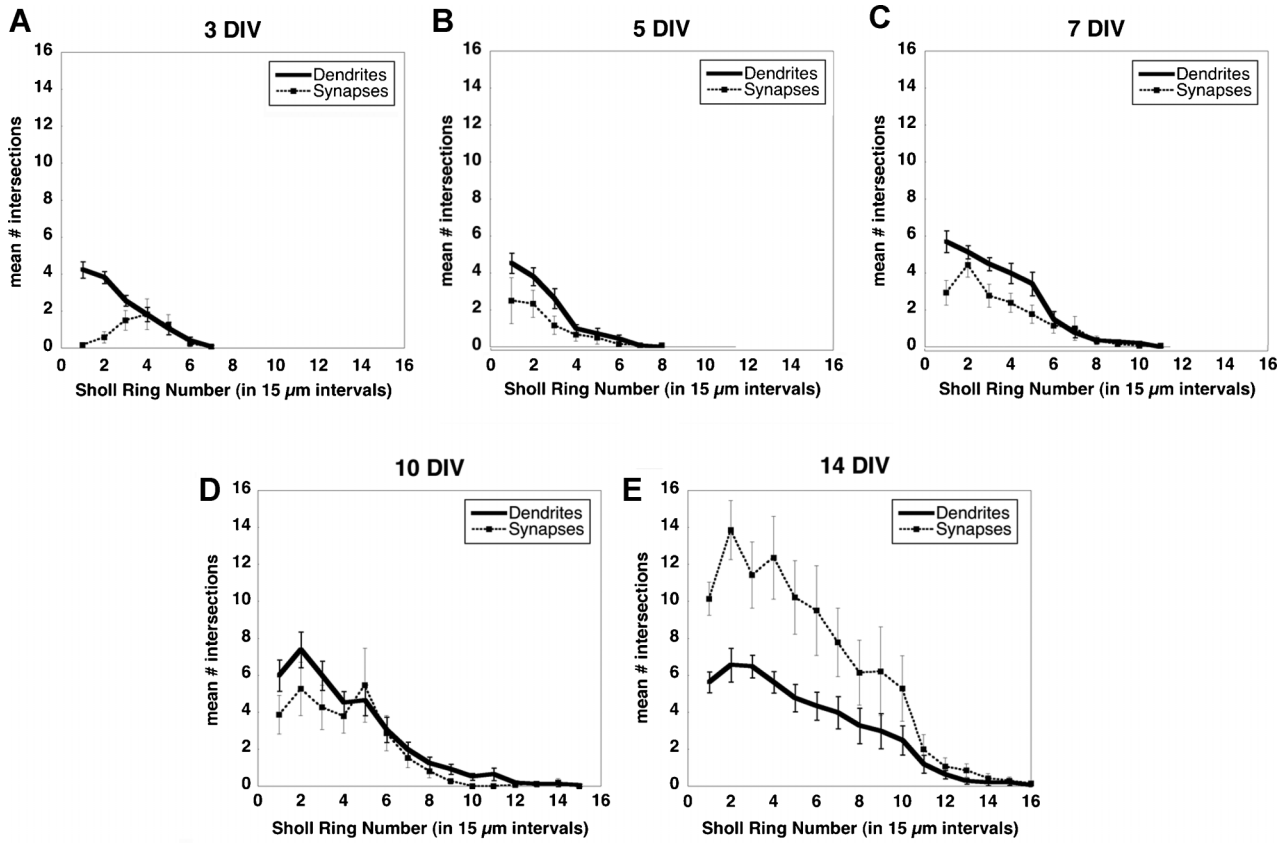

FIGURE 3 Quantitative analysis of the distribution of presynaptic contacts along dendrites from 3 to 14 DIV (Panels A–E) analyzed by density along Sholl rings (at 15 μm intervals). The heavy black bar represents the mean number of dendrite intersections at each ring/neuron, the hatched line shows the 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).

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

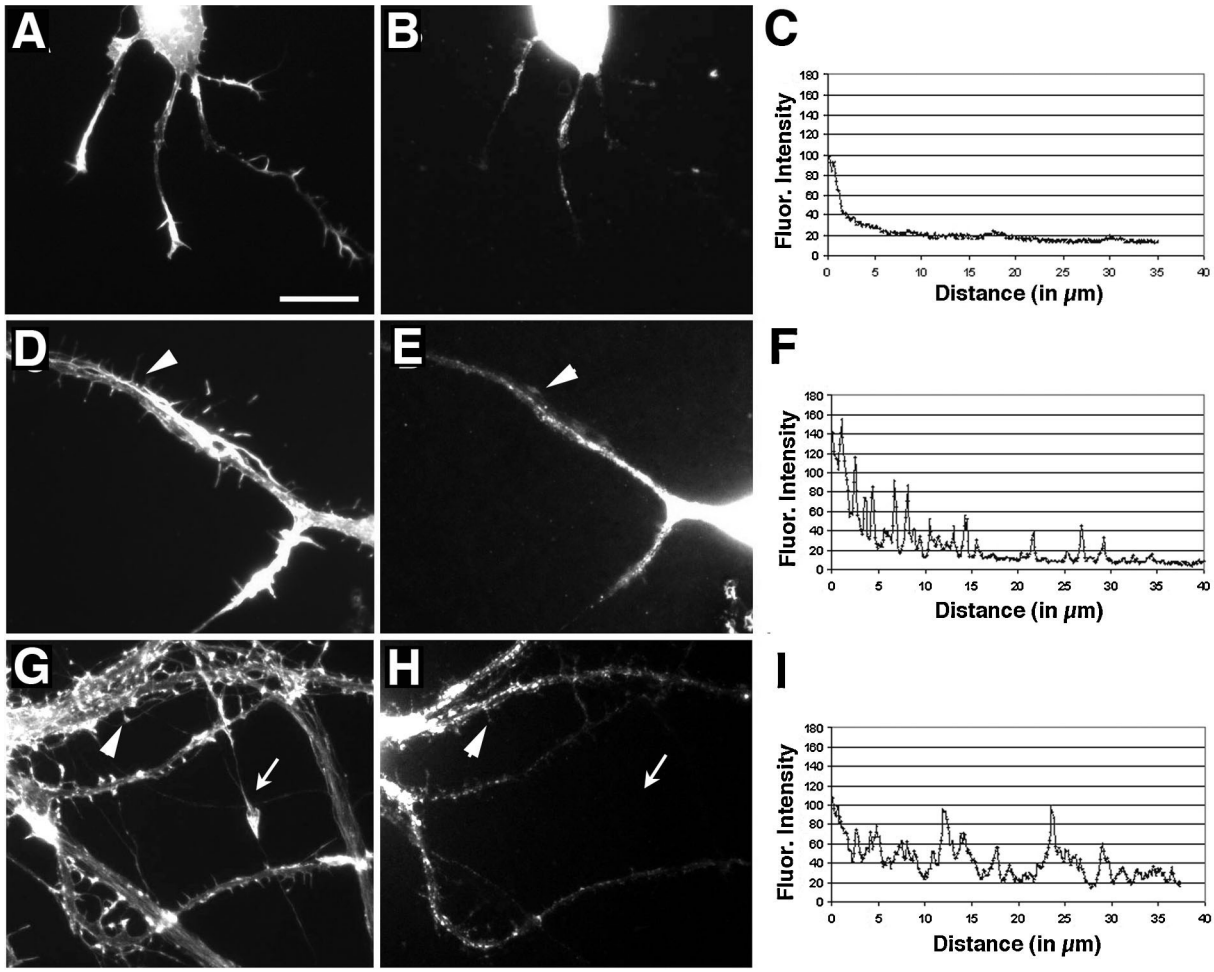

FIGURE 4 Development of localized protein synthesis machinery within dendrites, as detected using a fluorescent Nissl stain. Neurons at 1 (B), 4 (E), and 15 DIV (H) show the development of punctate Nissl staining, compared with the localization of polymerized actin (A,D,G), which is concentrated in presynaptic contacts, filopodia, and in dendritic spines. Line scans taken from a single dendrite of representative neurons at 1, 8, and 15 DIV (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.

4 DIV, Fig. 4B and E, with the neuron at 15 DIV, Fig. 4H). 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, and I and Tab. 1). Not only did Nissl staining become more clustered, but the distribution appeared to shift from the core to a more peripheral location within the dendritic shaft.

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

Days in vitro	% Correctly identified
1	80.00
4	69.23
8	73.33
11	68.75
15	90.00
18	100.00

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; Elmariah, 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

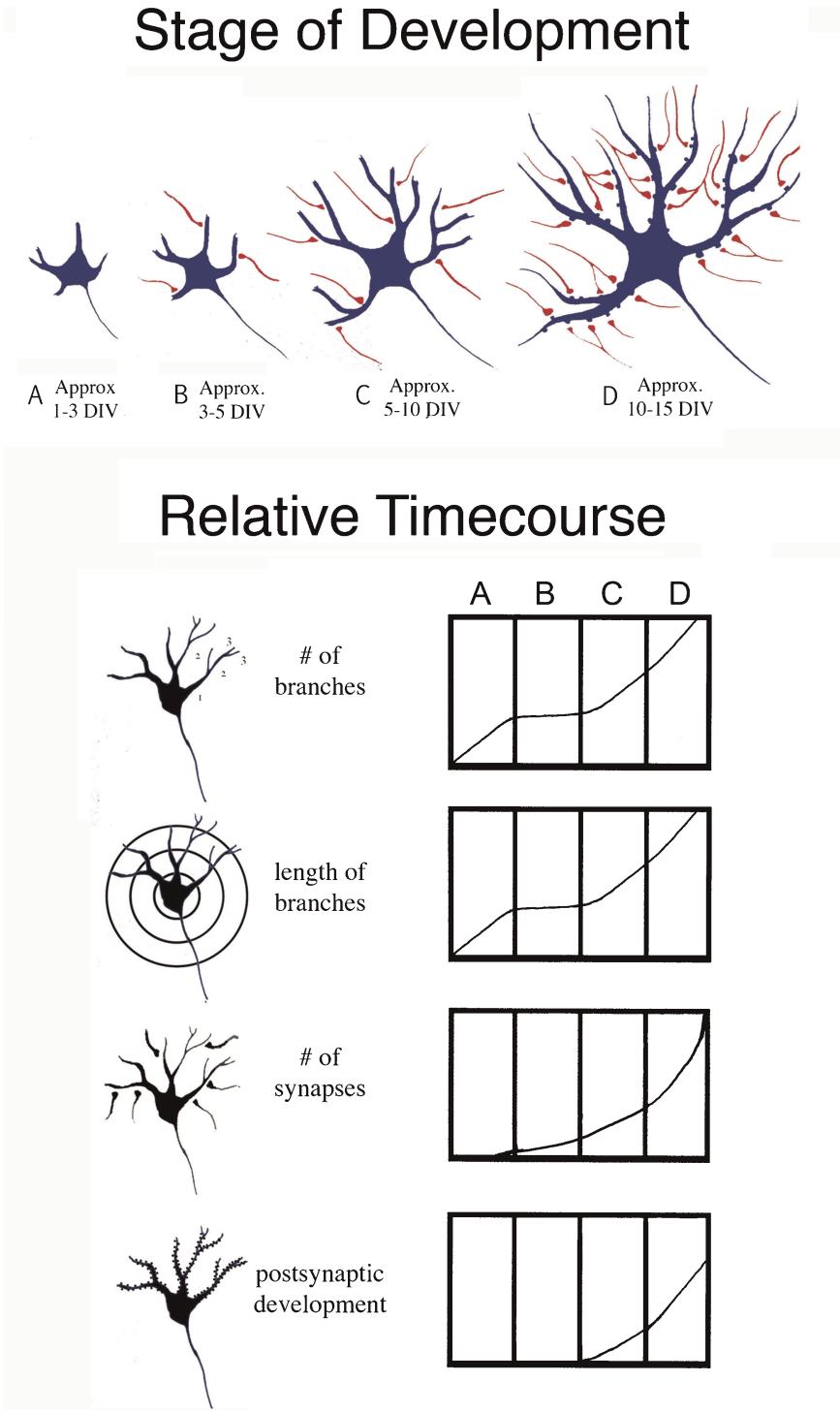

FIGURE 5 Notable phases of dendritic development are shown in the top panel, and the relative timetable for key events (e.g., dendritic outgrowth, branch formation, synapse formation, and postsynaptic specialization) is charted in the lower panel. Initial growth begins with the formation of immature 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-Studler & 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 dendrite 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

Supported by an NSF CAREER award to G.W., and NICHD R15 to G.W. and C.W. We thank Doug Hundley for sharing expertise on pattern recognition algorithms, and Sam Chasan for artwork. Author contributions—G.W., C.W., and E.G. contributed to the manuscript, G.W. designed and supervised all projects described, C.W., Nissl experiments, E.G., dendritic length analysis; I.E., Nissl analysis; M.A., dendritic length and synapse analysis.

REFERENCES

Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: Glia, the unacknowledged partner. Trends in Neuroscience, 22, 208–215.

Arikkath, J. (2009). Regulation of dendrite and spine morphogenesis and plasticity by catenins. Molecular Neurobiology, 40(1), 46–54.

Banker, G. A., & Goslin, K. (1998). Culturing nerve cells (2nd ed.). Cambridge, MA: MIT Press.

Banker, G. A., & Waxman, A. B. (1988). Hippocampal neurons generate natural shapes in cell culture. In R. J. Lasek & M. M. Black (Eds.), Intrinsic determinants of neuronal form and function (pp. 61–82). New York: Alan R. Liss, Inc.

Barker, A. J., Koch, S. M., Reed, J., Barres, B. A., & Ullian, E. M. (2008). Developmental control of synaptic receptivity. Journal of Neuroscience, 28, 8150–8160.

Barnes, A. P., & Polleux, F. (2009). Establishment of axon-dendrite polarity in developing neurons. Annual Review of Neuroscience, 32, 347–381.

Bartlett, W. P., & Banker, G. A. (1984). An electron microscopic study of the development of axons and dendrites of hippocampal neurons in culture. II. Synaptic relationships. Journal of Neuroscience, 4(8), 1954–1965.

Beckel-Mitchener, A., & Greenough, W. T. (2004). Correlates across the structural, functional, and molecular phenotypes of fragile X syndrome. Mental Retardation and Developmental Disabilities Research Reviews, 10(1), 53–59.

Benavides-Piccione, R., Ballesteros-Yanez, I., de Lagran, M. M., Elston, G., Estivill, X., Fillat, C., et al. (2004). On dendrites in Down syndrome and DS murine models: A spiny way to learn. Progress in Neurobiology, 74(2), 111–126.

Bourne, J. N., & Harris, K. M. (2008). Balancing structure and function at hippocampal dendritic spines. Annual Review of Neuroscience, 31, 47–67.

Black, J., Sirevaag, A., & Greenough, W. (1987). Complex experience promotes capillary formation in young rat visual cortex. Neuroscience Letters, 83, 351–355.

Brittis, P. A., Lemmon, V., Rutishauser, U., & Silver, J. (1995). Unique changes of ganglion cell growth cone behavior following cell adhesion molecule perturbations: A time-lapse study of the living retina. Molecular and Cellular Neuroscience, 6(5), 433–449.

Brittis, P. A., & Silver, J. (1995). Multiple factors govern intraretinal axon guidance: A time lapse study. Molecular and Cellular Neuroscience, 6, 413–432.

Bushong, E. A., Martone, M. E., & Ellisman, M. H. (2004). Maturation of astroctye morphology and the establishment of astrocyte domains during postnatal hippocampal development. International Journal of Developmental Neuroscience, 22, 73–86.

Christie, S. B., & DeBlas, A. L. (2003). GABAergic and glutamatergic axons innervate the axon initial segment and organize GABA(A) receptor clusters of cultured hippocampal pyramidal cells. Journal of Comparative Neurology, 456(4), 361–374.

Christopherson, K. S., Ullian, E. M., Stokes, C. C., Mullowney, C. E., Hell, J. W., Agah, A., et al. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell, 120(3), 421–433.

Comery, T. A., Harris, J. B., Willems, P. J., Oostra, B. A., Irwin, S. A., Weiler, I. J., et al. (1997). Abnormal dendritic spines in fragile X knockout mice: Maturation and pruning deficits. Proceedings of the National Academy of Sciences of the United States of America, 94, 5401–5405.

Craig, A., Blackstone, C., Huganir, R., & Banker, G. (1994). Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. Proceedings of the National Academy of Sciences of the United States of America, 92, 12373–12377.

Craig, A., Graf, E., & Linhoff, M. (2006). How to build a central synapse: Clues from cell culture. Trends in Neuroscience, 29(1), 8–20.

Craig, A. M., & Banker, G. (1994). Neuronal polarity. Annual Review Neuroscience, 17, 267–310.

Deitch, J. S., & Banker, G. A. (1993). An electron microscopic analysis of hippocampal neurons developing in culture: Early stages in the emergence of polarity. Journal of Neuroscience, 13(10), 82–90.

Dierssen, M., & Ramakers, G. J. A. (2006). Dendritic pathology in mental retardation: From molecular genetics to neurobiology. Genes Brain and Behavior, 5, 48–60.

Dotti, C. G., Sullivan, C. A., & Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. Journal of Neuroscience, 8, 1454–1458.

Eayrs, J. T., & Goodhead, B. (1959). Postnatal development of the cerebral cortex in the rat. Journal of Anatomy, 93, 385–402.

Elmariah, S., Oh, E., Hughes, E., & Balice-Gordon, R. (2005). Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABA(A) receptors. Journal of Neuroscience, 25(14), 3638–3650.

Eroglu, C., & Barres, B. A. (2010). Regulation of synaptic connectivity by glia. Nature, 468(7321), 223–231.

Esch, T., Lemmon, V., & Banker, G. (1999). Local presentation of substrate molecules directs axon specification by cultured hippocampal neurons. Journal of Neuroscience, 19(15), 6417–6426.

Fiala, J. C., Feinberg, M., Popov, V., & Harris, K. M. (1998). Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. Journal of Neuroscience, 18, 8900–8911.

Fletcher, T., Cameron, P., De Camilli, P., & Banker, G. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. Journal of Neuroscience, 11, 1617–1626.

Fletcher, T., DeCamilli, P., & Banker, G. (1994). Synaptogenesis in hippocampal cultures: Evidence indicating the axons and dendrites become competent to form synapses at different stages of neuronal development. Journal of Neuroscience, 14(11), 6695–6706.

Greenough, W., Black, J., & Wallace, C. (1987). Experience and brain development. Child Development, 58(3), 539–559.

Greenough, W. T., Hwang, H. M., & Gorman, C. (1985). Evidence for active synapse formation or altered postsynaptic metabolism in visual cortex of rats reared in complex environments. Proceedings of the National Academy of Sciences of the United States of America, 82, 4549–4552.

Greenough, W., Withers, G. S., & Wallace, C. S. (1990). Morphological changes in the nervous system arising from behavioral experience: What is the evidence that they are involved in learning and memory? In L. R. Squire & E. Lindenlaub (Eds.), The biology of memory, symposia medica hoescht (Vol. 23, pp. 158–192) Stuttgart-New York: F.K. Schattauder Verlag.

Hirokawa, N. (2006). mRNA transport in dendrites: RNAgranules, motors, and tracks. Journal of Neuroscience, 26, 7139–7142.

Hirokawa, N., & Takemura, R. (2005). Molecular motors and mechanisms of directional transport in neurons. Nature Reviews Neuroscience, 6(3), 201–214.

Holmaat, A., & Svoboda, K. (2009). Experience-dependent structural synaptic plasticity in the mammalian brain. Nature Reviews Neuroscience, 10, 647–659.

Huttenlocher, P. R. (1990). Morphometric study of human cerebral cortex development. Neuropsychologia, 28(6), 517–527.

Huttenlocher, P. R. (1991). Dendritic and synaptic pathology in mental-retardation. Pediatric Neurology, 7(2), 79–85.

Irwin, S. A., Galvez, R., & Greenough, W. (2000). Dendritic spine structural anomalies in fragile-X mental retardation syndrome. Cerebral Cortex, 10, 1038–1044.

Juraska, J. M., & Fifkova, E. (1979). A Golgi study of the early postnatal development of the visual cortex of the hooded rat. Journal of Comparative Neurology, 183, 247–256.

Kollins, K. M., Bell, R. L., Butts, M., & Withers, G. S. (2009). Dendrites differ from axons in patterns of microtubule stability and polymerization during development. Neural Development, 4:26.

Lardi-Studler, B., & Fritschy, J.-M. (2007). Matching of pre- and postsynaptic specializations during synaptogenesis. Neuroscientist, 13(2), 115–126.

Li, Z., Okamoto, K.-I., Hayashi, Y., & Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell, 119, 873–887.

Peng, Y. R., He, S., Marie, H., Zeng, S. Y., Ma, J., Tan, Z. J., et al. (2009). Coordinated changes in dendritic arborization and synaptic strength during neural circuit development. Neuron, 61, 71–84.

Polleux, F., Morrow, T., & Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature, 404, 567–573.

Purpura, D. P. (1975). Dendritic differentiation in human cerebral cortex: Normal and aberrant developmental patterns. Advances in Neurology, 12, 91–134.

Rodier, P. M. (1994). Vulnerable periods and processes during central nervous system development. Environmental Health Perspectives, 102, 121–124.

Rose, J., Jin, S. X., & Craig, A. (2009). Heterosynaptic molecular dynamics: Locally induced propagating synaptic accumulation of CaM kinase II. Neuron, 12(61), 351–358.

Sanchez, A. L., Matthews, B. J., Meynard, M. M., Hu, B., Javed, S., & Cohen-Cory, S. (2006). BDNF increases synapse density in dendritres of developing tectal neurons in vivo. Development, 133, 2477–2486.

Schuman, E. M., Dynes, J. L., & Steward, O. (2006). Synaptic regulation of translation of dendritic mRNAs. Journal of Neuroscience, 26, 7143–7146.

Sebeo, J., Hsiao, K., Bozdagi, O., Dumitriu, D., Ge, Y., Zhou, Q., et al. (2009). Requirement for protein synthesis at

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

Shaw, P., Kabani, N. J., Lerch, J. P., Eckstrand, K., Lenroot, R., Gogtay, N., Greenstein, D., Clasen, L., Evans, A., Rapoport, J. L., Giedd, J. N., & Wise, S. P. (2008). Neurodevelopmental trajectories of the human cerebral cortex. Journal of Neuroscience, 28(14), 3586–3594.

Shi, P., Shen, K., & Kam, L. C. (2007). Local presentation of L1 and N-cadherin in multicomponent, microscale patterns differentially direct neuron function in vitro. Journal of Neurobiology, 67(13), 1765–1776.

Sholl, D. A. (1956). Organization of the cerebral cortex. London: Methuen Press.

Sirevaag, A., Black, J., Shafron, D., & Greenough, W. (1988). Direct evidence that complex experience increases capillary branching and surface area in visual cortex of young rats. Brain Research, 471, 299–304.

Stevens, B. (2008). Neuron-astrocyte signaling in the development and plasticity of neural circuits. Neurosignals, 16(4), 278–288.

Steward, O. (1983a). Alterations in polyribosomes associated with dendritic spines during the reinnervation of the dentate gyrus of the adult rat. Journal of Neuroscience, 3, 177–188.

Steward, O. (1983b). Polyribosomes at the base of dendritic spines of central nervous system neurons—Their possible role in synapse construction and modification. Cold Spring Harbor Symposium on Quantitative Biology, 48, 745–759.

Svitkina, T., Lin, W. H., Webb, D. J., Yasuda, R., Wayman, G. A., Van Aelst, L., & Soderling, S. H. (2010). Regulation of the postsynaptic cytoskeleton: Roles in development, plasticity, and disorders. Journal of Neuroscience, 30(45), 14937–14942.

Swanwick, C. C., Murthy, N. R., Mtchedlishvili, Z., Sieghart, W., & Kapur, J. (2006). Development of gamma-aminobutyric acidergic synapses in cultured hippocampal neurons. Journal of Comparative Neurology, 495(5), 497–510.

Turner, A., & Greenough, W. (1985). Differential rearing effects on rat visual cortex synapses. I. Synaptic and neuronal density and synapses per neuron. Brain Research, 329, 195–203.

Volkmar, F. R., & Greenough, W. (1972). Rearing complexity affects branching of dendrites in the visual cortex of the rat. Science, 176, 1445–1447.

Wallace, C. S., Reitzenstein, J., & Withers, G. S. (2003). Diminished experiencedependent neuroanatomical plasiticy: Evidence for an improved biomarker of subtle neurotoxic damage to the developing rat brain. Environmental Health Perspectives, 111, 1294–1298.

Watson, R. E., DeSesso, J. M., Hurt, M. E., & Cappon, G. D. (2006). Postnatal growth and morphological development of the brain: A species comparison. Birth Defects Research (B), 77, 471–484.

Weiler, I. J., Irwin, S. A., Klintsova, A. Y., Spencer, C. M., Brazelton, A. D., Miyashiro, K., et al. (1997). Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proceedings of the National Academy of Sciences of the United States of America, 94, 5395–5400.

Weiler, I. J., Spangler, C. C., Klintsova, A. Y., Grossman, A. W., Kim, S. H., Bertaina-Anglade, V., et al. (2004). Fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses. Proceedings of the National Academy of Sciences of the United States of America, 101, 17504–17509.

Withers, G. S., & Banker, G. (1998). Characterizing and studying neuronal cultures. In G. Banker & K. Goslin (Eds.), Culturing nerve cells (2nd ed., pp. 113–151). Cambridge: MIT Press.

Withers, G. S., Higgins, D., Charette, M., & Banker, G. (2000). Bone morphogenetic protein-7 enhances dendritic growth and receptivity to innervation in cultured hippocampal neurons. European Journal of Neuroscience, 12(1), 106–116.

Withers, G. S., James, C. D., Kingman, C. E., Craighead, H. G., & Banker, G. A. (2006). Effects of substrate geometry on growth cone behavior and axon branching. Journal of Neurobiology, 66, 1183–1194.

Withers, G. S., Lambruschi, L., Brown, L., & Wallace, C. S. (2008). The absence of glia leads to increased dendritic growth in cultured hippocampal neurons. Society for Neuroscience Annual Meeting Abstracts, 524, 517.

Zhang, W., & Benson, D. L. (2002). Developmentally regulated changes in cellular compartmentation and synaptic distribution of actin in hippocampal neurons. Journal of Neuroscience Research, 69(4), 427–436.

Ziv, N. E., & Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron, 17(1), 91–102.