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Age-dependence in the homeostatic upregulation of hippocampal dendritic spine number during blocked synaptic transmission

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Abstract

Homeostatic regulation of spine number in mature hippocampal neurons results in more dendritic spines when synaptic transmission is blocked, providing a mechanism to compensate for diminished synaptic input. It is unsettled whether blockade of synaptic transmission also elevates spine number during development. To address this question, synaptic transmission was blocked in rat hippocampal slices during critical developmental stages of spine formation at postnatal days (P) 6–P22 and compared to adults. CA1 pyramidal cells were labeled with DiI and maintained for 5 h in one of three conditions, control artificial cerebrospinal fluid (ACSF), block media containing synaptic transmission antagonists in ACSF, or block media containing synaptic transmission antagonists in a nominally calcium-free ACSF with high magnesium. Slices were fixed in mixed aldehydes, sectioned, and the lateral dendrites were imaged in *stratum radiatum* with confocal microscopy. Dendritic spine density was quantified per unit length of dendrite. At P6–7 there were only a few protrusions emerging from the dendrites, which were predominantly filopodia-like in appearance. At both P11–12 and P15–16 there was a mixture of dendritic spines and filopodia-like structures. By P20–22 dendritic spines predominated and spine density was about 82% of the adult level. Dendritic spine density increased during blocked synaptic transmission at P20–22 as in adults, but was unchanged during blockade at younger ages. When extracellular calcium was nominally zero, dendritic spine density further increased on P20–22 dendrites as in adults. In contrast, spine density decreased along P11–12 dendrites under the nominally zero calcium condition. Under control conditions, dendritic protrusions were longer at P6–7 than at all other ages, which did not differ from one another. When synaptic transmission was blocked, dendritic protrusions further elongated at P6–7 only. Under the nominally zero calcium condition with blocked synaptic transmission, dendritic protrusions shortened at P11–12 only. These findings reveal age-dependent changes in the manifestation of homeostatic control of dendritic spines that could be mediated by maturational changes in mechanisms regulating postsynaptic calcium.

Keywords: Synaptogenesis; Structural plasticity; Hippocampal development; Homeostasis; Dendritic spines; CA1 pyramidal cell

1. Introduction

Neuronal networks are highly sensitive to changes in synaptic activity. Recent work shows that homeostatic mechanisms balance activity-dependent synaptic plasticity in neuronal networks. Homeostatic mechanisms maintain an optimal level of input to a neuron by regulating overall efficacy at excitatory synapses, by varying intrinsic neuronal excitability, and by altering synapse number (Harris, 1999; Kirov and Harris, 1999; Turrigiano, 1999; Turrigiano and Nelson, 2000; Burrone and Murthy, 2003). Sustained blockade or enhancement of synaptic transmission at developing neurons in culture results in a bi-directional regulation of synaptic strength and intrinsic excitability of neurons (Turrigiano et al., 1998; O'Brien et al., 1998; Lissin et al., 1998; Desai et al., 1999; Watt et al., 2000).

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Mature hippocampal neurons in slices have more synapses than in perfusion fixed brain (Kirov et al., 1999). Acute blockade of synaptic transmission in mature slices results in a further robust increase in dendritic spine number and length (Kirov and Harris, 1999; Kirov et al., 2004). These findings from mature neurons have been largely ignored because developing neurons in culture do not show changes in spine number during blocked synaptic transmission (Davis and Bezprozvanny, 2001; Burrone and Murthy, 2003).

It is not clear when neurons first develop homeostatic mechanisms to regulate total spine and synapse number. If homeostatic increases in spine number were to occur on immature neurons, then their low synaptic activity might wrongly accelerate synaptogenesis at times when synaptic pruning is required to sort out appropriate connections between neurons (Katz and Shatz, 1996). It should not be surprising then that the timing of homeostatic structural plasticity in a developing system appears to depend on brain region, experimental preparation, age and even location along the dendrite. Acute and organotypic hippocampal slices show evidence for homeostatic down regulation of synaptic efficacy as spines mature (De Simoni et al., 2003). Some in vivo studies suggest that prolonged blockade of synaptic transmission leads to more dendritic spines or filopodia-like protrusions on cortical or lateral geniculate nucleus neurons (Dalva et al., 1994; Rocha and Sur, 1995; McAllister et al., 1996). In addition, very early during development an increase in CA3 dendritic synapses has been detected after synaptic activity blockade in the intact neonatal (P3) hippocampus maintained in vitro (Lauri et al., 2003). Contrasting studies suggest that prolonged blockade of synaptic transmission results in a loss or no change in spine number on hippocampal neurons in culture (Papa and Segal, 1996; Kossel et al., 1997; Collin et al., 1997; Murthy et al., 2001). Other studies suggest that prolonged activation of synapses leads to more spines on developing cortical or hippocampal dendrites in culture (Annis et al., 1994; Papa and Segal, 1996). Recent studies show that filopodial dynamics depend not only on the age of cortical dendrites in culture, but whether the filopodia emerge from the dendritic growth cone or along the dendritic shaft in acute slices (Portera-Cailliau et al., 2003). Ultimately, some level of synaptic transmission is required to sustain dendritic spines on developing hippocampal neurons in organotypic cultures (McKinney et al., 1999).

Here, quantitative confocal microscopy was used to determine when acute blockade of synaptic transmission first affects spine number at important developmental stages during synaptogenesis in the hippocampus including P6–7, 11–12, 15–16, and 20–21. At P6–7, dynamic dendritic filopodia are prominent structures on hippocampal CA1 dendrites (Dailey and Smith, 1996; Fiala et al., 1998; Boyer et al., 1998; Dunaevsky et al., 1999). Some of the filopodia have synapses, although the majority of synapses occur directly on hippocampal dendritic shafts at this age (Fiala et al., 1998). Hippocampal dendrites begin to acquire dendritic spines around P11–12, although most of the synapses still occur on the dendritic shafts (Fiala et al., 1998; Harris, 1999). By P15–16, the shaft synapses begin to disappear and the synapses occur in roughly equal numbers on small, thin spines or large, mushroom spines (Harris et al., 1992). By P20–22, an adult-like complement of dendritic spines is present, though they are fewer in total number than in adults (Kirov et al., 1999). Hence, normal hippocampal spinogenesis undergoes dramatic changes during the first two weeks postnatal. Dendritic spines were imaged and quantified in hippocampal slices from developing rats during these key developmental stages to assess when blocking synaptic transmission in the presence or nominal absence of extracellular calcium begins to alter dendritic spine number. Some of this data has appeared in abstract form (Goddard et al., 2000).

2. Methods

2.1. Preparation of acute hippocampal slices

All procedures followed the National Institutes of Health guidelines for the care and use of laboratory animals and all efforts were made to minimize animal suffering and to reduce the number of animals used. Slices were prepared from young male rats of the Long Evans strain at P6–22 according to standard procedures (Harris and Teyler, 1984; Kirov et al., 1999). Animals were decapitated under halothane anesthesia and the left hippocampus was dissected free from the rest of the brain and bathed in ice cold, oxygenated control ACSF containing (in mM): 117 NaCl, 5.3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose, pH 7.4. Six to eight 400 μm slices from the middle third of the hippocampus were cut at 70° transverse to the long axis on a tissue chopper (Stoelting Co., Wood Dale, IL), which had been cooled to 4 °C. In order to compare measurements obtained from young slices to data from slices prepared from mature (P60–82) rats, we performed additional analyses of dendrites from adult hippocampal tissue prepared under similar experimental conditions described in Kirov and Harris (1999).

2.2. Experimental design

Two sets of slice conditions were compared. In the first set of experiments, all conditions contained normal calcium. Immediately after preparation the slices were

transferred into control ACSF or the block medium which contained ACSF with the sodium channel blocker tetrodotoxin (TTX, 1 μM) with the ionotropic glutamate receptors antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM), D,L-2-amino-5-phosphonovaleric acid (APV, 50 μM), and the metabotropic glutamate receptors antagonist, (S)-α -methyl-4-carboxyphenylglycine (MCPG, 500 μM). Corresponding adult hippocampal slices from Kirov and Harris (1999) included in this study were maintained in ACSF (control) or in ACSF with TTX only (block).

In the second set of experiments the block medium contained the same concentrations of activity antagonists, but calcium was omitted and the magnesium concentration was increased to 8 mM (Block/0 Ca^{2+}). Corresponding adult hippocampal slices from Kirov and Harris (1999) were maintained in similar conditions. All solutions were oxygenated and kept ice cold. Once all slices had been cut, they were transferred immediately into the recording chamber (Stoelting, Wood Dale, Illinois) via a wide-bore glass pipette. Slices were maintained on filter paper nets over wells in contact with either control or block medium at the interface of humidified 95% O_2/5% CO_2 atmosphere at 32 °C (pH 7.4).

TTX was acquired from Calbiochem (La Jolla, California), CNQX from Research Biochemicals (Natick, Massachusetts) and MCPG from Tocris Cookson (Bristol, UK). All other drugs and chemicals were from Sigma Chemical (St. Louis, Missouri). MCPG was solubilized at 100× final concentration in 1.1 eq. of NaOH. All other drugs were prepared at 1000× concentration in stock solutions.

2.3. Acute hippocampal slice labeling with DiI

Phenyl-substituted DiI (1,1'-dioctadecyl-5,5'-diphenyl-3,3,3',3'-tetramethylindocarbocyanine chloride) was obtained from Molecular Probes (Eugene, OR). The CA1 pyramidal cell layer of hippocampal slices was labeled with DiI-coated glass microelectrodes within 1 h of slice preparation as previously described (Kirov and Harris, 1999). DiI-coated microelectrodes were prepared by dissolving DiI in N,N-dimethylformamide (0.5% weight per volume), placing a droplet onto the tip of the microelectrode, and drying from 3 to 12 h at 60 °C. The quality of labeling was judged good when the dendrites were fully stained to their tapered tips.

2.4. Extracellular recording method

Approximately 4 h after the beginning of incubation a small concentric bipolar stimulating electrode (25 μm pole separation; Fred Haer, Brunswick, ME) was positioned in the middle of *s. radiatum* of the control slice. 100-μs-long pulses were delivered every 30 s and the field excitatory postsynaptic potentials (fEPSPs) were recorded in the middle of *s. radiatum* with a single extracellular recording electrode (glass micropipette filled with 120 mM NaCl) using a microelectrode amplifier (Model 1800, A-M Systems, Carlsborg, Washington). Signals were filtered at 5 kHz, digitized at 29 kHz using an interface board (RC Electronics, Santa Barbara, California) and analyzed online with the Scope software (RC Electronics). The slope function (mV per ms) of the fEPSP was measured from the steepest 400 μs segment of the negative field potential. Increasing stimulus intensity provided the input–output curves, and the half-maximal responses were used to monitor the stable fEPSPs. Slices maintained with activity antagonists were also tested to ensure that no synaptic response could be elicited, even at high stimulus intensities. Only experiments in which the control slices had a healthy sigmoidal input/output response function and a stable response at half maximal stimulation were used.

2.5. Confocal microscopy

After a total of 5 h, slices were fixed by immersion in mixed aldehydes (0.1% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4) with 8 s microwave irradiation producing a final temperature less than 37 °C (Jensen and Harris, 1989; Kirov and Harris, 1999). Slices were embedded in 4% low melting point agarose (Sigma) and sectioned at 45 μm with a vibrating blade microtome (VT1000 S, Leica Instruments GmbH, Nussloch, Germany) in 0.1 M PB. The middle two sections were mounted in PB on glass slides, coverslipped, sealed with nail polish and coded. Images were collected with a 63×/1.4 NA objective (Carl Zeiss, Jena, Germany) using the confocal laser scanning system LSM 510 (Carl Zeiss) attached onto the upright Axioscop 2 FS microscope (Carl Zeiss). The 543 nm emission line of a Helium-Neon ion laser was used for DiI excitation. Three-dimensional images of dendrites were taken at 0.25 μm increments with a nominal lateral spatial resolution of 20 pixels per 1 μm (512 × 512 pixel array). Z-stacks generally ranged from 1.5 to 2.5 μm in depth. Four scans per section were used to reduce noise. Tips of lateral branches of the apical dendrites were found in the middle of *s. radiatum* of area CA1 and the dendrite was photographed at 25–50 μm back from the tip along a segment that remained primarily in one focal plane.

2.6. Dendrite analyses

The Zeiss LSM 510 image examiner together with the custom software entitled *IGL Trace* (available for download at www.synapses.mcg.edu) were used for quantification. Distinctions between the different types

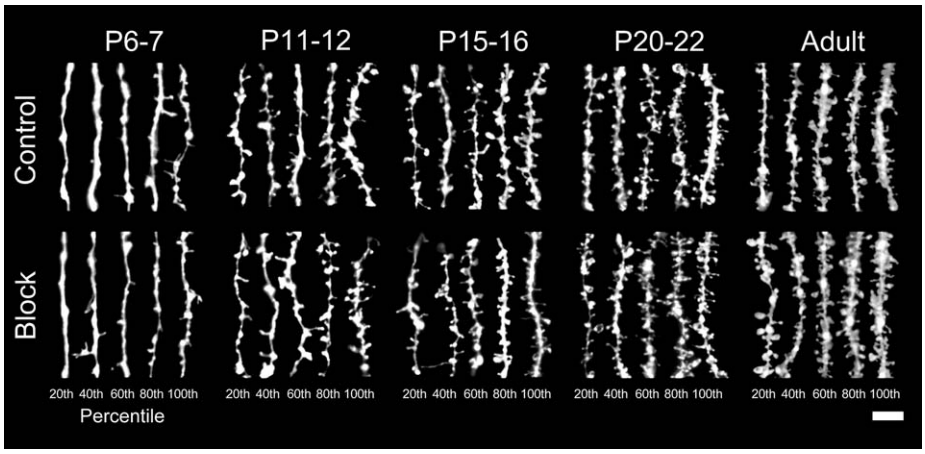

Fig. 1. Two-dimensional projection images at key developmental ages (heading) of dendritic segments from control slices (top row) and from slices with blocked synaptic transmission (bottom row). Dendritic segments are displayed by percentile rank (footer) in spine density in increments of twenty for each age and condition. Scale bar, 5 μm.

of dendritic spines or filopodia were not easily discerned with confocal microscopy, especially at the older ages; hence, for the purposes of this paper, all of the dendritic protrusions are referred to as "dendritic spines". All dendritic spines extending from the dendritic shaft on a two-dimensional projection image, which was made using a maximum brightness operation from a z-stack of images, were marked and counted (Kirov and Harris, 1999). The length of the dendritic segment was measured and the number of dendritic spines per unit length was computed and the relative increase or decrease in density was estimated. Dendritic spines obscured by the dendritic shaft or by one another obviously could not be counted, so the numbers provided must be considered as relative changes in spine density, not absolute values for spine density. The relative spine length was measured from its origin to the tip. Averaged cumulative spine length distributions were obtained by normalizing individual cumulative length distributions to the median length of the corresponding control distributions. Data from slices maintained at the same experimental conditions were combined and averaged by calculating the normalized lengths at fixed cumulative frequency intervals.

2.7. Statistical analysis

SigmaStat (Jandel, San Rafael, California) was used to compute one and two way ANOVA followed by Tukey's post-hoc method and Kruskal–Wallis one-way ANOVA on ranks, followed by Dunn's post-hoc test. A two-way ANOVA was used to distinguish the variation in spine densities and lengths between animals from changes produced by blocking synaptic transmission. The significance criterion was set at $p < 0.05$.

3. Results

Dendritic segments from hippocampal slices maintained in control or block medium are shown in Fig. 1. Dendritic spine density was quantified along 1149 dendritic segments imaged in slices from 21 animals across the five age groups (Fig. 2). All quantitative analyses were performed on coded images so that the experimenters were blind as to experimental conditions. Once the quantitative analyses were completed, the images were decoded, and sorted by age, experimental condition, and spine density. The five segments displayed in Fig. 1 are matched within 5% for percentile rank in spine density at each age and condition so that the den-

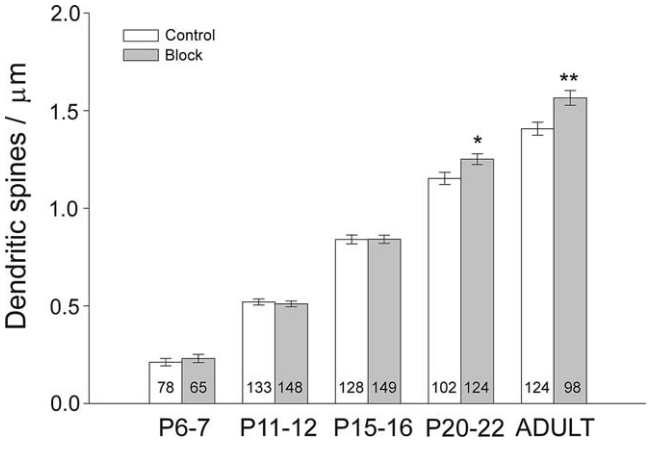

Fig. 2. Dendritic spine density in control and block slice conditions across age. The number of dendritic segments that were imaged and analyzed quantitatively in each age and condition are indicated within each bar. Dendrites were analyzed in at least two slices (control and block) from a total of 21 animals: 3 at P6–7, 5 at P11–12, 4 at P15–16, 4 at P20–22, and from 5 adult animals. Asterisks indicate significant differences between control and blocked slice conditions ($^{*}p < 0.02$; $^{**}p < 0.005$). Spine density is presented as mean ± s.e.m.

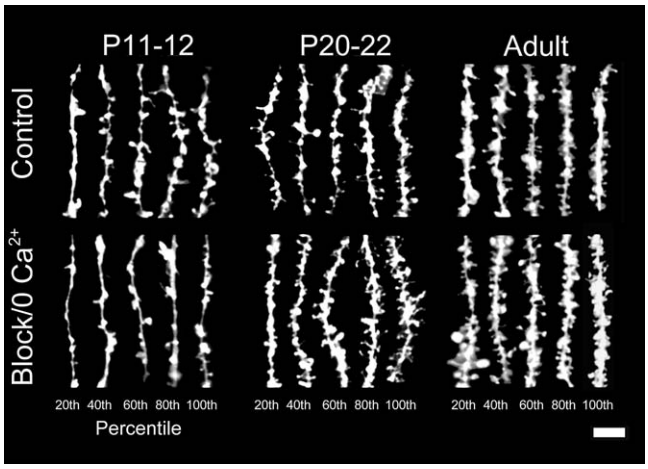

Fig. 3. Percentile-ranked images of dendritic segments in slices maintained in control (top row) or in block medium with activity antagonists and 0 mM Ca^{2+} and 8 mM Mg^{2+} (bottom row). Scale bar, 5 μm.

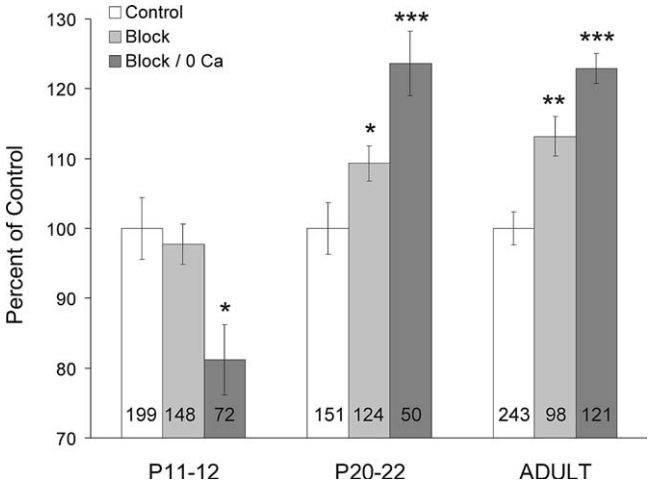

Fig. 4. Quantitative effect of combining synaptic activity antagonists in nominally calcium-free medium on dendritic spine density. At P11–12 spine density decreased while in P20–22 and adult slices spine density increased further above block alone. Numbers of dendritic segments in each condition are indicated within each bar. A total of 1206 dendritic segments from 25 animals are included. Asterisks indicate significant differences from the corresponding control condition ($^{*}p < 0.02$; $^{**}p < 0.005$; $^{***}p < 0.001$). Data are present as mean ± s.e.m.

drites can be compared visually between control and block conditions and across ages. Quantitatively, dendritic spine density increased steadily with age in the control slices (Fig. 2, $F_{4,563} = 282.48$, $p < 0.001$). Blocking synaptic transmission had no effect on spine density early during development at P6–16 (Figs. 1 and 2). Spine density increased during blocked synaptic transmission by P20–22 (Fig. 2, $F_{1,225} = 5.60$, $p < 0.02$) and this change was comparable to adults (Fig. 2, $F_{1,221} = 9.77$, $p < 0.005$).

Mature hippocampal dendrites became even more spiny when bathed in a nominally calcium free medium with or without activity antagonists (Kirov and Harris, 1999). Hence, a second set of experiments was done at P6–7, P11–12 and P20–22 to test whether the reduced calcium had similar effects on developing dendrites. In these experiments, the slices were incubated for 5 h in nominally calcium free ACSF containing high magnesium, TTX, APV, CNQX and MCPG (see Methods). Dendritic segments from control and blocked hippocampal slices in calcium free medium are shown by percentile rank in Fig. 3. Since so few spines are present even under control conditions at P6–7, only two animals were run in the Block/0 Ca^{2+} condition in this age group to confirm that there was no dramatic change in these nonspiny dendrites. Since the numbers were so low, these data are not included on the figures. In contrast to adults, spine density was decreased at P11–12 when synaptic transmission was blocked in a nominally calcium free medium and activity antagonists (Fig. 4, $F_{1,137} = 5.95$, $p < 0.02$). At P20–22 spine density showed a greater increase when slices were maintained in the nominally calcium free medium with activity antagonists (Fig. 4, $F_{1,98} = 13.37$, $p < 0.001$) comparable to adults (Fig. 4, $F_{1,239} = 52.70$, $p < 0.001$).

Dendritic spine length may also be altered by changes in synaptic transmission and calcium. During development, spines were significantly longer at P6–7 than at P11–12, 15–16 or 20–22 under the control condition (Fig. 5A). There were no significant differences in spine length between other age groups. Blocking synaptic transmission at P6–7 resulted in a significant increase in spine length (Fig. 5B). In contrast, blocking synaptic transmission at P11–12 and older ages had no effect on spine length (Fig. 5C). However, when synaptic transmission was blocked in nominally calcium free medium, the spines were shorter at age P11–12 only (Fig. 5D). These changes in spine length did not parallel changes in spine density confirming that the measured changes in spine density were not an artifact of changes in spine length affecting our ability to detect the spines.

4. Discussion

Acute blockade of synaptic transmission does not result in elevated spinogenesis during P6–16. This finding shows that during the critical early period of synaptogenesis and pruning the loss of synaptic activity does not trigger a homeostatic up-regulation in spine number. Instead homeostatic plasticity in spine number begins once the adult-like composition of dendritic spines is achieved around P20–22. These findings suggest that a substantial period of postnatal maturation

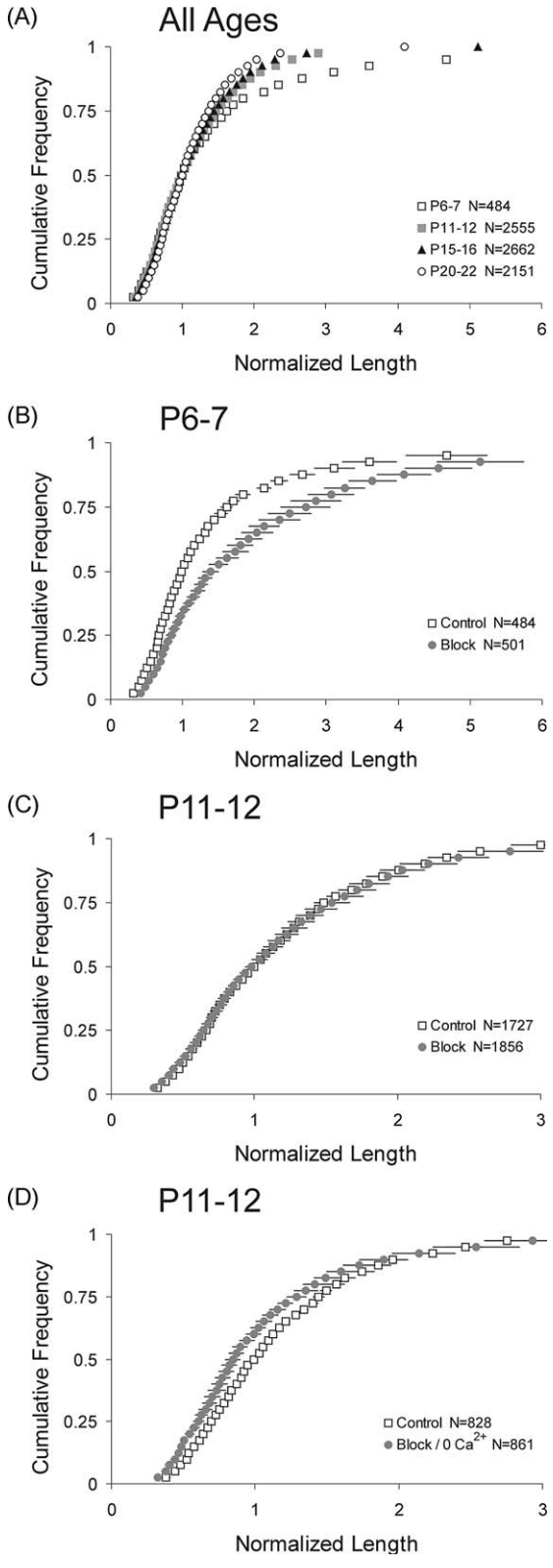

Fig. 5. Cumulative frequency in the length of dendritic spines. (A) In control ACSF spine length was significantly longer at P6–7 than all older ages ($p < 0.001$), which did not differ from one another. (B) At P6–7 the dendritic spines were longer in slices with blocked synaptic transmission (circles) relative to control slices (squares) ($p < 0.005$). (C) Blocking synaptic transmission alone did not affect spine length at P11–12. (D) Blocking synaptic transmission in a medium with 0 mM Ca^{2+} and 8 mM Mg^{2+} resulted in shorter dendritic spines at P11–12 ($p < 0.005$). A total of 11072 dendritic protrusion length measurements from 22 animals are included, with the specific number of spines that were measured for each condition indicated on the legend of each graph. Data are presented as mean ± s.e.m.

is required to initiate homeostatic regulation of dendritic spine number to maintain optimal synaptic activity on the neuron.

Counting dendritic spines on confocal images at P6–16 was easier than at older ages because the dendrites were less spiny. Thus, the elevation in spine number during blocking synaptic transmission at P20–22 and adult dendrites was not an artifact of age differences in detection. A higher spine density makes it more difficult to resolve neighboring dendritic spines, which would explain why the absolute spine density obtained through serial EM reconstructions are more than threefold greater than those counted on the confocal images from control slices (Kirov et al., 1999). This ceiling effect in detection of individual spines in confocal microscopy means that the absolute differences between the block and control conditions could be even greater in the older animals.

Homeostatic mechanisms other than changes in spine number appear to be sufficient to regulate synaptic input on immature neurons. Electrophysiological studies show that an optimal level of synaptic input is maintained on immature neurons in culture by increasing or decreasing the amplitude of both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-receptor (Turrigiano et al., 1998; O'Brien et al., 1998; Lissin et al., 1998) and N-methyl-D-aspartate (NMDA)-receptor-mediated currents at synapses (Watt et al., 2000). In addition, the amplitude of synaptic currents at immature hippocampal neurons is scaled down as synapse number increases with days in culture (Liu and Tsien, 1995). This scaling in synaptic strength is coupled in time to activity-dependent changes in postsynaptic glutamate receptors at developing synapses (Rao and Craig, 1997; Craig, 1998; O'Brien et al., 1998; Lissin et al., 1998; Liao et al., 1999). These findings suggest that under some developmental conditions, a homeostatic regulation of glutamate receptor number at existing or newly formed synapses is sufficient to sustain the optimal level of synaptic input needed by immature neurons, with little or no change in spine number. As the neuron matures, scaling in synapse number emerges as a supplemental homeostatic mechanism.

Complementary findings show that dendritic spines are lost from both immature and mature hippocampal neurons after excessive stimulation of synapses exposed

to epileptic seizures in vivo (Paul and Scheibel, 1986; Multani et al., 1994; Jiang et al., 1998; Swann et al., 2000), bicuculline or picrotoxin-induced seizures in hippocampal organotypic cultures (Müller et al., 1993; Drakew et al., 1996), or briefly to high concentrations of NMDA in hippocampal cell culture (Halpain et al., 1998). Thus, both immature and mature hippocampal neurons respond to excessive activation by removing spines thereby scaling down synaptic strength.

Long-term potentiation (LTP) differs from the global alterations in synaptic transmission discussed above, in that only a subset of synapses is stimulated (Bliss and Collingridge, 1993; Sanes and Lichtman, 1999; Grimwood et al., 2001). When local stimulation known to induce LTP is given to developing neurons in culture while living dendrites are imaged, new filopodia or dendritic spines can be seen to emerge or change shape (Hosokawa et al., 1995; Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999). In parallel, there also appears to be a loss of spines away from the site of LTP induction (Engert and Bonhoeffer, 1999). Reconstructions from serial electron microscopy suggest the possibility that new spines form during LTP in the immature hippocampus (Toni et al., 1999; Fiala et al., 2002; Ostroff et al., 2002; Harris et al., 2003). LTP induced in mature neurons results in no net change in total synapse or spine number, but here too, complementary changes could exist among synapses to balance total input on the neuron (e.g. Lee et al., 1980; Chang and Greenough, 1984; Desmond and Levy, 1990; Grabs et al., 1991; Sorra and Harris, 1998; Geinisman, 2000). Together, these morphological findings suggest that homeostatic mechanisms might also operate during LTP whereby the strengthening or gaining of some synapses is balanced by weakening or loss of other synapses on the same neuron.

There are some experimental conditions that have been found to increase spine or synapse number. For example, prolonged activation of neurons in the adult somatosensory barrel cortex for 24 h results in a temporary increase in total spine number. Subsequently, 4 days after stimulation, the excess spines are lost, but those remaining show a sustained increase in the frequency of spines supporting both an excitatory and an inhibitory synapse (Knott et al., 2002). Alternatively, during the first 3 days postnatal, prolonged blockade of synaptic transmission can enhance synaptogenesis in area CA3; electrophysiology shows these synapses are likely to be functional (Lauri et al., 2003). This study used the 2-section dissector approach which is not sufficient to identify dendritic spines or filopodia; however, at this early postnatal age it is probable that these synapses were on dendritic shafts. Thus the timing, location, and exact conditions will determine which types of synapses are increased.

There appear to be several ways to initiate homeostatic regulation of synaptic input to a neuron (Turrigiano and Nelson, 2000). A common underlying mechanism might be differences in response to changes in postsynaptic calcium (Matus, 2000; Yuste et al., 2000; Segal, 2001). In support of this hypothesis, our results show that mature and immature dendrites regulate spine number in opposite directions when extracellular calcium is nominally reduced to zero. Both P20 and mature neurons acquired even more dendritic spines under the calcium free condition than with synaptic activity antagonists alone. In contrast, P11–12 neurons lost spines under the calcium free condition, but the remaining spines were longer. An increase in length may reflect a return to a more filopodia-like state, which would be consistent with the findings in the developing system in vivo. For examples, when synaptic transmission was blocked with TTX more filopodia are seen both in the lateral geniculate nucleus (Dalva et al., 1994) and in the retina (Wong et al., 1991). At P11–12 the increase in length may also be consistent with the increase in filopodia, but with the lower calcium having a greater impact on spine loss. The in vivo TTX treatment did not involve lowered calcium. Thus, immature neurons require extracellular calcium to sustain dendritic spines. There is an optimal level of intracellular calcium that enhances actin polymerization and may cause the spine outgrowth (Fifkova, 1985; Janmey, 1994; Rao and Craig, 2000; Halpain, 2000). Perhaps under the reduced extracellular calcium mature neurons obtain this optimal level of calcium through intracellular stores or calcium buffering mechanisms, which leads to spine outgrowth. If these mechanisms are not sufficiently well developed in the immature dendrites then instead of stimulating spine outgrowth, some of the existing spines could collapse.

Acknowledgements

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