Published in final edited form as:

J Comp Neurol. 2011 November 1; 519(16): 3327–3345. doi:10.1002/cne.22728.

Spine Formation and Maturation in the Developing Rat Auditory Cortex

Scott J. Schachte^{1,2}, Joe Losh¹, Michael E. Dailey^{1,2}, and Steven H. Green^{1,2,*}

¹Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242-1109

²Program in Neuroscience, University of Iowa, Iowa City, Iowa 52242-1109

Abstract

The rat auditory cortex is organized as a tonotopic map of sound frequency. This map is broadly tuned at birth and is refined during the first 3 weeks postnatal. The structural correlates underlying tonotopic map maturation and reorganization during development are poorly understood. We employed fluorescent dye ballistic labeling (“DiOlistics”) alone, or in conjunction with immunohistochemistry, to quantify synaptogenesis in the auditory cortex of normal hearing rats. We show that the developmental appearance of dendritic protrusions, which include both immature filopodia and mature spines, on layers 2/3, 4, and 5 pyramidal and layer 4 spiny nonpyramidal neurons occurs in three phases: slow addition of dendritic protrusions from postnatal day 4 (P4) to P9, rapid addition of dendritic protrusions from P9 to P19, and a final phase where mature protrusion density is achieved (>P21). Next, we combined DiOlistics with immunohistochemical labeling of bassoon, a presynaptic scaffolding protein, as a novel method to categorize dendritic protrusions as either filopodia or mature spines in cortex fixed *in vivo*. Using this method we observed an increase in the spine-to-filopodium ratio from P9–P16, indicating a period of rapid spine maturation. Previous studies report mature spines as being shorter in length compared to filopodia. We similarly observed a reduction in protrusion length between P9 and P16, corroborating our immunohistochemical spine maturation data. These studies show that dendritic protrusion formation and spine maturation occur rapidly at a time previously shown to correspond to auditory cortical tonotopic map refinement (P11–P14), providing a structural correlate of physiological maturation.

INDEXING TERMS

DiOlistic; pyramidal; nonpyramidal; spine formation; synapse formation; dendritic protrusion

The peripheral and central components of the rat auditory system are anatomically connected prior to birth, including neural projections between the thalamus and auditory cortex (Gurung and Fritzsche, 2004). However, the functional properties of auditory neurons in the rat brain are immature at birth. The first auditory-evoked responses in rat auditory nuclei are recorded during the second postnatal week with mature auditory thresholds not achieved until the third postnatal week (Puel and Uziel, 1987; Geal-Dor et al., 1993; Metharate and Aramakis, 1999). Sound frequency in the primary auditory cortex is represented as a tonotopic gradient with neurons responsive to high-frequency sounds located rostrally and those responsive to low-frequency located caudally. This tonotopic

organization matures rapidly during the second and third postnatal week as broadly responsive neurons become tuned to a specific frequency (de Villers-Sidani et al., 2007).

A similar time course of functional maturation is established in the rat somatosensory and visual cortices where physiological maturation of pyramidal and nonpyramidal neuron excitability and receptive field size occurs up to 3 weeks postnatally (Franceschetti et al., 1998). This electrophysiological maturation coincides with the expansion of neuronal dendritic arbors and increases in spine density during the first 3 postnatal weeks (Miller, 1981; Petit et al., 1988). The cellular changes underlying electrophysiological maturation of the auditory cortex have yet to be clearly defined.

Dendritic spines, the primary location of excitatory neurotransmission in the cerebral cortex, contain a postsynaptic density (PSD) that organizes receptive, transductive, and downstream signal transduction components (Harris and Kater, 1994). Time-lapse imaging studies of organotypic hippocampal slice cultures have shown that filopodia and spines can be distinguished based on their motility: filopodia are motile, transient structures while spines are stable (Marrs et al., 2001). Moreover, increased spine stability is correlated with the presence of a postsynaptic density within the spine (Marrs et al., 2001).

One model for spine formation involves a developmental conversion of dendritic filopodia to mature spines. Indeed, during early cortical development dendrites are decorated with filopodia-like protrusions (Purpura, 1975), which gradually disappear as stubby spines become predominant (Morest, 1969). In vitro observations in organotypic hippocampal slice cultures, where neurons can be imaged and classified based on their motile behavior, provide further support for filopodium-to-spine conversion as an essential part of developmental synaptogenesis. Time-lapse imaging of fluorescent dyelabeled neurons in the slices reveal thin, long, and highly unstable filopodia, semistable protospines, and short and stable mature spines (Dailey and Smith, 1996). As the slice culture develops, a marked decrease in dendritic protrusion length and motility occur (Dailey and Smith, 1996; Marrs et al., 2001). In vitro imaging of PSDs, using fluorescently tagged postsynaptic density-95 (PSD95-GFP), indicated an $\approx 80\%$ colocalization of postsynaptic densities with presynaptic terminals. The presence of a PSD95-GFP punctum within a dendritic spine was correlated with decreased motility. Together, these in vitro data support a model of synaptogenesis involving developmental transition of dendritic protrusions from filopodia, which lack PSDs, to mature spines, which contain PSDs.

Here we describe morphological and structural components of synaptogenesis in the rat auditory cortex, providing an in vivo test of the above model of synaptogenesis. Using fluorescent dye ballistic labeling ("DiOlistics") (Gan et al., 2000; Benediktsson et al., 2005) alone or in combination with immunohistochemical detection of presynaptic terminals we quantified dendritic protrusions and the rate of dendritic spine maturation in vivo in pyramidal neurons of rat auditory cortical layers 2/3, 4, and 5 and in layer 4 spiny nonpyramidal neurons. The number of dendritic protrusions increased rapidly during the second postnatal week, coincident with hearing onset and maturation. Furthermore, we observed a concomitant shift from filopodia (no synapses) to spines (defined as dendritic protrusions with synapses) consistent with the model proposed above.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley dams were purchased from Harlan Sprague Dawley (Indianapolis, IN). Dams were monitored daily and the day of birth was designated postnatal day (P) 0. Both male and female neonates were used for these experiments. All procedures

were approved by the University of Iowa Institutional Animal Care and Use Committee and conform to National Institutes of Health (NIH) guidelines.

Tissue preparation for cytochrome oxidase and immunofluorescence

Cytochrome oxidase staining and cortical layer immunofluorescence were performed on P21 transcardially perfused rat brains. Rats were anesthetized with a ketamine (40 mg/kg)/xylazine (5 mg/kg) mixture and immediately exsanguinated by transcardial perfusion of phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Following an overnight postfixation in 4% PFA at 4°C, the brains were rinsed three times in PBS and placed into a sucrose infiltration gradient (10% sucrose in PBS for 24 hours; 30% sucrose in PBS for 24 hours). The brains were then embedded in OCT for sectioning on a cryostat. Because cytochrome oxidase and immunofluorescence required sections of different thickness, a set of three P21 rats were specifically designated for each analysis.

Cytochrome oxidase histochemistry was performed using a protocol modified from Wong-Riley et al. (1978). Coronal cryosections (50 μ m thickness) were preincubated in 10°C acetone followed by three rinses with 0.1 M PB + 10% sucrose. Slides were then transferred into a 0.05 M Tris buffer (pH 7.4), rinsed once in PB, and immersed in reaction solution (sodium phosphate buffer, sucrose, diaminobenzidine, cytochrome, catalase, and DMSO) at 37°C. Slides were removed once desired staining was achieved (90–120 minutes) and immediately postfixed in 4% PFA for 30 minutes. Slides were rinsed in 0.1 M PB + 10% sucrose, run through a dehydration gradient, and mounted on slides with gelmount.

Antibodies are described below and in Table 1. Immunohistochemical staining for the vesicular glutamate transporter 2 (VGluT2) along with a Nissl stain for cortical neurons was used as a guide to assign fluorescently labeled neurons to a specific cortical layer. Twenty- μ m-thick cryosections were rinsed in PBS, then incubated in 1.0% Triton-X 100 for 1 hour at room temperature (RT). Sections were blocked for 1 hour in 10% block buffer and incubated overnight with the anti-VGluT2 antibody (1:200) at 4°C. Sections were rinsed and incubated for 1 hour at RT with Alexa Fluor 568 goat antiguinea-pig IgG (Molecular Probes, Eugene, OR; A11075) secondary antibody. Slices were incubated for 30 minutes with NeuroTrace 530/615 red fluorescent Nissl stain (Molecular Probes; N-21482), a fluorescent derivative of the traditional Nissl stain that labels ribosomal RNA associated with the rough endoplasmic reticulum in neuronal perikarya and dendrites. Specificity of NeuroTrace was shown by the inhibition of staining by RNase pretreatment as well as by comparing its staining pattern to that of cresyl violet (Quinn et al., 1995).

Tract tracing

Retrograde and anterograde tract-tracing experiments were performed on brains from transcardially perfused rats at P21 (see above). Three brains were designated for both retrograde and anterograde tract tracing. Wedge-shaped filter paper was soaked in 200 μ l of methylene chloride-dissolved 1,1'-dioctadecyl-3,3',3'-tetramethyl-indo-carbocyanine perchlorate (DiI). Filter paper was allowed to dry and inserted into the gray matter of the presumptive auditory cortex (Fig. 1C) or the medial geniculate nucleus for retrograde and anterograde tract tracing, respectively. Brains were incubated at RT in PBS with 0.02% sodium azide for 3 weeks before being cut into 200–300- μ m-thick coronal vibratome sections. DiI was imaged using confocal microscopy (see below).

Ballistic labeling using lipophilic fluorescent dye (“DiOlistic labeling”)

The long chain lipophilic fluorescent dye DiI was coated on tungsten particles using a protocol modified from Gan et al. (2000) (Benediktsson et al., 2005). Two mg of DiI

(D-282; Molecular Probes) was dissolved in 200 μ l methylene chloride. Tungsten particles (50 mg, 1.0 μ m diameter, cat. no. 1652263, Bio-Rad, Hercules, CA) were placed on a glass slide. The dissolved dye was added to the tungsten, spread around the glass slide, and allowed to dry for 2 minutes. The DiI-coated tungsten particles were scraped into a vial, mixed with 3 ml of sterile distilled water, and sonicated until the solution appeared homogenous. Coating thickness was controlled by diluting the tungsten in more (for thinner coating) or less (for thicker coating) water. The solution was loaded into polyethylene tubing, allowed to settle, and removed, leaving behind the DiI-coated tungsten. The tubing was air-dried for 10 minutes and cut into 13 mm segments for the Bio-Rad Helios gene gun.

DiI-labeled dendritic protrusion measurements were made in P4, P9, P11, P16, P19, P21, P24, and P42 rats. Following transcardial perfusion with 4% PFA, intact brains were dissected from surrounding dura, divided into left and right hemispheres, and trimmed of brainstem tissue. Next, a coronal cut was made to remove the olfactory bulbs and approximately one-fourth of the rostral cortex. The newly exposed surface was then adhered to the vibratome mounting block with cyanoacrylate glue and a block of 2% agarose placed against the medial hemispheric surface to provide stability while sectioning. The mounted tissue was then immersed in cold PBS and cut into 300- μ m-thick coronal sections. Sections were postfixed in 4% PFA for 1–2 hours at RT, rinsed in PBS, and stored at 4°C in PBS with 0.02% sodium azide. Cortical slices were shot with DiI micro-tungsten particles using the gene gun at 60 psi through a nylon mesh (45 μ m diameter pore size; Small Parts, Logansport, IN, cat. no. CMN-1B). Shot slices were immersed in PBS and placed at RT where the DiI was allowed to diffuse throughout the labeled neurons for 24 hours prior to imaging.

A separate cohort of neonatal mice, sacrificed at P9, P16, and P21, were used for spine occupancy measurements. Auditory cortex slices (200–300 μ m thick) were prepared as described above, but shot with a fixable DiI variant (Cell Tracker CM-DiI; Molecular Probes, C7001) rather than DiI, incubated for 24 hours at 37°C, and fixed again with 4% PFA for up to 2 hours at RT. Slices containing CM-DiI labeled pyramidal neurons were mounted in a polyacrylamide/agarose gel for resectioning (Germroth et al., 1995). Slices were incubated overnight in a 4% polyacrylamide solution (29.6% monoacrylamide, 0.4% bis-acrylamide) in distilled water. After 24 hours, a 65°C solution of 2% low-melt agarose (Midwest Scientific) was mixed in equal volume with an RT 8% polyacrylamide solution to produce a 1% agarose/4% polyacrylamide solution maintained at 50–60°C. Slices were immersed and incubated in agarose/polyacrylamide for 2 hours at 50–60°C. Solidification of the gel was catalyzed by the addition of 2% ammonium persulfate (Fisher, Pittsburgh, PA) and TEMED (Bio-Rad). After cooling overnight at 4°C, gel blocks were mounted on the vibratome, cut into 100 μ m sections, and stored at 4°C in PBS until use.

Immunohistochemistry was performed on free-floating slices. The slices were washed three times in PBS and transferred to blocking buffer (PBS containing 1% horse serum) for 1 hour at RT. Sections were incubated overnight in blocking buffer containing the monoclonal mouse anti-Bassoon antibody diluted 1:400 in blocking buffer. The sections were washed three times with PBS and incubated overnight in 4°C in a goat antimouse Alexa Fluor 488 IgG secondary antibody. After three PBS rinses, sections were mounted in PBS containing penicillin/streptavidin.

DiOlistics randomly labels a small number of cells with lipophilic fluorescent dye, allowing clear visualization of even the smallest structures, such as dendrites and dendritic protrusions (Benediktsson et al., 2005). For all dendritic protrusion and spine occupancy experiments, clearly isolated DiI-labeled neurons were chosen for imaging and categorized based on cell layer location as well as morphological classification as pyramidal or

nonpyramidal. Because the density of dendritic protrusions may be lower in the vicinity of the soma, dendrite segments analyzed in this study extended distally starting from at least 50–100 μm from the cell soma. All dendrite segments were 40–90 μm in length. The total number of rats used and cells counted for dendritic protrusion density and for spine occupancy are indicated, respectively, in Tables 2 and 3.

Organotypic auditory cortical slice cultures

Organotypic auditory cortical tissue slices were prepared and grown on membrane filters. Rat pups aged P4 to P7 were killed by swift decapitation. Brains were dissected into cold (4°C) Hanks balanced salt solution (Gibco, Gaithersburg, MD) supplemented with dextrose (6 mg/ml), and immediately cut into 300- μm -thick coronal slices using a vibratome. Slices were placed onto Falcon polyethylene terephthalate-etched membrane culture inserts with 1 μm pores (Fisher). Tissue slices were maintained in culture medium (25% Hanks BSS, 50% MEM, 25% heat-inactivated horse serum, 2 mM glutamine, 0.044% NaHCO_3 , and 10 units/ml penicillin-streptomycin) in a 5% CO_2 humidified incubator (36°C) for 6–12 days before imaging. Medium was replaced three times a week.

A PSD95-GFP fusion protein, containing GFP fused in-frame to the C-terminus of PSD95 and driven by a cytomegalovirus promoter (donated by D. Bredt, UCSF, San Francisco, CA), was used for biolistic transfection of plasmid cDNA in cortical slice cultures. Biolistic transfection of neurons in tissue slices was accomplished using the Helios gene gun as previously described (Marrs et al., 2001; Zha et al., 2005). Colloidal gold particles (1 μm diameter; 8 mg) were combined with 0.05 M spermidine (100 μl), 1 M CaCl_2 (100 μl) and DNA (PSD95-GFP, 15–20 μg). Cortical slices were shot (70–80 p.s.i. at 2–3 mm distance) after 4–10 days in culture and examined 1–2 days later.

PSD95-GFP-transfected cortical slice cultures were immunostained for anti-synapsin. Slices were placed in culture media containing 4% PFA for either 10 minutes (36°C) or overnight (4°C). Tissues were rinsed in 0.01 M PBS, extracted with 1% Triton X-100 overnight, blocked sequentially in 50 mM NH_4Cl and 20% horse serum (30 minutes each), rinsed with PBS containing 1% horse serum (PBS + HS), and incubated for 10–16 hours (4°C) in affinity-purified anti-synapsin-1 polyclonal antibody (1:200) (Molecular Probes) in PBS + HS. Tissue slices were rinsed with PBS + HS, then incubated 10–16 hours (4°C) in Alexa-Fluor-568 conjugated goat antirabbit IgG secondary antibody (Molecular Probes) diluted 1:300 in PBS + HS before final rinse and mounting.

Antibody characterization

The monoclonal mouse anti-Bassoon antibody (Stressgen no. VAM-PS003) was raised against a recombinant fusion protein consisting of glutathione S-transferase (GST) and the amino acids 756–1,001 of the rat Bassoon protein. Specificity of this antibody was verified in both mouse and rat neurons as colocalizing with axon terminals as well as with piccolo, another presynaptic scaffolding protein (Fenster et al., 2000; tom Dieck et al., 2005; Regus-Leidig et al., 2009) as well as by loss of immunoreactivity in a Bassoon knockout mouse (Dick et al., 2003).

The anti-synapsin 1 antibody (Molecular Probes no. 486200/Invitrogen no. 51-5200) was raised against purified bovine brain synapsin 1 and epitope-affinity-purified from rabbit antiserum. In western blot analyses of rat brain tissue, the antibody stains a doublet at about 77 and 80 kDa, respectively corresponding to the expected position of synapsin 1 α and synapsin 1 β (manufacturer's technical information). Specificity of synapsin 1 immunostaining has been further verified by precise colocalization of synapsin 1 with postsynaptic density-95, a postsynaptic scaffolding protein, in rat cerebellum in vivo

(Castejon et al., 2004) and verified by us for rat hippocampal slice cultures (Marrs et al., 2001).

The anti-VGluT2 antibody (Chemicon, Temecula, CA; no. AB5907) is a guinea-pig polyclonal raised against a synthetic peptide corresponding to a region of the VGluT2 protein sequence, VQESAQDAYS YKDRDDYS, a sequence that does not overlap with that of VGluT1 or VGluT3. Specificity was demonstrated by elimination of immunostaining by blockade of the VGluT2 antiserum with the immunizing peptide (Hrabovszky et al., 2004; Stocker et al., 2006). We further verified antibody specificity by showing a pattern of a cortical layer staining using this antibody identical to that previously shown for VGluT2 immunoreactivity (Nahmani and Erisir, 2005; Kubota et al., 2007): specific labeling of cortical layers 1 and 4.

Confocal imaging

Images were captured using a Leica (Heidelberg, Germany) SP2 AOBS confocal microscope (inverted platform) equipped with an Ar laser (lines at 458, 476, 488, and 514 nm), HeNe laser lines (543 nm, 594 nm, and 633 nm), an acousto-optic beam splitter for simultaneous multiline excitation, and prismatic detection optics that support continuously variable detection bandwidths for maximum signal detection and channel separation. We used 20×/0.7NA PLAN APO and 63×/1.2NA water-immersion PLAN APO objective lenses. Cells containing DiI or CM-DiI were excited with the HeNe 543 nm laser line and emission collected between 560 nm and 700 nm. Detection of Alexa Fluor 488 secondary IgG was achieved by exciting with the Argon 488 nm laser line (with emission collected between 490 nm and 540 nm).

Organotypic cortical slice cultures were examined using the Kr laser and rhodamine filter set (590LP barrier filter). Simultaneous two-channel confocal imaging captured PSD95-GFP and Alexa-Fluor-568 fluorescence within the same tissue volumes. Stacks of 10–20 optical sections (1024 × 1024 pixel array) yielded voxel dimensions between 0.15 µm and 0.4 µm for the x-, y-, and z-axes.

Image processing and analysis

Adobe Photoshop (San Jose, CA) and NIH ImageJ (<http://rsb.info.nih.gov/ij/>) software were used for image processing and analysis, including contrast enhancement to assist in protrusion quantification as well as image rendering of DiI and bassoon photomicrographs for quantification of spine occupancy. The following dendritic properties were quantified: density of dendritic protrusions, colocalization of presynaptic terminals and dendritic protrusions for the purpose of identifying mature spines, length of dendritic protrusions, and dendritic arbor complexity. The measurements are described in detail in the Results.

For statistical analysis, the Mann–Whitney *U*-test was used to compare nonparametric data distributions and was conducted with SPSS 15.0 for Windows (Chicago, IL; licensed by the University of Iowa). Student's *t*-test with unequal variance was used to compare nonparametric data distributions and was conducted with the Microsoft Excel 2002 data analysis package. Error bars for both Mann–Whitney *U*-tests and Student's *t*-tests indicate the standard deviation.

Dendritic protrusion densities were quantified by offline processing of confocal images using NIH ImageJ software. Multi-z-plane confocal series images were projected into a single image and dendrite length measured (ranging from 50 to 90 µm in length). Individual dendritic protrusions were counted along the specified dendrite by scrolling through the z-plane series. Dendritic protrusions that were fully or partially visible in the x–y plane were

counted while those that projected only through the z-plane could not be clearly defined and were not counted. Each protrusion was marked in ImageJ to avoid double counting. The dendritic arborization of cortical neurons are still extending in neonatal mice and, especially at early timepoints, may be newly formed and short in length. Therefore, to consistently quantify protrusions across all of the chosen developmental timepoints, and to discount emerging dendrites, we liberally counted all DiI-labeled structures $<10\ \mu\text{m}$ in length extending from the dendritic shaft as protrusions. Generally, counts were made of protrusions on four to five neurons per rat and at least three rats per timepoint.

Length was measured only for protrusions entirely contained within a single optical section. Protrusion length was measured to the protrusion tip from its emergence from the dendritic shaft. In the event that a protrusion branched, its length was measured to the tip of the longest branch.

Bassoon density quantification was performed offline using ImageJ software. Single optical plane confocal images of bassoon immunofluorescence were counted using the analyze particle macro in ImageJ.

In all cases, images were coded prior to analysis to ensure that analyses were carried out in a blinded fashion with respect to postnatal age, neuron type, and dendrite category. Student's *t*-test with unequal variance was used for pairwise comparisons except for nonparametric data for which the Mann–Whitney *U*-test was used (SPSS 15.0 for Windows) In all cases error bars show standard deviation.

RESULTS

Confirmation of rat auditory cortex location

To confirm that quantitation of dendritic and spine morphology was restricted to auditory cortex neurons, we used four criteria to verify the anatomical location. First, we used established landmarks, in particular, locating the auditory cortex dorsal to the rhinal fissure. Second, the auditory cortex has higher levels of cytochrome oxidase (CO) than adjacent cortex (Wong-Riley et al., 1978, 1981). We visualized CO histochemically in P21 rat brains using 50- μm coronal cryosections. The region we identify as auditory cortex, extending dorsally several hundred μm from the rhinal fissure, does show higher CO activity (Fig. 1A). As previously reported (Wong-Riley et al., 1978, 1981) the highest CO activity in the auditory cortex is in the middle cortical layers (layer IV) (Fig. 1B).

Third, placing DiI-soaked filter paper wedges in the auditory cortex results in DiI-labeling of thalamocortical neurons in the medial geniculate nucleus (MGN), the source of ascending input to the auditory cortex (Fig. 1C). In addition to labeling the MGN, thalamocortical, and corticothalamic projections, this procedure labeled horizontal corticocortical projections, clearly defining the dorsal boundary for the auditory cortex. Fourth, DiI-soaked filter paper placed in the MGN labels the presumptive auditory cortex (Fig. 1D). High-resolution confocal microscopy of the auditory cortex showed clearly defined DiI-labeled cortical pyramidal neurons, confirming retrograde DiI labeling of the auditory cortex to MGN corticothalamic projection (Fig. 1E,F). Thalamocortical projections were also labeled, evident as axonal fibers penetrating the auditory cortex (Fig. 1E).

Categorization of dendrites for dendritic protrusion quantitative analysis

Auditory cortex layer 4 and adjacent layer 3 neurons receive thalamocortical inputs from the MGN; dendrites of pyramidal neurons in layers 2/3 and 5/6 receive cortical inputs (Smith and Populin, 2001). Each cortical layer has pyramidal and nonpyramidal neurons and the rate of synaptogenesis may vary among these types of neurons and among layers. Moreover,

pyramidal neurons possess distinct apical and basal dendrites. The single apical dendrite extends into upper (pial) layers while the basal dendritic arbor is generally restricted to the same cortical layer as the soma. Because of different types of inputs, the rate of synaptogenesis may vary between apical and basal dendrites. Here we quantified the rate of spine formation on auditory cortex dendrites, distinguishing between cortical layers, and among spiny nonpyramidal neuron dendrites, pyramidal neuron apical dendrites, and pyramidal neuron basal dendrites (Fig. 2). Representative images of DiI-labeled auditory cortical pyramidal and nonpyramidal neurons are shown in Figure 2B and 2C,D, respectively. Assignment of cells to specific cortical layers was assisted by coronal cryosections of P21 rat cortex immunostained for VGluT2, which labels cortical layers 1 and 4, and counterstained with Neurotrace, a fluorescent Nissl stain for neurons (Fig. 2A) (Kubota et al., 2007).

Note that the DiIs labels all dendritic protrusions and does not allow distinction between filopodia—operationally defined as dendritic protrusions without synapses—and spines—defined as dendritic protrusions with synapses (Marrs et al., 2001). For this reason, in the quantitation described in this section we refer only to “dendritic protrusions” without distinction between spines and filopodia and subsequently apply a test to establish the approximate proportion of spines to filopodia.

Quantitation of the developmental increase in the density of dendritic protrusions

Our preliminary observations indicated that the developmental increase in density of dendritic protrusions in rat auditory cortex layers 2/3, 4, and 5 occurs within the first 3 postnatal weeks. To establish the timecourse in detail we used rats euthanized at P4, P9, P11, P14, P16, P19, P21, and P42. An additional P24 timepoint was used for layer 4 neurons. Representative images of apical and basal dendrite segments from a layer 2/3 pyramidal neuron and a layer 4 spiny nonpyramidal neuron are shown, respectively, in Figure 3A–C. An increase in protrusion density can be observed between P4 and P21.

Quantitative analysis of dendritic protrusions on pyramidal neurons shows an ≈ 25 -fold increase in protrusion density from P4 to P21 (Fig. 4). The pattern of developmental increase in dendritic protrusions is similar among all of the dendrite types analyzed: apical and basal dendrites from layer 2/3, layer 4, and layer 5 pyramidal neurons as well as spiny nonpyramidal neurons (all spiny nonpyramidal neurons were grouped together for this analysis). For all, protrusions are added slowly between P4 and P9, followed by rapid addition between P11 and P19, acquisition of mature protrusion densities between P19 and P24, and little change after P21. Average protrusion density, standard deviation, number of cells analyzed, and number of rats are provided in Table 2.

Physiological responses of cortical neurons are mature by about P21 (Puel and Uziel, 1987; Geal-Dor et al., 1993; Metherate and Aramakis, 1999; Oswald and Reyes, 2008). To determine whether physiological maturation of cortical neurons correlates with maturation of dendritic morphology, we quantified dendritic protrusion density at P42 and compared it to that at P21 (Table 2, PFig. 4). Layer 5 apical dendrites showed a small but significant increase in protrusion density between P21 and P42 ($P = 0.017$, Mann–Whitney *U*-test) as did layer 4 pyramidal neuron apical ($P = 0.041$) and basal ($P < 0.001$) dendrites. Protrusion densities at P42 were not significantly different from P21 for layer 4 nonpyramidal neuron dendrites (Mann–Whitney *U*-test; $P = 0.732$), for layer 2/3 pyramidal neuron apical ($P = 0.310$), and basal dendrites ($P = 0.413$), nor for layer 5 pyramidal neuron basal dendrites ($P = 0.336$).

Because protrusion density for layer 4 pyramidal neuron apical dendrites at P21 was close to that at P42, we reasoned that these dendrites achieve morphological maturity close to P21.

Therefore, we quantified protrusion density for layer 4 neurons at P24. While layer 4 pyramidal neuron basal dendrite protrusion density remained significantly different ($P = 0.014$) between P24 and P42, apical dendrite protrusion density was not significantly different ($P = 0.897$). In summary, protrusion density matures by P21 for dendrites of layer 4 nonpyramidal neurons, dendrites of layer 2/3 pyramidal neurons, and for basal dendrites of layer 5 pyramidal neurons; by P24 for apical dendrites of layer 4 pyramidal neurons; and between the third and sixth postnatal week for layer 5 pyramidal neuron apical dendrites and for layer 4 pyramidal neuron basal dendrites.

Method for determining colocalization of presynaptic terminals and dendritic protrusion in confocal images

We next asked whether dendritic development in the auditory cortex takes place in a manner consistent with the hypothesis that spinogenesis occurs by conversion of filopodia to mature spines. To determine this, we require a definitive method to distinguish spines from filopodia on DiI-labeled dendrites. Although DiI alone does reveal filopodium-like and spine-like structures on dendrite segments at all ages between P4 and P21 (Fig. 3), the distinction is still ambiguous. We consider a mature spine to be a dendritic protrusion containing a synapse, based on our previous observation using cultured hippocampal slices that stability of a dendritic spine is tightly correlated with the presence of a PSD95 cluster within the spine (Marrs et al., 2001). Although immunoreactivity for PSD95 and other postsynaptic density markers is lost when the brain is fixed in situ, several presynaptic markers, e.g., bassoon (Altrock et al., 2003; tom Dieck et al., 2005), retain immunoreactivity and can be used to identify synapses. For example, Marrs et al. (2001) showed that $\approx 80\%$ of PSD95 immunofluorescent puncta are immediately adjacent to presynaptic terminals detected as synapsin-I immunofluorescent puncta. Thus, for the purposes of this analysis, we operationally define a mature spine as a dendritic protrusion immediately adjacent to a bassoon immunoreactive punctum.

Immunohistochemistry requires permeabilizing the cell membrane with detergent, which extracts lipophilic dyes, but by employing a fixable DiI variant (CM-DiI) we could combine DiOlistic labeling with immunofluorescence. Auditory cortical slices from P9, P16, and P21 rats were DiOlistically labeled, as above, with CM-DiI, and immunolabeled with a monoclonal antibody to the presynaptic protein bassoon, chosen for its resistance to the extensive fixation required.

Quantitation of spine occupancy was performed on high-magnification confocal image stacks consisting of optical sections ("z-sections") spaced $0.8\ \mu\text{m}$ apart in the Z-axis through a single dendrite segment. Three adjacent optical sections (Fig. 5A–I) were combined in ImageJ to generate a "flattened" projection image representing a Z-axis depth of $1.6\ \mu\text{m}$ (Fig. 5J–L). As shown in the figure, this was performed in parallel for the corresponding CM-DiI and Bassoon image channels. Each CM-DiI projection image was processed in Adobe Photoshop to expand the perimeter of the CM-DiI fluorescence by $0.2\ \mu\text{m}$ and this expanded image was used to create a mask (Fig. 5M). The reason for choosing a criterion of $0.2\ \mu\text{m}$ is discussed below. This mask was superimposed on the corresponding bassoon immunofluorescence image (Fig. 5N) to eliminate all bassoon immunofluorescence that did not fall within a $0.2\ \mu\text{m}$ "envelope" of the fluorescently labeled dendrite segment (Fig. 5O). Thus, only bassoon puncta that were located within $0.2\ \mu\text{m}$ of a fluorescently labeled dendrite in the XY plane and within $0.8\ \mu\text{m}$ in the Z-axis were visible and counted as synapses on that dendrite. Following image processing, dendritic protrusions with adjacent bassoon puncta were considered "occupied" and counted as spines. Spine counts were performed blindly on sections of dendrites $40\text{--}90\ \mu\text{m}$ in length.

To determine the maximum distance between a PSD and a presynaptic terminal we used biolistic transfection of a PSD95-GFP expression construct into auditory cortex slice cultures to directly label PSDs, as previously described (Zha et al., 2005). We combined this with immunofluorescence for a presynaptic protein, in this case synapsin, to directly observe colocalization of pre- and postsynaptic densities—shown for auditory cortex in Figure 6A–C. Within any 2- μm diameter circle centered on a postsynaptic density (visualized as a PSD95-GFP punctum), peak synapsin fluorescence colocalizes with peak PSD95-GFP fluorescence in the Z-axis (Fig. 6D,E). We determined the distance in increments of 0.8 μm —the spacing between z-sections—between synapsin and PSD95-GFP fluorescence peaks for 58 synapses on seven neurons and plotted this as a cumulative histogram (Fig. 6F). This shows that ~90% of PSD95-GFP clusters are within 1.6 μm (two z-sections) of a presynaptic immunofluorescent punctum.

In vivo fixed cortical tissue has a higher density of pre-synaptic terminals relative to cultured cortical slices, increasing the likelihood that there will be some colocalization with dendritic protrusions by chance. This suggests that allowing a distance of 1.6 μm as a criterion for proximity may not be sufficiently stringent. To test this, we compared colocalization of dendritic protrusions with the actual corresponding bassoon image to colocalization with randomly chosen bassoon images (Fig. 7A,B). We expect that for randomly chosen bassoon images the fraction of protrusions that colocalize with bassoon should be significantly less than for the corresponding bassoon image. However, when the criterion for colocalization was that the bassoon punctum be within a 1.6 μm envelope of the dendrite perimeter there was no significant difference between the corresponding bassoon image and a random one (Fig. 7C). As the criterion envelope is reduced, in 0.2- μm increments, the percentage of dendritic protrusions colocalizing with bassoon puncta drops slightly when superimposition is with the corresponding bassoon image but drops considerably when superimposition is with a random bassoon image, thereby increasing the difference between the number of correct and the number of random matches. The greatest difference was observed with the minimal perimeter expansion of 0.2 μm , at which 38% of protrusions colocalized with random bassoon puncta but 75% of protrusions colocalized with corresponding bassoon puncta. We therefore chose a proximity of 0.2 μm in the XY-plane as the most stringent criteria for establishing bassoon colocalization with dendritic protrusions. A criterion of 0.8 μm was used in the Z-axis because this is the minimum spacing between optical sections.

We used these criteria to ensure that protrusions that are counted as spines are indeed spines. This also helps compensate for the possibility that some dendritic spines that possess a postsynaptic density are not necessarily apposed to a presynaptic terminal (Marrs et al., 2001; Christopherson et al., 2005). However, because the percentage of dendritic protrusions colocalizing with corresponding bassoon puncta is reduced at 0.2 μm in the XY-plane and 0.8 μm in the Z-axis relative to 1.6 μm , use of these criteria likely results in an undercount of the number of occupied dendritic protrusions (mature spines). The percentage of occupied dendritic protrusions may be slightly higher than our count, although, because the same criteria are used throughout, the relative proportions are the same for all ages and conditions.

Given the increasing difference between colocalization of actual and random bassoon puncta with decreasing proximity criterion distance, it might be supposed that a criterion distance of <0.2 μm would be preferable. However, this could increase the undercount while, at best, only slightly reducing any possible false positives. Moreover, confocal optical sections are not infinitely thin. Even in a single optical section, two points that are precisely overlapping could potentially be as far apart as the thickness of the optical section (which is dependent on the objective numerical aperture and pinhole setting and can be >0.2 μm). Thus, a low

“background” level of false positives can be attributed to the z-dimension and cannot be eliminated by further reduction of the mask below 0.2 μm .

Quantitation of a developmental increase in dendritic spines

With the criteria and methods developed above, we quantified the fraction of dendritic protrusions that are spines, i.e., occupied by presynaptic terminals. Figures 5 and 7A are examples of fluorescently labeled dendrite images superimposed on the corresponding images of bassoon immunofluorescence, showing colocalization of dendritic protrusions and presynaptic terminals. Table 3 shows quantitation of colocalization in such images for four types of auditory cortex pyramidal neuron dendrites—layer 2/3 apical, layer 2/3 basal, layer 5 apical, and layer 5 basal dendrites—at P9, P16 and P21. The table includes the mean percentage of dendritic protrusions occupied by presynaptic terminals, i.e., the percentage of dendritic protrusions that are spines, standard deviation, and sample sizes. The data are plotted in Figure 8. Figure 8A shows combined quantitative data for all dendritic protrusions, regardless of cortical layer or dendrite type. The percentage of dendritic protrusions that are spines increased significantly ($P < 0.001$) from $\approx 40\%$ at P9 to $\approx 70\%$ at P16. There was a small but significant decrease ($P = 0.010$) in the percentage of spines between P16 and P21.

Separating out the different types of dendrites reveals rather similar developmental change among these types (Fig. 8B): there is a significant increase in the percentage of dendritic protrusions that are spines between P9 and P16 for layer 2/3 apical ($P < 0.001$), layer 2/3 basal ($P < 0.001$), layer 5 apical ($P < 0.001$), and layer 5 basal dendrites ($P = 0.003$). From P16 to P21, no significant change in percentage of spines was seen on layer 2/3 apical ($P = 0.806$), layer 5 apical ($P = 0.689$), and layer 5 basal dendrites ($P = 0.059$). A small but significant decrease was observed in layer 2/3 basal ($P = 0.003$) dendrites.

To determine the extent to which the increase in the fraction of protrusions that are spines can be attributed to an increase in the number of presynaptic puncta, we quantified the spatial density of bassoon immunofluorescence at P9, P16, and P21. This analysis revealed an increase in bassoon puncta between P9 and P16, but not between P16 and P21 (Fig. 8C). However, the increased bassoon density between P9 and P16 (134%) only partially accounts for the concomitant 4-fold increase in occupied protrusions (440%).

In summary, these data show that the ratio of spines to filopodia among dendritic protrusions increases dramatically between P9 and P16, concomitant with the rapid increase in dendritic protrusion density. Dendritic protrusions and spines continue to emerge until $\approx P19$, indicating a period of slower, yet continuous, spine maturation that extends beyond the second postnatal week.

Developmental changes in dendritic protrusion morphology

Conversion of filopodia to mature spines, indicated by the data above, might be expected to be correlated with a change in morphology (Marrs et al., 2001) because filopodia tend to be longer than mature dendritic spines (Harris et al., 1992). To this end, we measured length of individual protrusions on apical and basal dendrites of layer 2/3, 4, and 5 auditory cortical pyramidal neurons and layer 4 nonpyramidal neurons from P9, P16, and P21 rats (Fig. 9). The length measurements are plotted as cumulative histograms in Figure 9A–G. Decreasing length corresponds to a shift of the curve to the left. The mean length and standard deviation for each dendrite and neuron type are shown in Figure 9H,I.

Protrusion length decreases significantly between P9 and P16 on apical and basal dendrites of layer 2/3, 4, and 5 pyramidal neurons and by P21 on layer 4 spiny non-pyramidal neuron

dendrites. Specifically, Figure 9A–D,H shows length data for layer 2/3 and 5 pyramidal neurons. Layer 2/3 apical dendrite protrusion length at P16 ($1.62 \pm 0.09 \mu\text{m}$; $n = 267$) was significantly shorter than at P9 ($1.85 \pm 0.19 \mu\text{m}$; $n = 179$; $P < 0.001$). There was no further change in protrusion length at P21 ($1.55 \pm 0.10 \mu\text{m}$; $n = 327$) ($P = 0.517$). Layer 2/3 basal dendrite protrusion length at P16 ($1.52 \pm 0.15 \mu\text{m}$; $n = 179$) was significantly shorter than at P9 ($2.03 \pm 0.42 \mu\text{m}$; $n = 160$) ($P < 0.001$) but did not differ from P21 ($1.47 \pm 0.17 \mu\text{m}$; $n = 629$; $P = 0.066$). Layer 5 apical dendritic protrusion length at P16 ($1.46 \pm 0.08 \mu\text{m}$; $n = 117$) was significantly shorter than at P9 ($2.95 \pm 1.70 \mu\text{m}$; $n = 77$; $P < 0.001$) with no further change in length at P21 ($1.46 \pm 0.15 \mu\text{m}$; $n = 258$; $P = 0.859$). Layer 5 basal dendritic protrusion length at P16 ($1.72 \pm 0.08 \mu\text{m}$; $n = 284$) was significantly shorter than at P9 ($2.18 \pm 0.50 \mu\text{m}$; $n = 108$; $P < 0.001$) with no further change in length at P21 ($1.48 \pm 0.18 \mu\text{m}$; $n = 443$; $P = 0.025$).

Protrusions on layer 4 pyramidal neurons similarly decreased in length during the second postnatal week (Fig. 9E–G,I). Layer 4 basal dendrite protrusion length at P16 ($1.55 \pm 0.01 \mu\text{m}$; $n = 256$) was significantly shorter than at P9 ($2.11 \pm 0.23 \mu\text{m}$; $n = 151$; $P < .001$) with no further change in length at P21 ($1.49 \pm 0.09 \mu\text{m}$; $n = 292$; $P = 0.232$). Layer 4 apical dendrite protrusion length at P16 ($1.42 \pm 0.17 \mu\text{m}$; $n = 186$) was significantly shorter than at P9 ($1.84 \pm 0.36 \mu\text{m}$; $n = 184$; $P = 0.001$) with no further change in length at P21 ($1.45 \pm 0.09 \mu\text{m}$; $n = 219$; $P = 0.905$).

Layer 4 nonpyramidal neurons showed a decrease in length of dendritic protrusions that was delayed relative to the pyramidal neurons described above. Protrusion length of layer 4 nonpyramidal neurons did not change significantly from P9 ($1.95 \pm 0.42 \mu\text{m}$; $n = 173$) to P16 ($1.71 \pm 0.24 \mu\text{m}$; $n = 139$; $P = 0.398$) but was significantly shorter by P21 ($1.58 \pm 0.21 \mu\text{m}$; $n = 124$; $P = 0.011$).

To investigate whether stubby spines are more likely than longer spines to have synapses, we analyzed layer 5 basal dendrites for a correlation between dendritic protrusion length and the presence or absence of a bassoon immunoreactive puncta (Table 4). Our data show similar protrusion length between occupied and unoccupied protrusions at P9, P16, and P21. At P21 there is a trend for a longer mean length for unoccupied protrusions but there is high variation in spine length across all timepoints and the trend is not statistically significant.

DISCUSSION

Developmental increase in dendritic protrusion density

In the altricial rat, cortical maturation is finalized postnatally. We show here that the developmental increase in dendritic protrusions occurs with a similar timecourse in auditory cortex as it does in somatosensory and visual cortices, even though the peripheral sensory receptors mature at different times. Cortical pyramidal and nonpyramidal neurons expand their dendritic arbors and increase protrusion density during the first 3 postnatal weeks (Miller, 1981; Petit et al., 1988). Specifically, in auditory cortex we show an increase in protrusion density on layers 2/3, 4, and 5 pyramidal neurons during the first 3 weeks postnatal, with most protrusions added P11–P21. The mature protrusion density in auditory cortex pyramidal neurons is similar to that in the somatosensory and visual cortices, including higher density on layer 2/3 than on layer 5 pyramidal neurons (Miller, 1981; Petit et al., 1988).

In layer 4 we quantified protrusion density on spiny nonpyramidal neurons as well as on pyramidal neurons. Layer 4 receives the bulk of direct sensory relays from the medial geniculate nucleus with pyramidal neurons being the primary recipient of direct thalamocortical projections (Smith and Populin, 2001; Broicher et al., 2010). We show that

acquisition of dendritic protrusions occurs with a similar timecourse on nonpyramidal and pyramidal neurons. Maximal protrusion density is lower on nonpyramidal than on pyramidal neurons. However, spine density on nonpyramidal neurons is highly variable, evident in the large standard deviations in the numbers at ages P19 (Fig. 4), consistent with the report of Kawaguchi et al. (2006) that specific subpopulations of nonpyramidal neurons can be distinguished by spine density. Because the variability among neurons is much less prior to P19, it is possible that functional divergence emerges later, after the period of rapid addition of protrusions.

A previous study (Nicolai, 1981) of auditory cortex layer 5 pyramidal neuron apical dendrites used Golgi labeling and found a significantly lower density of dendritic protrusions than we report as well as a different, biphasic, timecourse of protrusion development. It is unlikely that these discrepancies are due to the use of different spine labeling methods. Our use of DiI yields values similar to those obtained in Golgi-labeled somatosensory and visual cortical pyramidal neurons, with respect to density and development of dendritic protrusions. Rather, it is more likely that a decline in protrusion density between P12 and P24 reported by Nicolai et al. (1981) results from an inability to resolve short stubby protrusions in brightfield images of Golgi-stained material. Indeed, our data show a reduction in mean protrusion length between P9 and P16, which corresponds temporally with the reported decline in protrusion density (Nicolai, 1981).

Synaptogenesis and spine maturation

Neuronal development is characterized by a gradual decline in the number of filopodial dendritic protrusions (Purpura, 1975) and increase in the number of stubby spines (Morest, 1969; Grutzendler et al., 2002; Trachtenberg et al., 2002). Time-lapse observations of cultured neonatal hippocampal neurons (Okabe et al., 2001) or hippocampal slices (Marrs et al., 2001) indicate that conversion of a motile filopodia to a stable mature spine coincides with a postsynaptic density, visible as a PSD95-GFP fluorescent punctum, translocating to or forming in the dendritic protrusion. This is indicative of synapse formation as the PSD95-GFP puncta colocalize with pre-synaptic terminals (Marrs et al., 2001; Fig. 6) evident as bassoon-immunoreactive puncta. Thus, formation of dendritic protrusions and maturation of filopodia into spines are separable events, with the latter indicative of synaptogenesis.

In the current study, in addition to establishing the timecourse of generation of dendritic protrusions, we determined the timecourse of spine maturation and synaptogenesis. To do so we developed a method to combine DiOlastic labeling and immunofluorescence to quantify dendritic protrusions adjacent to bassoon-labeled presynaptic terminals, i.e., structural synapses, on layers 2/3 and 5 pyramidal neuron basal and apical dendrites. With fixed tissue, motility cannot be used to distinguish filopodia from spines. Rather, using the correlation between stability and presence of a synapse established in the time-lapse studies summarized above (Marrs et al., 2001; Okabe et al., 2001), we can operationally define filopodia as dendritic protrusions that lack adjacent pre-synaptic terminals and spines as protrusions with adjacent presynaptic terminals (bassoon-immunoreactive puncta).

The data presented here show an increase in the fraction of dendritic protrusions with synapses, i.e., mature spines, from P9 to P16, the same period during which there is the largest increase in protrusion number. These data highlight the significance of this period in cortical synaptogenesis. Not only do dendrites extend protrusions at an elevated rate but these are being converted to synapse-bearing spines at such a high rate that the ratio of spines to filopodia increases despite the increase in protrusion density. Subsequently, protrusion density continues to increase slowly until ~P21 but the ratio of mature spines to filopodia does not change, indicating that synapses form at the same rate as dendrites extend protrusions.

We observed a 440% increase in the number of dendritic protrusions occupied by bassoon-immunoreactive puncta over a time interval, P9 and P16, when there is a much more modest (134%) increase in the total number of bassoon-immunoreactive puncta. This implies that a large fraction of the bassoon puncta is not associated directly with dendritic spines, a fraction particularly large prior to P9. These immunoreactive puncta may be attributable to the immunohistochemical detection of nonsynaptic bassoon clusters, located in piccolo-bassoon transport vesicles (PTVs) (Shapira et al., 2003; Bresler et al., 2004; Dresbach et al., 2006); PTVs and synaptic vesicle protein transport vesicles (STVs) shuttle active zone proteins to presynaptic sites and are dynamic during early development (Sabo et al., 2006). Excess nonsynaptic PTVs may precede most synaptogenesis events only to be coupled with a postsynaptic partner during the period of rapid spinogenesis identified in this study.

Developmental changes in spine morphology

Spine maturation involves a decrease in mean length of dendritic protrusions, evident in time-lapse studies (Dailey and Smith, 1996) and electron microscopy (Harris et al., 1992). This is presumably due, at least in part, to a reduced ratio of filopodia to spines, given that the latter are shorter. Therefore, we used spine morphology as an additional measure of spine maturity. Our data show a decrease in dendritic protrusion length between P9 and P16 in layers 2/3, 4, and 5 pyramidal neurons and layer 4 nonpyramidal neurons with no further change after P16. These data strengthen the conclusion reached from our observations of filopodium and spine density that P9–P16 is the principal period for cortical synaptogenesis and spinogenesis.

Critical period

Hearing onset in the rodent begins in the second postnatal week. Hearing-evoked activity in the central auditory nuclei is first recorded during the second postnatal week with mature auditory thresholds achieved during the third postnatal week (Puel and Uziel, 1987; Geal-Dor et al., 1993; Metherate and Aramakis, 1999). Immediately following hearing onset, P11–P14, is the auditory critical period, a restricted window of time during which the functional tonotopic organization of the auditory cortex can be manipulated by environmental auditory stimuli (Zhang et al., 2001; Nakahara et al., 2004; Norena et al., 2006; de Villers-Sidani et al., 2007). For example, constant exposure to a specific sound frequency during the second and third postnatal week results in its overrepresentation in the auditory tonotopic map (Zhang et al., 2001); elimination of patterned auditory input during the second and third postnatal weeks by constant exposure to broadband noise results in an immature tonotopic map (Chang and Merzenich, 2003; Speechley et al., 2007). Expression of the NR1 subunit of the N-methyl-D-aspartic acid receptor (NMDAR) increases rapidly between P7 and P14 (Lu et al., 2007), suggesting changes in synaptic physiology during the critical period.

Our data show that the most intensive period of spinogenesis and synaptogenesis is between P9 and P16. These data provide a structural correlate to electrophysiological data showing concomitant rapid functional reorganization of the cortical tonotopic representation and maturation of response properties in the auditory cortex (Metherate and Aramakis, 1999) during the critical period. The coincidence of hearing onset and the observed rapid structural changes in auditory cortical neuron dendritic protrusions suggests that hearing onset may be causal to these changes. A critical question remains as to whether early auditory activity is necessary for spinogenesis and synaptogenesis in the auditory cortex.

Acknowledgments

Grant sponsor: National Institutes of Health (NIH); Grant number: DC02961 (to S.H.G.); Grant sponsor: American Heart Association Heartland Predoctoral fellowship (to S.S.); Grant sponsor: University of Iowa Biosciences Initiative (to M.E.D. and S.H.G.).

LITERATURE CITED

- Altrock WD, tom Dieck S, Sokolov M, Meyer AC, Sigler A, Brakebusch C, Fassler R, Richter K, Boeckers TM, Potschka H, Brandt C, Loscher W, Grimberg D, Dresbach T, Hempelmann A, Hassan H, Balschun D, Frey JU, Brandstatter JH, Garner CC, Rosenmund C, Gundelfinger ED. Functional inactivation of a fraction of excitatory synapses in mice deficient for the active zone protein bassoon. *Neuron*. 2003; 37:787–800. [PubMed: 12628169]
- Benediktsson AM, Schachtele SJ, Green SH, Dailey ME. Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *J Neurosci Methods*. 2005; 141:41–53. [PubMed: 15585287]
- Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE. Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. *J Neurosci*. 2004; 24:1507–1520. [PubMed: 14960624]
- Broicher T, Bidmon HJ, Kamuf B, Coulon P, Gorji A, Pape HC, Speckmann EJ, Budde T. Thalamic afferent activation of supragranular layers in auditory cortex in vitro: a voltage sensitive dye study. *Neuroscience*. 2010; 165:371–385. [PubMed: 19840834]
- Castejon OJ, Fuller L, Dailey ME. Localization of synapsin-I and PSD-95 in developing postnatal rat cerebellar cortex. *Brain Res Dev Brain Res*. 2004; 151:25–32.
- Chang EF, Merzenich MM. Environmental noise retards auditory cortical development. *Science*. 2003; 300:498–502. [PubMed: 12702879]
- Christopherson KS, Ullian EM, Stokes CC, Mullen CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell*. 2005; 120:421–433. [PubMed: 15707899]
- Dailey ME, Smith SJ. The dynamics of dendritic structure in developing hippocampal slices. *J Neurosci*. 1996; 16:2983–2994. [PubMed: 8622128]
- de Villers-Sidani E, Chang EF, Bao S, Merzenich MM. Critical period window for spectral tuning defined in the primary auditory cortex (A1) in the rat. *J Neurosci*. 2007; 27:180–189. [PubMed: 17202485]
- Dick O, tom Dieck S, Altrock WD, Ammermuller J, Weiler R, Garner CC, Gundelfinger ED, Brandstatter JH. The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. *Neuron*. 2003; 37:775–786. [PubMed: 12628168]
- Dresbach T, Torres V, Wittenmayer N, Altrock WD, Zamorano P, Zuschratter W, Nawrothki R, Ziv NE, Garner CC, Gundelfinger ED. Assembly of active zone precursor vesicles: obligatory trafficking of presynaptic cytomatrix proteins Bassoon and Piccolo via a trans-Golgi compartment. *J Biol Chem*. 2006; 281:6038–6047. [PubMed: 16373352]
- Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B, Garner AM, Kaempf U, Kindler S, Gundelfinger ED, Garner CC. Piccolo, a presynaptic zinc finger protein structurally related to bassoon. *Neuron*. 2000; 25:203–214. [PubMed: 10707984]
- Franceschetti S, Sancini G, Panzica F, Radici C, Avanzini G. Postnatal differentiation of firing properties and morphological. *Neuroscience*. 1998; 83:1013–1024. [PubMed: 9502243]
- Gan WB, Grutzendler J, Wong WT, Wong RO, Lichtman JW. Multicolor “DiOlistic” labeling of the nervous system using lipophilic dye combinations. *Neuron*. 2000; 27:219–225. [PubMed: 10985343]
- Geal-Dor M, Freeman S, Li G, Sohmer H. Development of hearing in neonatal rats: air and bone conducted ABR thresholds. *Hear Res*. 1993; 69:236–242. [PubMed: 8226345]
- Germroth PG, Gourdie RG, Thompson RP. Confocal microscopy of thick sections from acrylamide gel embedded embryos. *Microsc Res Tech*. 1995; 30:513–520. [PubMed: 7541260]
- Grutzendler J, Kasthuri N, Gan WB. Long-term dendritic spine stability in the adult cortex. *Nature*. 2002; 420:812–816. [PubMed: 12490949]

- Gurung B, Fritsch B. Time course of embryonic midbrain and thalamic auditory connection development in mice as revealed by carbocyanine dye tracing. *J Comp Neurol*. 2004; 479:309–327. [PubMed: 15457503]
- Harris KM, Kater SB. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci*. 1994; 17:341–371. [PubMed: 8210179]
- Harris KM, Jensen FE, Tsao B. Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci*. 1992; 12:2685–2705. [PubMed: 1613552]
- Hrabovszky E, Turi GF, Kallo I, Liposits Z. Expression of vesicular glutamate transporter-2 in gonadotropin-releasing hormone neurons of the adult male rat. *Endocrinology*. 2004; 145:4018–4021. [PubMed: 15205380]
- Kawaguchi Y, Karube F, Kubota Y. Dendritic branch typing and spine expression patterns in cortical nonpyramidal cells. *Cereb Cortex*. 2006; 16:696–711. [PubMed: 16107588]
- Kubota Y, Hatada S, Kondo S, Karube F, Kawaguchi Y. Neo-cortical inhibitory terminals innervate dendritic spines targeted by thalamocortical afferents. *J Neurosci*. 2007; 27:1139–1150. [PubMed: 17267569]
- Lu J, Cui Y, Cai R, Mao Y, Zhang J, Sun X. Early auditory deprivation alters expression of NMDA receptor subunit NR1 mRNA in the rat auditory cortex. *J Neurosci Res*. 2007; 86:1290–1296. [PubMed: 18041094]
- Marrs GS, Green SH, Dailey ME. Rapid formation and remodeling of postsynaptic densities in developing dendrites. *Nat Neurosci*. 2001; 4:1006–1013. [PubMed: 11574832]
- Metherate R, Aramakis VB. Intrinsic electrophysiology of neurons in thalamorecipient layers of developing rat auditory cortex. *Brain Res Dev Brain Res*. 1999; 115:131–144.
- Miller M. Maturation of rat visual cortex. I. A quantitative study of Golgi-impregnated pyramidal neurons. *J Neurocytol*. 1981; 10:859–878. [PubMed: 6171624]
- Morest DK. The differentiation of cerebral dendrites: a study of the post-migratory neuroblast in the medial nucleus of the trapezoid body. *Z Anat Entwicklungsgesch*. 1969; 128:271–289. [PubMed: 4899900]
- Nahmani M, Erisir A. VGluT2 immunocytochemistry identifies thalamocortical terminals in layer 4 of adult and developing visual cortex. *J Comp Neurol*. 2005; 484:458–473. [PubMed: 15770654]
- Nakahara H, Zhang LI, Merzenich MM. Specialization of primary auditory cortex processing by sound exposure in the “critical period. *Proc Natl Acad Sci U S A*. 2004; 101:7170–7174. [PubMed: 15118079]
- Nicolai B. The postnatal development of the lamina V pyramidal cells in the temporal cortex of the albino rat. *J Hirnforsch*. 1981; 22:351–381. [PubMed: 7310113]
- Norena AJ, Gourevitch B, Aizawa N, Eggermont JJ. Spectrally enhanced acoustic environment disrupts frequency representation in cat auditory cortex. *Nat Neurosci*. 2006; 9:932–939. [PubMed: 16783369]
- Okabe S, Miwa A, Okado H. Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J Neurosci*. 2001; 21:6105–6114. [PubMed: 11487634]
- Oswald AM, Reyes AD. Maturation of intrinsic and synaptic properties of layer 2/3 pyramidal neurons in mouse auditory cortex. *J Neurophysiol*. 2008; 99:2998–3008. [PubMed: 18417631]
- Petit TL, LeBoutillier JC, Gregorio A, Libstug H. The pattern of dendritic development in the cerebral cortex of the rat. *Brain Res*. 1988; 469:209–219. [PubMed: 3401798]
- Puel JL, Uziel A. Correlative development of cochlear action potential sensitivity, latency, and frequency selectivity. *Brain Res*. 1987; 465:179–188. [PubMed: 3440200]
- Purpura DP. Dendritic differentiation in human cerebral cortex: normal and aberrant developmental patterns. *Adv Neurol*. 1975; 12:91–134. [PubMed: 1155280]
- Quinn B, Toga AW, Motamed S, Merlic CA. Fluoro nissl green: a novel fluorescent counterstain for neuroanatomy. *Neurosci Lett*. 1995; 184:169–172. [PubMed: 7715839]
- Regus-Leidig H, Tom Dieck S, Specht D, Meyer L, Brandstatter JH. Early steps in the assembly of photoreceptor ribbon synapses in the mouse retina: the involvement of precursor spheres. *J Comp Neurol*. 2009; 512:814–824. [PubMed: 19067356]

- Sabo SL, Gomes RA, McAllister AK. Formation of pre-synaptic terminals at predefined sites along axons. *J Neurosci.* 2006; 26:10813–10825. [PubMed: 17050720]
- Shapira M, Zhai RG, Dresbach T, Bresler T, Torres VI, Gundelfinger ED, Ziv NE, Garner CC. Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. *Neuron.* 2003; 38:237–252. [PubMed: 12718858]
- Smith PH, Populin LC. Fundamental differences between the thalamocortical recipient layers of the cat auditory and visual cortices. *J Comp Neurol.* 2001; 436:508–519. [PubMed: 11447593]
- Speechley WJ, Hogsden JL, Dringenberg HC. Continuous white noise exposure during and after auditory critical period differentially alters bidirectional thalamocortical plasticity in rat auditory cortex in vivo. *Eur J Neurosci.* 2007; 26:2576–2584. [PubMed: 17970743]
- Stocker SD, Simmons JR, Stornetta RL, Toney GM, Guyenet PG. Water deprivation activates a glutamatergic projection from the hypothalamic paraventricular nucleus to the rostral ventrolateral medulla. *J Comp Neurol.* 2006; 494:673–685. [PubMed: 16374796]
- tom Dieck S, Altmann WD, Kessels MM, Qualmann B, Regus H, Brauner D, Fejtova A, Bracko O, Gundelfinger ED, Brandstätter JH. Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. *J Cell Biol.* 2005; 168:825–836. [PubMed: 15728193]
- Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature.* 2002; 420:788–794. [PubMed: 12490942]
- Wong-Riley MT, Merzenich MM, Leake PA. Changes in endogenous enzymatic reactivity to DAB induced by neuronal inactivity. *Brain Res.* 1978; 141:185–192. [PubMed: 414822]
- Wong-Riley MT, Walsh SM, Leake-Jones PA, Merzenich MM. Maintenance of neuronal activity by electrical stimulation of unilaterally deafened cats demonstrable with cytochrome oxidase technique. *Ann Otol Rhinol Laryngol Suppl.* 1981; 90(2 Pt 3):30–32. [PubMed: 6261663]
- Zha XM, Green SH, Dailey ME. Regulation of hippocampal synapse remodeling by epileptiform activity. *Mol Cell Neurosci.* 2005; 29:494–506. [PubMed: 15953736]
- Zhang LI, Bao S, Merzenich MM. Persistent and specific influences of early acoustic environments on primary auditory cortex. *Nat Neurosci.* 2001; 4:1123–1130. [PubMed: 11687817]

Figure 1.

Confirmation of auditory cortex by cytochrome oxidase reaction and by anterograde and retrograde labeling. **A:** The auditory cortex is defined by elevated cytochrome oxidase staining above the rhinal fissure (arrow) and extending dorsally (arrowhead). **B:** Magnified image of the area between the arrows in A. **C:** Placement of the lipophilic dye, DiI, into the auditory cortex of P21 rats labels efferent and afferent projections, as well as the ventral (v) and dorsal (d) medial geniculate nuclei (MGN). **D:** DiI (magenta) placed in the MGN labels both the ventral (v) and dorsal (d) MGN, afferent and efferent projections from the thalamus, and the auditory cortex. The slice is counter-stained with Syto61 (green), a nuclear stain. **E:** High-magnification confocal projection image of boxed area in D, showing DiI-labeled cortical pyramidal neurons and thalamocortical axons in the auditory cortex. **F:** Higher-magnification image of the layer 5 cortical pyramidal neuron in the boxed area in E. Scale bars = 1.5 mm in A; 700 μ m in B; 600 μ m in C,D; 200 μ m in E; 50 μ m in F.

Figure 2.

DiI-ballistic labeling of pyramidal and nonpyramidal neurons in all auditory cortical layers.

A: VGLUT2 labeling (green) of layers 1 and 4 of auditory cortex along with a NeuroTrace counterstain for neurons (magenta) allows distinction between cortical layers 1, 2/3, 4, 5/6.

B: A layer 2/3 cortical pyramidal neuron showing the typical single apical dendrite and multiple basal dendrites. **C,D:** Images of two spiny nonpyramidal neurons exemplify the morphological diversity of the nonpyramidal population. Scale bars = 300 μm in A; 50 μm in B; 25 μm in C,D.

Figure 3.

Ontogeny of dendritic morphology in rat auditory cortex. Representative images of dendrite segments from DiI-labeled layer 2/3 basal (A–E), layer 2/3 apical (F–J) dendrites, and layer 4 spiny nonpyramidal (H–O) neurons at P4, 9, 16, 19, and 21. Note increase in morphological complexity over time. Scales bar = 5 μ m.

Figure 4. Quantitation of dendritic protrusion density in auditory cortex pyramidal and spiny nonpyramidal neurons. Mean density of dendritic protrusions on layer 2/3 (A), layer 5 (B), and layer 4 (C) auditory cortex pyramidal neurons. Density on apical dendrites is represented by the gray line; density on basal dendrites by the black line. Mean density of dendritic protrusions for layer 4 spiny nonpyramidal neurons is shown in D. Error bars show standard deviation.

Figure 5.

Colocalization of fluorescently labeled dendritic protrusions and presynaptic terminals (bassoon immunoreactive puncta). A typical basal dendrite segment from a P21 layer 5 pyramidal neuron is used here to illustrate how we quantified colocalization of dendritic protrusions (DiI-labeled) and presynaptic terminals (bassoon immunofluorescent puncta). DiI and bassoon images are captured in the same optical section and merged. CM-DiI fluorescence (magenta; **A,D,G**) is shown on the left, bassoon immunofluorescence (green; **B,E,H**) in the center, and a merged image on the right (**C,F,I**). Three serial confocal optical sections, spaced 0.8 μm apart on the Z-axis, are projected together to generate an image with a depth in the Z-axis of 1.6 μm (**J-L**). **M**: Mask generated by expanding the perimeter of the CM-DiI fluorescence by 0.2 μm . **N**: Mask superimposed on the bassoon image. **O**: all bassoon immunofluorescence that does not fall within the boundary of the mask is eliminated facilitating identification of dendritic protrusions occupied or unoccupied by a presynaptic terminal. Scale bar = 5 μm .

Figure 6.

Colocalization of postsynaptic densities and presynaptic terminals in cultured auditory cortex slices. Organotypic auditory cortex slices from P5 rat were cultured for 5–6 days before Diolistic transfection of PSD95-GFP, fixation, and synapsin immunohistochemistry. **A–C:** Projection images of a typical confocal z-stack of seven optical sections spaced 0.8 μm apart in the Z-axis: anti-synapsin immunofluorescence is shown in A; PSD95-GFP fluorescence in B, and the merged synapsin (magenta) and GFP (green) fluorescence in C for the region in the outlined rectangle in B. The individual composite optical sections are shown in **D**. A 2- μm diameter circle was drawn centered on a PSD95-GFP-labeled postsynaptic density (D) and the total green and magenta fluorescence within the circle was plotted as a function of location within the z-stack (**E**). Note colocalization of the synapsin and GFP fluorescence peaks within a z-section. A cumulative histogram showing distance between synapsin and PSD95-GFP puncta in the Z-axis for 58 puncta analyzed is shown in **F**. Scale bars = 5 μm in A–C; 3 μm in D.

Figure 7.

Distance criterion for colocalization of dendritic protrusions and presynaptic terminals in auditory cortex. Inclusion of an increasing number of bassoon-immunoreactive puncta as a result of expanding the inclusion envelope of a fluorescently labeled dendrite is shown for a dendrite image superimposed on its corresponding bassoon image (A) and on a randomly chosen bassoon image (B). The mean percentage of dendritic protrusions with bassoon-immunoreactive puncta within the inclusion envelope is plotted for each of the conditions in C. Error bars show standard deviation. Scale bar = 5 μm in A,B.

Figure 8.

Dendritic protrusions acquire presynaptic partners rapidly during the second postnatal week.

A: Mean percentage of dendritic protrusions colocalizing with presynaptic terminals combined for all dendrite types. ******There is a significant increase between P9 and P16 ($P < 0.001$). *****A small but significant decrease in spine occupancy was observed between P16 and P21 ($P = 0.010$). **B:** Mean percentage of dendritic protrusions colocalizing with presynaptic terminals grouped according to dendrite type: apical and basal dendrites from layer 2/3 and layer 5 pyramidal neurons. *****The increase in occupancy between P9 and P16 for all dendrite categories is significant (Mann–Whitney U -test: layer 2/3 apical, $P < 0.001$; layer 2/3 basal, $P < 0.001$; layer 5 apical, $P < 0.001$; layer 5 basal, $P = 0.003$). Error bars show standard

deviation. **C:** Dot plot of bassoon density/ μm^2 at P9, P16, and P21 indicating a significant increase in bassoon puncta between P9 and P16. Each point represents a single field of view and the solid bar indicates mean bassoon density (n = fields of view (rat)).

Figure 9.

Protrusion length decreases during the second postnatal week. Dendritic protrusion length was quantified on layer 2/3 and 5 pyramidal (P) neurons (A–D,H) as well as layer 4 pyramidal and spiny nonpyramidal (NP) neurons (E–G,I). Protrusion length for pyramidal neuron apical (Api) and basal (Bas) dendrites are plotted separately. Protrusion length was measured at P9, P16, and P21 and plotted as cumulative histograms (A–G) and as mean protrusion length and standard deviation (H–I) for each timepoint. *Statistically significant differences: $P < 0.001$ for all except layer 4 NP for which $P = 0.011$ (Mann–Whitney U -test).

TABLE 1

Antibody Details

Antibody	Immunogen	Dilution	Host	Company
Bassoon	Fusion protein of glutathione-s-transferase and a fragment of rat bassoon (amino acids 756–1,001)	1:400	Mouse	Stressgen #VAM-PS003
Synapsin-1	Purified bovine brain synapsin 1	1:200	Rabbit	Molecular Probes #486200/ Invitrogen #51-5200
VGluT2	A synthetic peptide with the sequence VQESAQDAYSYKDRDDYS	1:200	Guinea pig	Chemicon #AB5907

TABLE 2

Dendritic Protrusion Density and Sample Size for Postnatal Rats

	Layer 5 apical		Layer 5 basal		Layer 2/3 apical		Layer 2/3 basal	
	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)
P4	0.0727 $\pm$ 0.0210	12(3)	0.0771 $\pm$ 0.0201	9(3)	0.0626 $\pm$ 0.0103	12(3)	0.0538 $\pm$ 0.0275	27(3)
P9	0.1516 $\pm$ 0.0987	16(4)	0.1438 $\pm$ 0.0966	22(4)	0.1478 $\pm$ 0.0362	15(3)	0.1348 $\pm$ 0.0455	26(4)
P11	0.1876 $\pm$ 0.0479	13(3)	0.1228 $\pm$ 0.0330	15(2)	0.2031 $\pm$ 0.0448	17(3)	0.2039 $\pm$ 0.0723	26(4)
P14	0.4238 $\pm$ 0.0957	16(4)	0.4526 $\pm$ 0.0652	15(4)	0.6346 $\pm$ 0.1594	11(4)	0.5422 $\pm$ 0.0303	25(4)
P16	0.6983 $\pm$ 0.1061	13(4)	0.6307 $\pm$ 0.0680	16(4)	0.8945 $\pm$ 0.1142	14(3)	0.7526 $\pm$ 0.0738	21(3)
P19	0.9747 $\pm$ 0.1933	12(4)	0.8224 $\pm$ 0.0740	15(3)	1.2909 $\pm$ 0.0932	12(3)	1.0978 $\pm$ 0.1659	19(3)
P21	0.9833 $\pm$ 0.2608	16(4)	0.8752 $\pm$ 0.1085	25(4)	1.2983 $\pm$ 0.1885	15(4)	1.1541 $\pm$ 0.1999	33(4)
P42	1.2036 $\pm$ 0.2066	22(4)	1.0473 $\pm$ 0.1387	29(5)	1.4341 $\pm$ 0.1459	14(3)	1.2178 $\pm$ 0.1421	19(3)

	Layer 4 apical		Layer 4 basal		Layer 4 nonpyramidal	
	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)	Protrusions/ $\mu\text{m} \pm \text{SD}$	Cell (rat)
P4	0.1056 $\pm$ 0.0314	20(4)	0.1080 $\pm$ 0.0439	18(5)	0.1153 $\pm$ 0.0612	13(5)
P9	0.2514 $\pm$ 0.0041	28(3)	0.1710 $\pm$ 0.0432	24(3)	0.1771 $\pm$ 0.0072	25(3)
P11	0.3239 $\pm$ 0.0585	23(3)	0.3658 $\pm$ 0.0698	21(3)	0.2993 $\pm$ 0.0242	12(3)
P14	0.7314 $\pm$ 0.0652	20(3)	0.6348 $\pm$ 0.0404	24(3)	0.5607 $\pm$ 0.0818	17(3)
P16	0.9796 $\pm$ 0.2358	12(3)	0.9222 $\pm$ 0.1517	27(6)	0.6488 $\pm$ 0.2380	13(5)
P19	1.4242 $\pm$ 0.1555	17(3)	1.2066 $\pm$ 0.2298	18(3)	0.9230 $\pm$ 0.1735	12(3)
P21	1.4190 $\pm$ 0.1376	23(7)	1.0958 $\pm$ 0.2101	33(8)	0.7686 $\pm$ 0.3905	32(8)
P24	1.6070 $\pm$ 0.0553	18(4)	1.4627 $\pm$ 0.1033	19(4)	0.6425 $\pm$ 0.3279	4(2)
P42	1.7065 $\pm$ 0.4044	20(2)	1.6774 $\pm$ 0.1140	22(2)	0.5835 $\pm$ 0.3439	12(2)

Density is number of dendritic protrusions/ μm . For each timepoint the mean number of cells for which protrusions were counted is given as well as (in parentheses) the number of individual rats used. Typically, 4–5 cells were analyzed in each rat.

TABLE 3

Spine Occupancy and Sample Size for Postnatal Rats

	P9		P16		P21	
	% spine $\pm$ SD	Dendritic protrusions (rats)	% spine $\pm$ SD	Dendritic protrusions (rats)	% spine $\pm$ SD	Dendritic protrusions (rats)
Apical 5	50 $\pm$ 18%	71 (5)	72 $\pm$ 10%	260 (5)	73 $\pm$ 6%	278 (4)
Basal 5	41 $\pm$ 5%	140 (6)	69 $\pm$ 5%	460 (5)	65 $\pm$ 9%	1253 (6)
Apical 2/3	38 $\pm$ 13%	226 (5)	74 $\pm$ 4%	630 (4)	65 $\pm$ 4%	689 (5)
Basal 2/3	42 $\pm$ 7%	231 (5)	76 $\pm$ 11%	483 (5)	69 $\pm$ 11%	1766 (6)
All spines	41 $\pm$ 23%		74 $\pm$ 11%		69 $\pm$ 11%	

Percent spines is the percentage of dendritic protrusions ($\pm$ standard deviation) identified as spines by colocalization with presynaptic terminals. The number of dendritic protrusions scored and the number of individual rats (in parentheses) used for the determination of percentage is in the adjacent column.

TABLE 4

Average Length of Bassoon-Occupied and -Unoccupied Dendritic Protrusions

	Bassoon occupied	Bassoon unoccupied
Age (<i>n</i> = rat)	$\mu\text{m} \pm \text{SEM}$ (<i>n</i> = protrusions)	$\mu\text{m} \pm \text{SEM}$ (<i>n</i> = protrusions)
P9 (3)	1.62 ± 0.863 (33)	1.58 ± 0.805 (54)
P16 (4)	1.71 ± 0.691 (57)	1.75 ± 0.783 (45)
P21 (5)	1.51 ± 0.743 (78)	1.66 ± 0.737 (43)

Protrusion occupancy is identified as colocalization with bassoon-labeled presynaptic terminals. The number of dendritic protrusions scored is in parentheses; next the mean and SEM. The number of individual rats (in parentheses) is located next to the age of the animal. This analysis was performed on the basal dendrites of layer 5 pyramidal neurons.