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Maturation of Intrinsic and Synaptic Properties of Layer 2/3 Pyramidal Neurons in Mouse Auditory Cortex

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Abstract

We investigated the development of L2/3 pyramidal cell (PC) circuitry in juvenile mice from postnatal day 10 (P10) to P29. Using whole cell recordings in an in vitro thalamocortical slice preparation, we examined the connection architecture and intrinsic and synaptic properties of PCs. The excitatory connections between PCs were highly localized: the probability of connection between PCs declined with intersomatic distance from 0.18 to about 0.05 over 150 μm , but did not vary with age. However, the mean and variance of the intrinsic and synaptic properties of PCs changed dramatically between P10 and P29. The input resistance, membrane time constant, and resting membrane potential decreased, leading to reduced neural excitability in older animals. Likewise, there were age-dependent decreases in the amplitude and decay time of the excitatory postsynaptic potentials as well as short-term synaptic depression. Both the intrinsic and synaptic properties underwent a transitional period between P10 and P18 prior to reaching steady state at P19–P29. We show that these properties combine to produce age-related differential synaptic responses to low- and high-frequency synaptic input that may contribute to differences in auditory processing during development.

INTRODUCTION

Neurons in the auditory pathway exhibit several prominent changes during development. In rodents, hearing onset occurs in the second week of life between postnatal day 10 (P10) and P12 (Ehret and Romand 1976). Auditory experience, particularly between P10 and P14, can dramatically influence subcortical and cortical development and lead to persistent changes in information processing over the lifetime of the animal (Chang et al. 2003; de Villers-Sidani et al. 2007; Seidl and Grothe 2005; Zhang et al. 2001). However, throughout the auditory pathway, the maturation of cellular and network properties extends beyond this critical period of enhanced plasticity. In brain stem and midbrain auditory nuclei, the subthreshold and suprathreshold responses change dramatically between P14 and P29 (Ehret and Romand 1992; Ene et al. 2003; Joshi and Wang 2002; Romand and Ehret 1990; Sanes and Constantine-Paton 1985a,b; Song 2006; Sprengle et al. 2001; Wu and Oertel 1987; Yu et al. 2007). Similarly, the intrinsic membrane properties of neurons in the medial geniculate nucleus of the thalamus are mature by P14 (Tennigkeit et al. 1998), but glutamate receptor subunit composition changes significantly from P7 through P25 (Hsieh et al. 2002).

The development of the primary auditory cortex (AI) follows a timeline similar to that of the subcortical auditory areas. The tonotopic map of rats is considered mature between P14 and P22 (Chang et al. 2003; de Villers-Sidani et al. 2007; Zhang et al. 2001). However, between

P20 and P35 the bandwidths of excitatory receptive fields continue to decrease and the ability of neurons to follow repetitive stimuli is enhanced (Chang et al. 2003, 2005). At the cellular level, the input resistance and membrane time constant have been shown to decrease with age between P8 and P21 (Metherate and Aramakis 1999). In addition, age-dependent changes in synaptic properties have been shown using in situ hybridization and extracellular stimulation (Aramakis et al. 2000; Hsieh et al. 2002). However, these studies (Aramakis et al. 2000; Hsieh et al. 2002; Metherate and Aramakis 1999) pooled results from different neural types and across layer 2/3 (L2/3) and L4. Consequently, the identities, locations, and contributions of specific neurons and network interactions are unknown. Moreover, it is unclear whether these properties have reached steady-state values by P21.

In the present study, we expand on previous results by investigating the specific circuit architecture and developmental timelines of the intrinsic and synaptic properties of L2/3 pyramidal cells (PCs). These neurons integrate information from the middle layers (L3–L5), from other L2/3 PCs within and across cortical columns, and from the contralateral hemisphere (Code and Winer 1985; Linden and Schreiner 2003; Ojima et al. 1991; Winer 1984, 1985). Recurrent connections among L2/3 PCs consist of a substantial proportion of the excitatory input to L2/3 PCs and may enhance the integration of stimulus features and broaden subthreshold receptive fields (Kaur et al. 2004; Ojima and Murakami 2002). In addition, the differential expression of short-term synaptic depression or facilitation at L2/3 PC synapses has been postulated to contribute to stimulus-selective responses in L2/3 neurons (Atzori et al. 2001; Barbour and Wang 2003; Oswald et al. 2006; Wang et al. 2005). The influence of recurrent PC–PC circuits on auditory processing likely depends on the spatial extent of the connections; the intrinsic excitability of neurons; and the strength, duration, and short-term plasticity of the synaptic responses. Moreover, if these properties vary with age, then L2/3 PC circuits in young and older animals likely use different strategies for processing acoustic stimuli.

We performed simultaneous whole cell recordings from up to four PCs in an in vitro slice preparation of AI from juvenile mice (P10–P29). We show that there were dramatic changes in the intrinsic properties of the neurons between P10 and P29 that resulted in an overall reduction in neural excitability. The probability of connection between PCs depended on the distance between somas but did not vary with age. Neither the strength nor the latency of synaptic responses depended on intersomatic distance. However, the amplitude, decay time, and short-term synaptic depression of excitatory postsynaptic potentials (EPSPs) decreased between P10 and P29. Furthermore, we show that PC–PC connections, regardless of age, constitute a single population of depressing synapses. These results are contrary to previous findings that suggest there are two populations of PC–PC connections that express differential short-term synaptic plasticity (Atzori et al. 2001). Finally, we show that the decay time of the EPSP and short-term synaptic plasticity combine to produce age-related differential synaptic responses to low- and high-frequency stimulation. We speculate that these changes in the intrinsic and synaptic properties of PCs contribute to the age-dependent firing properties of AI neurons in response to acoustic stimuli.

METHODS

Slice preparation

Auditory thalamocortical slices were prepared from Swiss Webster mice (P10–P29) as described in Cruikshank et al. (2002). All surgical procedures followed the guidelines determined by the New York University Animal Welfare Committee. Mice were anesthetized with halothane and decapitated. The brain was exposed and two coronal cuts were made to remove the anterior 25% of the brain and the cerebellum. The brain was then removed from the skull and immersed, anterior cut down, in ice-cold oxygenated (95% O₂–

5% CO₂) artificial cerebrospinal fluid (ACSF, in mM: 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1.0 MgCl₂, 25 dextrose, and 2 CaCl₂). A third cut was made with an approximately 15° medial-to-lateral slant to remove the dorsal portion of the brain, leaving one hemisphere mostly intact. The brain was then glued, dorsal surface down, onto the vibraslice stage such that the intact hemisphere faced the blade. The brain was quickly submerged in ice-cold ACSF and horizontal slices (300 µm) were made. One or two slices were obtained that contained primary auditory cortex (AI), the medial geniculate nucleus (MG), and the thalamocortical fiber tract. The slices were maintained in ACSF at 37°C for 30 min then allowed to sit at room temperature (20–22°C) prior to recording (minimum 2 h). Recording temperature ranged from 29 to 34°C.

Electrophysiology

Neurons were visualized using infrared-differential interference contrast (IR-DIC) microscopy (Olympus). At low magnification the MG was located medial to the hippocampus and posterior to the lateral geniculate and AI was located lateral to the anterior half of the hippocampus (see Cruikshank et al. 2002). Only slices containing a significant portion of the MG and AI were used. Stimulating the MG and recording responses in L3/4 of auditory cortex verified the location of AI. Layer 2/3 is the cell-dense region 100–300 µm below the pia. At higher magnification, pyramidal cells were identified by a distinct apical dendrite that extended toward the pial surface. Whole cell current-clamp recordings were made simultaneously from up to four neurons (Amplifier; Dagan, Minneapolis, MN). Pipettes were pulled from borosilicate glass (2.0 OD) on a Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA) to a resistance of 3–10 MΩ. The intracellular solution consisted of (in mM) 130 K-gluconate, 5 KCl, 2 MgCl₂, 4 ATP-Mg, 0.3 GTP, 10 HEPES, and 10 phosphocreatine (all chemicals from Sigma, St. Louis, MO).

Stimulation

A series of hyperpolarizing and depolarizing step currents were injected to measure the input resistance, time constant, and action potential (AP) responses of each cell. To determine whether pairs of neurons were connected, one neuron was stimulated with five suprathreshold current pulses (0.5–1 nA, 5-ms pulse width) delivered at 10 Hz. In synaptically connected cells distinct EPSPs could be evoked in the target cell. Average membrane responses were compiled from 25 to 50 trials. The intertrial interval was sufficiently long (3–5 s) to ensure full recovery from short-term plasticity.

Biocytin fills

To verify cell identity, 0.5% biocytin was added to the intracellular solution. Following recording, slices were fixed in 4% paraformaldehyde. The slices were rinsed with phosphate-buffered saline (PBS), quenched with 1% H₂O₂ in a 10% MeOH-PBS solution, permeabilized in Triton-X-100, and then exposed to an avidin-peroxidase complex (ABC kit, Vector Laboratories, Burlingame; CA). The slices were rinsed (with PBS) and reacted with 3,3-diaminobenzidine, then rinsed again. Finally, slices were mounted onto slides with Fluoromount for microscopy.

Analysis and statistics

Presynaptic stimulation successfully evoked an EPSP if 1) the onset of EPSP was within 4 ms of the presynaptic AP initiation; 2) the EPSP amplitude (baseline to peak) was a minimum of 0.10 mV; and 3) the EPSP peaked within 10–20 ms of onset. Otherwise, the trial was considered a failure with an “amplitude” of 0 mV. The failure rate was determined as the number of failed trials divided by the total number of trials. The reported EPSP amplitudes for any given pair and all traces shown are the average over all trials including

failures. The reported failure rates, EPSP amplitudes, latencies, and both rise and decay time constants are for the first EPSP in the train.

The time constants for the rise (τ_r) and decay (τ_d) of the EPSP were obtained by fitting the normalized (peak – baseline = 1) average EPSP, excluding failures, by a difference of two exponentials

$$f(t; A, \tau_1, \tau_2) = A(e^{t/\tau_d} - e^{t/\tau_r}) \quad (1)$$

Only EPSPs that were well fit by Eq. 1 ($\chi^2 < 1.75$, $n = 119$, average χ^2 : 0.72 ± 0.57) were chosen for analysis.

All statistics are reported as mean $\pm$ SD and significance was assessed using a two-tailed, unpaired Student's *t*-test, unless otherwise stated.

RESULTS

Whole cell current-clamp recordings were made from 703 pyramidal cells (PCs) in L2/3 of AI. Under IR-DIC, PCs had a distinct apical dendrite that extended to L1. Several neurons were filled with biocytin to confirm their identity (data not shown). We measured the intrinsic membrane and synaptic properties and quantified the connection probability of L2/3 PCs from mice ranging in age from P10 to P29. Both the intrinsic and synaptic properties undergo a transition between P10 and P18 before reaching steady-state values at P19–P29. The transitional period was divided arbitrarily into an early phase (P10–P14), which corresponds to the critical period in AI of rats (de Villers-Sidani et al. 2007), and a later phase (P15–P18). Unless otherwise stated, all statistical comparisons are between the three epochs: P10–P14, P15–P18, and P19–P29.

Age-related changes in the intrinsic properties of pyramidal neurons

The intrinsic membrane properties changed dramatically between P10 and P18, but did not significantly differ between P19 and P29 (Fig. 1, A–C). The resting membrane potential (V_m), input resistance (R_n), and membrane time constant (τ_m) were maximal at P10–P14 (V_m : -70 ± 6 mV; R_n : 306 ± 76 M Ω ; τ_m : 38 ± 9 ms) and decreased significantly to reach steady-state values at P19–P29 (V_m : -81 ± 5 mV; R_n : 139 ± 34 M Ω ; τ_m : 15 ± 5 ms, $P < 0.01$; Fig. 1, A–C, Table 1). This was accompanied by a reduction in the variability (SD) of these properties during the P19–P29 epoch (Fig. 1, A–C, Table 1).

Although the AP threshold (-37 ± 5 mV, membrane potential at peak of the second derivative) was constant (Table 1), the AP properties changed with age. The AP width decreased significantly between P10–P14 (1.9 ± 0.5 ms) and P19–P29 (1.2 ± 0.3 ms, $P < 0.01$, Table 1, Fig. 1D). Likewise, the afterhyperpolarization (AHP), measured as the difference in membrane potential from AP threshold to the minimum of the AHP, decreased between P10–P14 (16 ± 3 mV) and P19–P29 (12 ± 3 mV, $P < 0.01$, Table 1). Finally, the AP height (measured from threshold) did not change significantly between P10–P14 (51 ± 9 mV), P15–P18 (48 ± 9 mV), and P19–P29 (54 ± 10 mV, Table 1).

The plots of the average AP frequency (Hz $\pm$ SE) versus input current (*F/I* curve) also changed between P10 and P29 (Fig. 1E). The rheobase current required to evoke APs increased from 0.07 ± 0.06 nA (P10–P14) to 0.26 ± 0.09 nA (P19–P29, Table 1). PCs from the youngest animals (P10–P11) had the highest firing rates at low input currents, but also had the lowest maximal firing rates (~ 35 Hz) and reached depolarization block at 0.4 – 0.5 nA (Fig. 1E). The range of firing rates increased between P12 and P14 and was significantly

broader by P15–P18. In addition, there was a significant rightward shift in the F/I curve (measured by comparing firing rates between P10–P11 and P15–P18 at 0.1 and 0.2 nA, Fig. 1E, $**P < 0.01$). At P19–P29, the F/I curves shifted further to the right, given that firing rates at both 0.2 and 0.6 nA were significantly less than those at P15–P18 (Fig. 1E, $**P < 0.01$). These results suggest neural excitability decreases with age between P10 and P29. Since the AP threshold does not vary with age (Table 1), these changes in excitability likely reflect the decreases in V_m and R_n during development.

Patterns of connectivity

To determine the connectivity patterns between PCs, simultaneous whole recordings were made from up to four neurons and the connections between each cell were tested sequentially (see METHODS). We recorded from 703 PCs, tested a total of 1,381 putative PC–PC connections, and found 152 connected pairs. The probability of connection (P_C) was calculated by dividing the number of confirmed connections (N_C) by the total number of connections tested (N_T). The P_C over the pooled data set was 0.11 ± 0.01 (proportion of connected pairs $\pm [P_C(1 - P_C)]/N_T^{1/2}$) and did not vary with age (P10–P14: 0.11 ± 0.02 ; P15–P18: 0.11 ± 0.03 ; and P19–P29: 0.13 ± 0.02). The probability of finding reciprocal connections (RC) between pyramidal cells was low (nine pairs, P_{RC} : 0.014).

The P_C varied with the distance (μm) between the neural somata (Fig. 2A). Distances between pairs were binned in 20- μm increments from 0 to 140 μm and the P_C was calculated for each bin. The P_C declined from 0.18 at ≤ 20 μm to about 0.05 at 140 μm . The spatial connectivity pattern was obtained by plotting the (x, y) coordinates of the postsynaptic cell centered on the presynaptic neuron for both connected (black circles) and unconnected pairs (+) (Fig. 2B). The connection probability was generally symmetrical around the presynaptic cell. Longer connection distances were found in the horizontal direction (± 150 μm), although this likely reflects sampling bias: vertical distances were bounded by L1 and the cell-sparse L4 region (± 100 μm).

Characterization of EPSPs

The developmental timeline of the synaptic properties was similar to that of the intrinsic properties. The synaptic properties in the transitional phase, P10–P18, significantly differed from those at the steady state, P19–P29. A summary of the age-dependent synaptic properties is presented in Table 2.

The EPSP amplitudes ranged from 0.1 to 2.6 mV and there was a significant decrease in EPSP amplitude between P10–P14 (1.0 ± 0.6 mV) and P19–P29 (0.6 ± 0.4 mV, $P < 0.01$, Fig. 3A). The EPSP amplitudes did not vary with the intersomatic distance (R : -0.04 , Fig. 3B). The coefficient of variation ($CV = SD/\text{mean}$) of EPSP amplitudes increased from 0.49 ± 0.24 (P10–P14) to 0.64 ± 0.29 (P19–P29, $P < 0.05$, Fig. 3C) and decreased with increasing synaptic strength (Fig. 3D).

The latency, defined as the time difference between the onset of the EPSP and the onset of the presynaptic AP (Fig. 4A), was 1.6 ± 0.6 ms, (Fig. 4B) and did not vary with intersomatic distance (R : 0.11, Fig. 4C) or age. The average time-to-peak of the EPSP, taken as the difference between the maximum of the EPSP and its onset, was 9.5 ± 3.2 ms (Fig. 4D) and also did not vary with age.

To obtain the time constants for the rise (τ_r) and decay (τ_d) of the EPSP, we fit the average EPSP with a difference of two exponentials (gray dashed line, Fig. 4A; see METHODS). Although τ_r did not change with age (2.9 ± 1.9 ms, Fig. 4E), the decay of the EPSP (τ_d) decreased significantly between P10–P14 (44 ± 15 ms) and P19–P29 (23 ± 9 ms, $P < 0.01$) (Fig. 4, F and G). τ_d was highly correlated with V_m , τ_m , and R_n (R : 0.92, 0.83, and 0.93, respectively,

Fig. 4H). In a subset of PC pairs we performed voltage-clamp recordings to investigate whether age-dependent changes in V_m resulted in differential *N*-methyl-D-aspartic acid (NMDA) receptor activation and thus differences in τ_d . We found that NMDA currents were recruited at much higher holding potentials (-30 to -40 mV) than the resting membrane potential (-60 to -80 mV, Fig. 4G). The decays of the synaptic currents recorded at -60 and -80 mV were fit with a single exponential. The time constants did not differ significantly between holding potentials of -60 (6 ± 2 ms) and -80 mV (5 ± 2 ms, $n = 6$), suggesting that NMDA currents do not substantially affect τ_d at these resting potentials. Thus the age-related changes in synaptic decay shown in Fig. 4, *F* and *G* are primarily attributed to associated changes in the intrinsic properties rather than differential NMDA-receptor activation.

Short-term synaptic plasticity

The majority of synapses exhibited frequency-dependent short-term synaptic depression. Low stimulation frequencies (10 and 20 Hz) resulted in weak depression of EPSPs (Fig. 5, *A* and *E*), but 40- and 80-Hz stimulation yielded significantly stronger depression (Fig. 5*E*, *inset*: $**P < 0.1$, $*P < 0.05$). At all ages and for all stimulation frequencies the average paired-pulse ratios [PPR = (amplitude of EPSP₂)/(amplitude of EPSP₁)] were < 1 and only a small number of connections ($n = 11$) showed paired-pulse facilitation (PPR: 1.1–1.7, Fig. 5*B*). The average PPR (10 Hz) increased significantly between P10–P14 (0.68 ± 0.18) and P15–P18 (0.83 ± 0.21 , $P < 0.01$) and again by P19–P29 (0.95 ± 0.20 , $P < 0.01$, Fig. 5, *B* and *C*, Table 2).

The age-related differences in short-term synaptic depression depended on both stimulation frequency and the number of stimulus pulses. In response to 20- or 40-Hz stimulus trains, the PPR of the first two pulses was significantly lower in P10–P14 than that in P15–P18 animals, but did not differ between P15–P18 and P19–P20 animals (Fig. 5*C*). However, synapses from the oldest age group (P19–P29) were significantly less depressed by the fifth EPSP compared with younger groups (10–40 Hz, Fig. 5*D*). Higher stimulation frequencies (i.e., 80 Hz) produced comparable PPR and depression of the fifth pulse in both P15–P18 and P19–P29 (Fig. 5, *C* and *D*).

Relationship between synaptic reliability and short-term synaptic plasticity

We assessed synaptic reliability by evaluating the failure rate (number of trials that failed to evoke an EPSP divided by the total number of trials; see METHODS) of each connection. The distribution of failure rates was bimodal (Fig. 6*A*); more than half of the connections ($n = 85$) had failure rates < 0.10 and the remaining pairs ($n = 67$) had failure rates that ranged from about 0.20 to 0.80. The median and mean failure rates were significantly greater between P19–P29 versus P10–P14 and P15–P18 (medians: see Fig. 6*A*; means: P10–P14: 0.14 ± 0.21 ; P15–P18: 0.16 ± 0.19 ; P19–P29: 0.25 ± 0.23 , $P < 0.05$, Mann–Whitney test; Fig. 6*B*). Synapses with low failure rates tended to have higher-amplitude EPSPs than those with high failure rates (Fig. 6*C*). This was true when failures are included (shown) or excluded (not shown) from the average amplitude.

A previous report suggested that PC–PC connections in L2/3 of AI can be divided into two populations based on failure rates, amplitude, and degree of short-term synaptic depression (Atzori et al. 2001). According to this study, low-probability connections (LPCs) have high failure rates, smaller EPSP amplitudes, and are nondepressing (PPR ≥ 1), whereas high-probability connections (HPCs) have low failure rates, larger EPSP amplitudes, and tend to depress (PPR < 1). We tested whether our data could be statistically grouped into distinct LPC and HPC populations. We restricted our analyses to animals aged P19 to P29 when the synaptic properties were stable. We divided the connections into two groups: low failure

(LF) connections that failed in <0.2 of trials and high failure (HF) connections with rates >0.2 . The EPSP amplitudes of LF connections were significantly greater (0.77 ± 0.41 mV) than those of HF connections (0.18 ± 0.10 mV, $P < 0.01$). However, the PPRs (10 and 20 Hz) did not significantly differ between the two groups (Table 3). Contrary to what has been described previously, connections, regardless of age, could not be grouped into distinct populations based on the correlations between PPR and failure rate (Fig. 6D).

In a subset of pairs ($n = 20$) we recorded synaptic responses to paired-pulse stimuli (10 and 20 Hz) in both current clamp (CC) and voltage clamp (VC) (Fig. 6E). The PPRs recorded in CC were highly correlated with those obtained in VC in the same pairs ($R: 0.72$, slope 0.85 ± 0.14 ; Fig. 6F). The PPRs measured with the two techniques were not significantly different [$P = 0.40$ (10 Hz), 0.86 (20 Hz), paired t -test].

Age-dependent synaptic integration

The temporal properties of the synapse shape the transfer of information between neurons. Long EPSP decay times promote the summation of EPSPs during repetitive stimulation and effectively lengthen the synaptic integration time window. On the other hand, short-term depression reduces the magnitude of the summed current. We investigated how EPSP decay times and depression combine to influence the level of sustained excitation at the synapse in response to physiological firing frequencies (Fig. 1) for the P10–P14, P15–P18, and P19–P29 age groups. Figure 7A shows the postsynaptic response to 20-Hz (*top*) and 40-Hz (*bottom*) stimulation of the presynaptic cell at three ages (individual pairs, black lines): P11 (*left*), P17 (*middle*), and P24 (*right*). The mean responses for P10–P14, P15–P18, and P19–P29 (gray lines) are also shown. To compare across connections with differing initial EPSP amplitudes, all traces were normalized such that the amplitude of the first EPSP is equal to 1 (dashed horizontal lines). EPSP summation was then quantified by integration of the mean response for each pair. The youngest age group (P10–P14) had the longest EPSP decays (Fig. 4) but also the strongest depression (Fig. 5). This resulted in a strong transient response at the onset of stimulation with minimal summation that quickly decayed. Alternatively, the oldest age group (P19–P29) had the shortest EPSP decays and minimal depression. The reduction in depression resulted in peak synaptic responses that were consistent across repeated stimuli at both 20 and 40 Hz. However, the decrease in the EPSP decay limited summation at stimulation frequencies <40 Hz. In contrast, EPSP summation is significantly greater in the P15–P18 group (gray bars) than that in both older (P19–P29: black bars, 20 and 40 Hz, $P < 0.01$) and younger groups (P10–P14: white bars, 40 Hz, $P < 0.05$, Fig. 7B). This is attributable to the combination of moderate age-related decreases in both synaptic decay and depression between P15 and P18.

DISCUSSION

The role of L2/3 PC–PC circuits in auditory processing depends on the spatial extent of the connections; the intrinsic membrane properties; and the strength, duration, and short-term plasticity of the synaptic responses. Here we have characterized the probability of connection in local PC–PC circuits and the maturation of the intrinsic and synaptic properties of L2/3 pyramidal neurons.

Development of the intrinsic and synaptic responses in pyramidal neurons

Our results show that the intrinsic and synaptic properties of L2/3 pyramidal cells are in transition from hearing onset (P10/P11) through P18 and reach steady-state values between P19 and 29. This results in dramatic changes in the responses of PCs to both step-current injections and synaptic input between P10 and P29.

At P10–P14 the input resistance (R_n), membrane time constant (τ_m), resting membrane potential (V_m), action potential (AP) width, and afterhyperpolarization (AHP) of L2/3 PCs were maximal. These factors likely contribute to the high excitability of these neurons in response to low-input currents. Between P10 and P18 the R_n , τ_m , and V_m , as well as the AP width and AHP, significantly decreased. The mean and variance of these properties did not differ between P19 and P29, suggestive of a steady state. There was also an overall decrease in neural excitability and a rightward shift in the F/I curves of neurons aged P19–P29 compared with younger neurons.

Comparable age-related changes in the subthreshold intrinsic properties and AP shapes have been reported for neurons in AI (Kotak et al. 2007; Metherate and Aramakis 1999) and other cortical areas (Frick et al. 2007; Kasper et al. 1994; Maravall et al. 2004; McCormick and Prince 1987; Zhang 2004). These changes might, in part, be due to differential recruitment of potassium (K^+) conductance during development. Age-dependent changes in K^+ currents have been reported in the cochlea and auditory brain stem (Kanjhan et al. 2004; Nakamura and Takahashi 2007) as well as in cortical pyramidal cells (Kang et al. 1996) and interneurons (Du et al. 1996; Tansey et al. 2002).

At the synapses between PCs, the reliability, amplitude, and paired-pulse depression of the EPSPs decreased significantly between P10–P14 and P19–P29. Comparable developmental changes in presynaptic release probabilities, EPSP amplitudes, and short-term synaptic plasticity have been reported in somatosensory (Frick et al. 2007; Reyes and Sakmann 1999; Yanagisawa et al. 2004) and visual (Ramoia and Sur 1996) cortices, as well as in the striatum (Choi and Lovinger 1997; Ou et al. 1997), cerebellum (Pouzat and Hestrin 1997), and hippocampus (Muller et al. 1989). These differences might be due to differential regulation of presynaptic calcium levels (Ali and Nelson 2006; Rozov et al. 2001) or developmental changes in presynaptic metabotropic glutamate receptors (Chen and Roper 2004).

The decay of the EPSP (τ_d) also decreased significantly between P10–P14 and P19–P29. τ_d was correlated with the resting potential, membrane time constant, and input resistance of the postsynaptic cell, suggesting that associated changes in the intrinsic properties of PCs contribute to the synaptic decay. It is also possible that the age-dependent transition from NR2B (slow kinetics) to NR2A (faster kinetics) subunits in NMDA receptors (Cull-Candy et al. 2001; Hsieh et al. 2002) contribute to a reduction in synaptic decay constants with age. However, the low resting membrane potentials and the concentration of magnesium (Mg^{2+}) in the recording solutions limit the influence of NMDA-receptor activation on τ_d in the present study.

These results are consistent with the timeline for developmental changes throughout the auditory pathway. In subcortical auditory areas, neural responses to acoustic stimuli occur as early as P10 and the response thresholds, latencies, amplitudes, and tuning properties mature between P14 and P35 (Ehret and Romand 1992; Romand and Ehret 1990; Sanes and Constantine-Paton 1985a,b; Song 2006; Sprenkle et al. 2001; Wu and Oertel 1987; Yu et al. 2007). In AI, adultlike tonotopic maps arise between P14 and P22 (Chang et al. 2003; de Villiers-Sidani et al. 2007; Zhang et al. 2001), whereas the excitatory receptive fields and the ability of neurons to follow repetitive stimuli mature by P35 (Chang et al. 2003, 2005). In many of these studies, the proper development of neural responses was dependent on acoustic stimulation. Likewise, the transition from high excitability to low excitability and changes in the intrinsic and synaptic properties of L2/3 PCs shown here require acoustic stimulation. In animals that experience moderate to severe hearing loss from P10, the intrinsic and synaptic properties of L2/3 PCs recorded between P18 and P23 resembled those of younger neurons (Kotak et al. 2005; Xu et al. 2007).

Comparison with previous results in AI and other cortical areas

Our results are consistent with those reported in somatosensory, visual, and motor cortices where L2/3 PC–PC connections have amplitudes on the order of 1 mV, low failure rates, and exhibit short-term synaptic depression (Feldmeyer et al. 2006; Reyes and Sakmann 1999; Thomson et al. 2002). In AI, EPSP amplitudes ranged from 0.1 to 2.6 mV; the average failure rate of PC–PC connections was low (~ 0.20), with the majority of synapses having failure rates < 0.5 ; and 91% of synapses showed paired-pulse depression at all stimulation frequencies.

Our results are in contrast to a previous study of synaptic responses in L2/3 PC pairs in AI, which reported an average failure rate of 0.87, with only 36% of connections exhibiting short-term synaptic depression (Atzori et al. 2001). PC–PC connections were previously classified in two distinct populations based on failure rates, EPSC amplitudes, and short-term plasticity. We performed a similar analysis but were unable to classify PC–PC connections into two populations based on these parameters.

This discrepancy does not appear to arise from differences in experimental procedures. The slice angle might introduce biases due to planar or regional differences in connectivity. However, the probability of connection in parasagittal slices (neocortex; Holmgren et al. 2003) and horizontal slices (AI; present study) did not differ from coronal slices (Atzori et al. 2001; Holmgren et al. 2003). Our recordings were performed under current clamp, whereas those of Atzori et al. were under voltage clamp. However, we found no differences in the PPRs measured with either technique. Finally, our recordings were conducted at 29–34°C, whereas those of Atzori et al. (2001) were at 22°C. Low recording temperatures substantially reduce the probability of transmitter release (P_r) at cortical synapses (Hardingham and Larkman 1998; Volgushev et al. 2004), increase the proportion of weak, unreliable synapses, and thus might reduce the expression of short-term depression (Zucker 1989; Zucker and Regehr 2002). However, this does not explain the differences between AI and somatosensory cortex presented in Atzori et al. (2001). In both studies there is a pool of weak, unreliable synapses and one of strong, highly reliable synapses. It remains to be determined whether these function as distinct populations.

Contributions of L2/3 PC–PC connections in AI cortical networks

Generally, the probability of connection (P_C) between L2/3 PCs is between 0.10 and 0.20 (Atzori et al. 2001; Feldmeyer et al. 2006; Holmgren et al. 2003; Mason et al. 1991; Thomson et al. 2002; Yoshimura et al. 2005b). In AI, the axonal arborizations of L2/3 PCs suggest two pools of connections: highly localized ($< 200 \mu\text{m}$) and long-range ($> 500 \mu\text{m}$) connections (Ojima et al. 1991). We show that the probability of PC–PC connection is highest (0.18) among nearby neurons ($< 20 \mu\text{m}$ apart) and declines to < 0.05 with distances $> 80 \mu\text{m}$. These are most likely local, intracolumnar interactions among PCs. Although connection probabilities may be subject to undersampling and false negatives due to axonal truncation, the probability of excitatory connection between L2/3 PCs and fast-spiking interneurons that are $< 100 \mu\text{m}$ apart is high (0.5–0.7) and does not decline (Holmgren et al. 2003; Oswald and Reyes, data not shown). This suggests that the low P_C between PCs is not due to axon truncation.

In other cortical areas, local PC–PC connectivity within L2/3 has been correlated with interlaminar network interactions (Kampa et al. 2006; Yoshimura et al. 2005a,b). For instance, connected L2/3 PC–PC pairs are more likely to receive common input from L4 (Yoshimura et al. 2005b), whereas L5 neurons are more likely to receive inputs from unconnected L2/3 neurons (Kampa et al. 2006). It is interesting to note that connected PC–PC pairs in these previous studies were generally separated by $< 35 \mu\text{m}$, which coincides

with the region of highest connection probability in this study. If similar interlaminar connectivity patterns exist in AI, then the high probability of connection among nearby PCs could locally amplify feedforward inputs from L4 (Douglas and Martin 2007; Douglas et al. 1995; Liu et al. 2007).

Implications for auditory processing during development

The age-related changes in the intrinsic and synaptic properties of AI PCs might contribute to developmental differences in neural responses to acoustic stimuli recorded in vivo. Low intrinsic firing rates may, in part, underlie the minimal responsiveness of cortical neurons to acoustic stimuli early in the critical period (P10–P11; de Villers-Sidani et al. 2007). In addition, the combination of longer synaptic decays and strong synaptic depression could reduce the ability of young neurons to follow repetitive stimuli (Chang et al. 2005). Between P15 and P18 the moderate decreases in the EPSP decay constant and synaptic depression enhance the summation of excitatory inputs both within a single connection and across pairs. The combination of a broader range of firing rates and synaptic summation may boost interactions among L2/3 neurons over a variety of inputs and contribute to the broadly tuned responses recorded in vivo (Chang et al. 2003; Zhang et al. 2001). In older mice (P19–P29), PCs have shorter synaptic decay constants and decreased short-term depression, which might enhance temporal fidelity and the ability to follow repetitive low-frequency stimuli (Bao et al. 2004; Chang et al. 2005; Saeb et al. 2007). In addition, since EPSP summation is limited to higher stimulus frequencies, these synapses can be selective for stimuli that produce high firing rates. This, combined with the reduced intrinsic excitability of older neurons, might help narrow tuning curves in older animals (Chang et al. 2003, 2005). Our results provide insight into the development of L2/3 PC circuitry in AI. Further studies are required to determine the exact role of these circuits in auditory processing throughout the lifetime of the animal.

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FIG. 1.

Development of the intrinsic properties of pyramidal neurons. Plots of membrane time constant (A), input resistance (B), and the resting membrane potential (C) vs. postnatal day age from P10 to P29 (filled circles: mean $\pm$ SD). D, left: action potential (AP) responses to rheobase current injections for pyramidal cells (PCs) from animals age P10 (0.05 nA, top), P16 (0.1 nA, middle), and P20 (0.2 nA, bottom). Scale: vertical: 40 mV; horizontal: 200 ms. Right: the 1st AP at P10 (gray) and P20 (black) were aligned at AP threshold to show the decrease in AP width with age. Scale: vertical: 20 mV; horizontal: 10 ms. E: the mean $\pm$ SE firing frequency vs. input current (F/I) curves for animals P10–P11 (open circles), P15–P18 (gray circles), and P19–P29 (black circles). Most neurons in the P10–P11 age group reached

depolarization block by 0.5 nA. There was an increase in the range of firing rates in P15–P18 and P19–P29 neurons. Finally, there was a significant rightward shift in the F/I curves and decrease in excitability with age as indicated by the reduction in firing rates with age at 0.1 and 0.2 nA (P10–P11 vs. P15–P18, $**P < 0.01$) and at 0.2 and 0.6 nA (P15–P18 vs. P19–P29, $**P < 0.01$).

FIG. 2.

Distance-dependent changes in connection probability. *A*: probability of connection (P_C) for PC–PC pairs plotted against the distance between somas (20-μm bin width). *B*: the (x , y) coordinates of the postsynaptic cell with respect to the presynaptic neuron located at (0, 0) for all tested (+) and connected pairs (filled circles). *Inset*: schematic of the slice orientation with L1 at the top of the field of view; the x - and y -axes correspond to the vertical distance and horizontal distances within L2/3.

FIG. 3.

Strength of PC–PC connections. *A*: excitatory postsynaptic potential (EPSP) amplitude vs. age for individual pairs at P10–P14 (open circles), P15–P18 (gray circles), P19–P29 (black circles), and the average EPSP amplitude $\pm$ SD for each age group (open squares). There was a significant decrease in EPSP amplitude between P10–P14 and P19–P29 (** $P < 0.01$). *B*: EPSP amplitude vs. distance between somata (μm). *C*: coefficient of variation (CV) for EPSP amplitudes vs. age for individual pairs at P10–P14 (open circles), P15–P18 (gray circles), P19–P29 (black circles), and the mean CV $\pm$ SD for each age group (open squares). There was a significant increase in CV between P10–P14 and P19–P29 (* $P < 0.05$). *D*: CV vs. EPSP amplitude for each connection.

FIG. 4.

Time course of the EPSPs. **A**: presynaptic action potential (AP, thin black line) and mean evoked EPSP (thick black line) in a PC-PC pair. The EPSP amplitudes were normalized to 1 and the EPSPs were fit by a difference of 2 exponentials (gray dashed line) to obtain time constants for the rise (τ_r) and decay (τ_d). Scale bars: horizontal: 20 ms (AP and EPSP); vertical: 40 mV (AP) and 0.5 normalized units (EPSP). The latency was defined as the difference between the onset of the AP (vertical, dashed line) and the onset of the EPSP (arrowhead). **B**: distribution of latencies over all pairs. **C**: latency vs. distance between somata. **D**: the distribution of EPSP peak times (difference between the onset of the EPSP and the maximum) for all pairs. **E**: the distribution of EPSP rise constants (τ_r). **F**: the

synaptic decay constant (τ_d) vs. postnatal day age for individual pairs (open circles) and the mean $\pm$ SD for each age (filled circles). *G*: the distributions of EPSP decay constants (τ_d) for P10–P14 (open bars), P15–P18 (gray bars), and P19–P29 (black bars). τ_d decreased significantly with age (** $P < 0.01$). *H*: the mean τ_d plotted against the mean resting membrane potential (V_m , *left*), membrane time constant (τ_m , *middle*), and mean input resistance (R_n , *right*) for each postnatal day. τ_d was positively correlated with V_m (R : 0.92), τ_m (R : 0.83), and R_n (R : 0.93, black fit lines). Error bars $\pm$ SD. *I*: voltage- and current-clamp recordings of evoked excitatory postsynaptic currents (EPSCs) and EPSPs in the same PC–PC pair. *Left*: EPSCs recorded at different holding potentials (HPs) from -80 to -30 mV. *Right, top*: the same EPSCs shown *left*, normalized to the peak amplitude, for 3 holding potentials: -80 mV (black), -60 mV (gray), and -30 mV (dashed). *Right, bottom*: normalized EPSPs recorded in the same pair at -60 mV (gray) and -80 mV (black). The resting membrane potential of the postsynaptic cell was -68 mV. Scale bars: horizontal: 20 ms, vertical: 100 pA.

FIG. 5.

Short-term synaptic plasticity at different stimulation frequencies. *A*: average EPSPs from 3 PC–PC pairs recorded at P11 (*left*), P18 (*middle*), and P28 (*right*) in response to presynaptic cell stimulation consisting of 5 pulses delivered at 10, 20, 40, or 80 Hz. Horizontal scale bars: 100 ms. Vertical scale bars: 0.5 mV. *B*: paired-pulse ratio (PPR = EPSP₂/EPSP₁) vs. postnatal day age for each connection (filled triangles) and average PPR $\pm$ SD for each age (open circles) in response to 10-Hz stimulation. *C*: the average PPR significantly increases between P10–P14 (white bars) and P15–P18 (gray bars) in response to 10-, 20-, and 40-Hz stimulation and between P15–P18 and P19–P29 (black bars) in response to 10-Hz presynaptic stimulation (** $P < 0.01$). *D*: average short-term synaptic depression of the 5th EPSP in the train relative to the 1st EPSP for the P10–P14 (white bars), P15–P18 (gray bars), and P19–P29 (black bars) age groups. Depression of the 5th pulse was significantly greater in younger mice (P10–P14, P15–P18) than that in older mice in response to 10-, 20-, and 40-Hz stimulation. Error bars are $\pm$ SD. ** $P < 0.01$, * $P < 0.05$. *E*: the average short-term depression $\pm$ SE vs. the time of each impulse for PC pairs in mice P19–P29. The depression did not significantly differ between 10-Hz (filled circles) and 20-Hz (open circles) stimulation frequencies. Higher stimulation frequencies (40 Hz, filled squares, and 80 Hz, open squares) resulted in significant increases in synaptic depression at all points over lower frequencies (table, *inset*, ** $P < 0.01$, * $P < 0.05$).

FIG. 6.

The reliability of PC-PC connections. **A**: histograms of failure rates (# failures/# trials) in P10-P14 (open bars, $n = 27$), P15-P18 (gray bars, $n = 85$), and P19-P29 (black bars, $n = 37$) animals. Each distribution is presented as the proportion of pairs in each age group. The median of each distribution is shown (dashed lines). **B**: the mean failure rates at P10-P14 (open bars) and P15-P18 (gray bars) are lower than those in older animals (P19-P29, black bars, $*P < 0.05$, Mann-Whitney U test). **C**: mean EPSP amplitude vs. failure rate at P10-P14 (open circles), P15-P18 (gray circles), and P19-P29 (black circles). **D**: the PPR vs. failure rate for each connection at P10-P14 (open circles), P15-P18 (gray circles), and P19-P29 (black circles). Points that are above/below the horizontal dashed line are indicative of

facilitating/depressing synapses. The vertical dashed line delineates low failure (LF) connections from high failure (HF) connections. *E*: evoked EPSPs (*black traces*) and EPSCs (*gray traces*) recorded in the same PC–PC pair in response to 10-Hz (*top*) and 20-Hz (*bottom*) presynaptic stimulation. Scale bars: horizontal: 50 ms; vertical: 1 mV (EPSPs), 100 pA (EPSCs). *F*: the PPRs recorded in voltage clamp (VC) vs. those recorded in current clamp (CC) for the same pair during 10-Hz (filled circles) and 20-Hz (open circles) paired-pulse stimulation. The solid black line is the unity line.

FIG. 7.

Summation of EPSPs during 20- and 40-Hz stimulation during development. *A*: the mean responses to presynaptic stimulation at 20 Hz (*top*) and 40 Hz (*bottom*) in individual pairs (black lines) and over each age group [gray lines, P10–P14 (*left*), P15–P18 (*middle*), and P19–P29 (*right*)]. The traces are normalized such that the amplitude of the 1st EPSP is equal to 1 (horizontal dashed lines); horizontal scale bar: 100 ms. Synapses in young mice (P10–P14) show transient responses with minimal summation to both 20- and 40-Hz stimulation. Summation is significantly increased in the P15–P18 age group compared with older and younger age groups. At P19–P29, the synapses follow 20-Hz stimulation with minimal decrement in amplitude but significant summation occurs only during 40-Hz stimulation. *B*:

summation in response to 20- and 40-Hz stimulation is quantified as the area under the normalized traces at P10–P14 (white bars), P15–P18 (gray bars), and P19–P29 (black bars). Error bars are $\pm$ SD; ** $P < 0.01$, * $P < 0.05$.

TABLE 1

Intrinsic membrane and action potential properties (P10–P29)

Property	Age, Days		
	P10–P14 (n = 56)	P15–P18 (n = 66)	P19–P29 (n = 66)
R_{in} , M Ω	306 $\pm$ 76	244 $\pm$ 64**	139 $\pm$ 34**††
τ_{m} , ms	38 $\pm$ 9	26 $\pm$ 7**	15 $\pm$ 5**††
V_{m} , mV	−70 $\pm$ 6	−75 $\pm$ 6**	−81 $\pm$ 5**††
HW, ms	1.9 $\pm$ 0.5	1.8 $\pm$ 0.5	1.2 $\pm$ 0.3**††
AHP, mV	16 $\pm$ 2	14 $\pm$ 3**	12 $\pm$ 3**††
Height, mV	52 $\pm$ 9	48 $\pm$ 16	54 $\pm$ 10
Rheobase, nA	0.07 $\pm$ 0.06	0.10 $\pm$ 0.06**	0.26 $\pm$ 0.09**††
Threshold, mV	−37 $\pm$ 4	−37 $\pm$ 5	−37 $\pm$ 5

Values are means $\pm$ SD. R_{in} , input resistance; τ_{m} , membrane time constant; V_{m} , resting membrane potential; HW, action potential width at half maximum; AHP, afterhyperpolarization. Significance:

** $P < 0.01$ for adjacent columns (i.e., P10–P14 vs. P15–P18 and P15–P18 vs. P19–P29);

†† $P < 0.01$ for P10–P14 versus P19–P29.

TABLE 2

Summary of age-dependent synaptic properties

Property	Age, Days		
	P10–P14 (n = 27)	P15–P18 (n = 85)	P19–P29 (n = 37)
Amplitude, mV	1.0 ± 0.6	0.8 ± 0.5	0.6 ± 0.4 ^{††}
Fail rate	0.14 ± 0.21	.16 ± 0.19	0.25 ± 0.23 ^{*†}
CV	0.49 ± 0.24	0.60 ± 0.31	0.64 ± 0.3 [†]
τ_d , ms	44 ± 15	37 ± 13 ^{**}	24 ± 9 ^{**††}
PPR, 10 Hz	0.68 ± 0.18	0.80 ± 0.22 ^{**}	0.94 ± 0.20 ^{**††}

Values are means ± SD. CV, coefficient of variation; τ_d , EPSP decay constant; PPR, paired-pulse ratio. Significance:

^{**} $P < 0.01$,

^{*} $P < 0.05$ for adjacent columns (i.e., P10–P14 vs. P15–P18 and P15–P18 vs. P19–P29);

^{††} $P < 0.01$,

[†] $P < 0.05$ for P10–P14 versus P19–P29.

TABLE 3

Paired-pulse ratios (PPR) in LF versus HF connections (P19–P29)

Frequency	PPR $\pm$ SD		<i>t</i> -Test (<i>P</i> Value)
	LF	HF	
10 Hz	0.89 $\pm$ 0.15 (<i>n</i> = 17)	0.91 $\pm$ 0.22 (<i>n</i> = 15)	0.72
20 Hz	0.95 $\pm$ 0.37 (<i>n</i> = 14)	0.95 $\pm$ 0.49 (<i>n</i> = 13)	1.0

Values are means $\pm$ SD. LF, low failure connection; HF, high failure connection.