Transient Coupling between Infragranular and Subplate Layers to Layer 1 Neurons Before Ear Opening and throughout the Critical Period Depends on Peripheral Activity

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Cortical layer 1 (L1) contains a diverse population of interneurons that can modulate processing in superficial cortical layers, but the intracortical sources of synaptic input to these neurons and how these inputs change over development and with sensory experience is unknown. We here investigated the changing intracortical connectivity to L1 in the primary auditory cortex (A1) of mice of both sexes in vitro slices across development using laser-scanning photostimulation. Before postnatal day (P)10, L1 cells receive excitatory input from within L1, L2/3, L4, and L5/6 as well as from subplate. Excitatory inputs from all layers increase, especially from L4, and peak during P10–P16, around the peak of the critical period for tonotopy. Inhibitory inputs followed a similar pattern. Functional circuit diversity in L1 emerges after P16. In adults, L1 neurons receive ascending inputs from L2/3 and L5/6, but only few inputs from L4. The transient hyperconnectivity from deep layers but not L2/3 is absent in deaf mice. Our results demonstrate that deep excitatory and superficial inhibitory circuits are tightly linked in early development and might provide a functional scaffold for the layers in between. These results suggest that early thalamically driven spontaneous and sensory activity in subplate can be relayed to L1 from the earliest ages on and shape L1 connectivity from deep layers. Our results also reveal a period of high transient columnar hyperconnectivity after ear opening coinciding with the critical period, suggesting that circuits originating in deep layers might play a key role in this process.

Key words: cerebral cortex; critical period; layer 1; subgranular; subplate; transient

Significance Statement

L1 contains a diverse population of interneurons that can modulate processing in superficial cortical layers but the sources of synaptic input to these neurons and how these inputs change over development is unknown. We found that during the critical period a large fraction of excitatory inputs to L1 originated in L5/6 and the cortical subplate. This hyperconnectivity is absent in deaf mice. Our results directly demonstrate that deep excitatory and superficial inhibitory circuits are tightly linked in early development and might provide a functional scaffold for the layers in between.

Introduction

Layer 1 (L1) is unique in the neocortex in that most L1 neurons are GABAergic interneurons, showing diversity in firing patterns and expression of molecular markers such as neuron-derived neurotrophic factor (NDNF), 5HT3, and others (Winer and Larue, 1989; Hestrin and Armstrong, 1996; Soda et al., 2003; Jiang et al., 2013; Muralidhar et al., 2013; Ma et al., 2014; Lee et al., 2015; Tremblay et al., 2016; Schuman et al., 2019). In adults, L1 neurons can have a profound impact on the sensorily evoked responses of neurons in other layers whose dendritic tufts reside within L1, especially L2/3 cells (Chu et al., 2003; Muñoz et al., 2017). L1 is the target of diverse intracortical inputs, including
cross-modal inputs, as well as subcortical neuromodulatory inputs (Levitt and Moore, 1978; Martin et al., 1989; Chu et al., 2003; Zhu and Zhu, 2004; Alitto and Dan, 2012; Ibrahim et al., 2016; Roth et al., 2016; Mesik et al., 2019). Multiple anatomic studies showed that there are axonal projections from deep layers into L1 in development as well as in adults (Kasper et al., 1994; Clancy and Cauller, 1999; Larsen and Callaway, 2006; Romand et al., 2011; Viswanathan et al., 2017; Abs et al., 2018). These axons can potentially target dendrites of many neurons present in L1. Moreover, axonal terminals from subplate neurons (SP) are also present in L1 during development (Friauf et al., 1990; Viswanathan et al., 2017; Ghezzi et al., 2021), and stimulation of subplate neurons results in inhibitory responses in Cajal-Retzius cells in L1 (Myakhar et al., 2011), suggesting that subplate neurons can directly influence L1 neurons. However, the precise intracortical innervation of L1 neurons and the development of the functional excitatory and inhibitory circuits to L1 neurons is unknown.

The topographic representation of sensory stimulus properties in the cerebral cortex emerges during development, and sensory experience can shape this process (Hensch, 2004; Feldman, 2009; Schreiner and Polley, 2014). Developing thalamocortical projections first target and activate subplate neurons (Friauf and Shatz, 1991; Hangar et al., 2002; Higashi et al., 2002; Zhao et al., 2009; Kanold and Luhmann, 2010; Wess et al., 2017; Kanold et al., 2019). Subplate neurons relay this activity to L4 but also to L1 (Zhao et al., 2009; Kanold and Luhmann, 2010; Deng et al., 2017; Viswanathan et al., 2017). It has been known that inputs to L1 have the potential to play a key role in development (Che et al., 2018), and L1 has been proposed to play a role in critical period plasticity (Takeşian et al., 2018). However, the underlying circuit mechanisms and how sensory inputs affect cortical circuits of L1 neurons during development have remained unknown.

To identify presynaptic inputs to L1 and how these inputs develop and are modulated by peripheral activity, we performed laser-scanning photostimulation (LSPS) combined with whole-cell patch-clamp recording in primary auditory cortex (A1) L1 neurons in thalamocortical slices from postnatal day (P)5 to P32 mice and measured the spatial pattern of excitatory and inhibitory connections. To assess the effects of sensory experience we also recorded from pups deficient in otoferlin (OTOF-KO), a gene that is required for synchronous release from hair cells, the knock-out of which results in deafness (Roux et al., 2006; Mukherjee et al., 2021). We found that in wild-type mice before ear opening (P5–P9) L1 neurons receive excitatory input from all layers, especially L2/3 and L5/6. Most L1 neurons (2/3 of L1 neurons) receive cortical subplate inputs. Inhibitory inputs mostly originate from L1, L2/3, L5/6, and from within subplate. The excitatory inputs from all layers transiently increased at P10–16, especially from L4. In adult, L1 neurons receive most excitatory and inhibitory input from within L1, L2/3, as well as superficial L5. Moreover, the functional circuits to L1 neurons diversify during development. We find that in OTOF-KO animals, connections from L4, L5/6, and subplate to L2/3 are reduced and do not change over development, whereas inputs from within L2/3 increase.

Together, our results indicate that distinct circuit topologies exist after ear opening in rodents and that this topology requires hearing. In particular, (1) L1 neurons receive inputs from all layers including subplate, (2) L4 inputs decrease after the second postnatal week, (3) specific L1 subcircuits emerge after the second postnatal week resulting in circuit sparsification, and (4) these circuit changes do not occur in OTOF-KO animals. Together these findings suggest that infragranular circuits as well as subplate may play a role in controlling activity and plasticity in the superficial cortex before and during the critical period.

Materials and Methods

Animals. All animal procedures were approved by the University of Maryland and Johns Hopkins University Animal Care and Use Committee. We used male and female mice (C57BL/6j background, The Jackson Laboratory) and Otoferlin-KO mice (from Tobias Moser, University of Göttingen, bred in house). Mice were raised in 12 h light/12 h dark conditions.

Slice preparation. Mice (P6–P32) were deeply anesthetized with isoflurane (Halocarbon). A block of brain containing A1 and the medial geniculate nucleus was removed, and thalamocortical slices (500 μm thick) were cut on a vibrating microtome (Leica) in ice-cold artificial (ACSF) containing the following (in mM): 130 NaCl, 3 KCl, 1.25 KH2PO4, 20 NaHCO3, 10 glucose, 1.3 MgSO4, and 2.5 CaCl2, pH 7.35–7.4, in 95% O2/5% CO2. The cutting angle was −15° from the horizontal plane (lateral raised), and A1 was identified as described previously (Cruikshank et al., 2002; Zhao et al., 2009; Meng et al., 2015). Slices were incubated for 1 h in ACSF at 30°C and then kept at room temperature. Slices were held in a chamber on a fixed-stage microscope (Olympus BX51) for recording and superfused (2–4 ml/min) with high-Mg2+ ACSF recording solution at room temperature to reduce spontaneous activity in the slice. The recording solution contained the following (in mM): 124 NaCl, 5 KCl, 1.23 NaH2PO4, 26 NaHCO3, 10 glucose, 4 MgCl2, and 4 CaCl2. The location of the recording site in A1 was identified by landmarks (Cruikshank et al., 2002; Zhao et al., 2009; Viswanathan et al., 2012; Meng et al., 2014, 2015, 2017b, 2019, 2021).

Electrophysiology. Whole-cell recordings from L1 cells were performed with a patch-clamp amplifier (MultiClamp 700B, Molecular Devices) using pipettes with input resistance of 4–9 MΩ. Data acquisition was performed with National Instruments AD boards and custom software (Éphus; Suter et al., 2010), which was written in MATLAB (MathWorks) and adapted to our setup. Voltages were corrected for an estimated junction potential of 10 mV. Electrodes were filled with the following (in mM): 115 cesium methanesulfonate (CsCH3SO3), 5 NaF, 10 EGTA, 15 CaCl2, 3.5 MgATP, and three QX-314, pH 7.25, 300 mOsM. Cesium and QX314 block most intrinsic active conductances and thus make the cells electrophysiologically compact. Biocytin or Neurobiotin (0.5%) was added to the electrode solution as needed. Series resistances were typically 20–25 MΩ.

LSPS. LSPS experiments were performed as previously described (Meng et al., 2015; Viswanathan et al., 2017; Meng et al., 2017b, 2017c). Gated geniculate (GG) Caged-glycerol–loaded OTOF-KO mice (Muralidharan et al., 2016) (0.5–1mM) was added to the ACSF. This compound has no effect on neuronal activity without UV light (Muralidharan et al., 2016). UV laser light (500 mW, 355 nm, 1 ms pulses, 100 kHz repetition rate; DSPS Lasers) was split by a 33% beamsplitter (CVI Melles Griot), attenuated by a Pockels cell (Conoptics), gated with a laser shutter (NM Laser), and coupled into a microscope via scan mirrors (Cambridge Technology) and a dichroic mirror. The laser beam in LSPS enters the slice axially through the objective (Olympus 10×, 0.3 NA/airwater) and has a diameter of <20 μm. Laser power at the sample is <25 mW. We typically stimulated up to 30 × 25 sites spaced 40 μm apart, enabling us to probe areas of 1 mm2; such dense sampling reduces the influence of potential spontaneous events. Repeated stimulation yielded essentially identical (correlation 0.9 ± 0.05 SEM, n = 4) maps (Fig. 1b). Stimuli were applied at 0.5–1 Hz. Analysis was performed essentially as described previously with custom software written in MATLAB (Meng et al., 2014, 2015, 2017a, 2017b, 2019). Activation profiles of neurons across cortical layers during these ages show that LSPS resolution does not vary (Meng et al., 2019, 2021). To detect monosynaptically evoked postsynaptic currents (PSCs), we detected PSCs with onsets in an ~50 ms window after the stimulation. This window was chosen based on the observed spiking latency under our recording conditions (Meng et al., 2015; Viswanathan et al., 2017; Meng et al., 2018) and to identify presynaptic inputs to L1 and how these inputs develop and are modulated by peripheral activity, we performed laser-scanning photostimulation (LSPS) combined with whole-cell patch-clamp recording in primary auditory cortex (A1) L1 neurons in thalamocortical slices from postnatal day (P)5 to P32 mice and measured the spatial pattern of excitatory and inhibitory connections. To assess the effects of sensory experience we also recorded from pups deficient in otoferlin (OTOF-KO), a gene that is required for synchronous release from hair cells, the knock-out of which results in deafness (Roux et al., 2006; Mukherjee et al., 2021). We found that in wild-type mice before ear opening (P5–P9) L1 neurons receive excitatory input from all layers, especially L2/3 and L5/6. Most L1 neurons (2/3 of L1 neurons) receive cortical subplate inputs. Inhibitory inputs mostly originate from L1, L2/3, L5/6, and from within subplate. The excitatory inputs from all layers transiently increased at P10–16, especially from L4. In adult, L1 neurons receive most excitatory and inhibitory input from within L1, L2/3, as well as superficial L5. Moreover, the functional circuits to L1 neurons diversify during development. We find that in OTOF-KO animals, connections from L4, L5/6, and subplate to L2/3 are reduced and do not change over development, whereas inputs from within L2/3 increase.

Together, our results indicate that distinct circuit topologies exist after ear opening in rodents and that this topology requires hearing. In particular, (1) L1 neurons receive inputs from all layers including subplate, (2) L4 inputs decrease after the second postnatal week, (3) specific L1 subcircuits emerge after the second postnatal week resulting in circuit sparsification, and (4)
Figure 1. Optical circuit mapping of sources of input to L1 cells. a, Infrared image of a brain slice with patch pipette on an L1 neuron at P12. Stimulation grids are indicated by the blue dots. Left, The black bars are the layer boundaries (Pia, boundary between L1 and L2/3, boundary between L2/3 and L4, boundary between L4 and L5/6, and the boundary between L6 and subplate (SP)). Scale bar, 100 µm. b, Relative position of recorded cells within L1 for different age groups (P5–9, blue; P10–16, red; P20–23, orange; P28–32, purple). There is no significant difference between different groups (multicomparison, P5–9 vs P10–16, p = 0.64; P5–9 vs P20–23, p = 1; P5–9 vs P28–32, p = 0.97; P10–16 vs P20–23, p = 0.77; P10–16 vs P28–32, p = 0.89; P20–22 vs P28–32, p = 0.98). c, Whole-cell voltage-clamp recordings with holding potentials at −70 mV (left) and 0 mV (right) to investigate excitatory and inhibitory synaptic connections, respectively. Shown are example traces evoked by photostimulation at different locations. The solid blue line indicates the time of photostimulation. The dashed blue line marks 8 ms poststimulus, which is the minimal latency for synaptic responses. The dashed green line marks the end of the 50 ms event analysis window. d, Spike latencies in each layer from cell-attached recordings; 80% of spikes triggered by UV laser for L2/3, L4, and L5/6 neurons are within 50 ms windows after laser onset. e, Mean and SEM of the distance between the spike activation sites to the recorded cells with respect to spike latency for different age groups. f, Cumulative distribution function plots of the recorded spontaneous rate for different age groups. Rates were low and similar between groups (*p > 0.05). g, Exemplar cell from P14 mouse. Pseudocolor maps show EPSC (top) and IPSC (bottom) charge at each stimulus location. White circle indicates the soma location. Black pixels indicate stimulation sites that evoked direct responses. Horizontal bars indicate layer borders. Scale bar, 200 µm. h, Repeated maps in two exemplar cells from P13 and P17. Scale bar, 200 µm. i, Individual maps of connection probability.
al., 2017b, 2019), showing that photostimulation of L2/3, L4, and L5/6 cells at P5–9, P10–16, and P28–32 evokes mostly single action potentials within a 50 ms time window (Fig. 1f) and that photostimulation caused spiking when applied within a 120 μm range from targeted cells (Fig. 1e; Meng et al., 2019). Our recordings are performed at room temperature and in high-Mg2+ solution to reduce the probability of polysynaptic inputs. Traces containing a short-latency (<8 ms) direct response were discarded from the analysis (black colors in color-coded maps) as were traces that contained longer latency inward currents of long duration (>50 ms). The short-latency currents could sometimes be seen in locations surrounding (<100 μm) areas that gave a direct response. Occasionally some of the direct responses contained evoked synaptic responses that we did not separate out, which leads to an underestimation of local short-range connections. Cells that did not show any large (>100 pA) direct responses were excluded from the analysis as these could be astrocytes. It is likely that the observed PSCs at each stimulation location represent the activity of multiple presynaptic cells. We measured both peak amplitude and transferred charge; transferred charge was measured by integrating the PSC. Although the transferred charge might include contributions from multiple events, our prior studies showed a strong correlation between these measures (Viswanathan et al., 2012; Meng et al., 2014, 2015, 2019). Layer boundaries were determined from the infrared pictures.

Stimulus locations that showed PSC were deemed connected, and we derived binary connection maps. We aligned connection maps for each neuron in the population and averaged connection maps to derive a spatial connection probability map. In these maps the value at each stimulus location indicates the fraction of neurons that received input from these stimulus locations. Layer boundaries were determined for each cell from the infrared pictures (Viswanathan et al., 2012; Meng et al., 2014, 2015, 2019). For display purposes only we average the layer boundaries across the population and indicate this on the spatial probability maps. We derived layer-specific measures. Input area is calculated as the area within each layer that gave rise to PSCs. Mean charge is the average charge of PSCs from each stimulus location in each layer. Intralaminar integration distance is the extent in the rostrocaudal direction that encompasses connected stimulus locations in each layer. We calculated an excitatory/inhibition (E/I) balance index in each layer for measures of input area and strength as (E − I)/(E + I), thus (AreaE − AreaI)/(AreaE + AreaI), resulting in a number that varied between −1 and 1 with 1 indicating dominant excitatory and −1 indicating dominant inhibition.

Spatial connection probability maps show the average connection pattern in each group. To visualize the diversity of connection patterns over the population of neurons in each group, we calculated Fano factor maps. For each responsive spatial location, we calculated the Fano factor of the PSC as σ2/μ and plotted maps of the Fano factor.

To compare the large-scale connectivity between cells in each group we calculated the spatial correlation of the binary connection maps in each group by calculating the pairwise cross-correlations (Meng et al., 2019).

Morphology. Biocytin-filled cells were processed as reported previously (Zhao et al., 2009; Deng et al., 2017; Meng et al., 2019; Sheikh et al., 2011). Cells filled with biocytin were stained and reconstructed (Neurolucida, version 2019, MBF Bioscience). Cell morphology was analyzed using the built-in Sholl analysis in Neurolucida.

Statistics. Results are plotted as means ± SEM unless otherwise indicated. Populations from WT and OTOF or NDNF were compared with a rank sum or Student’s t test (based on Lilliefors test for normality) and deemed significant if p < 0.05. ANOVA (MATLAB function anova1), followed by a multicomparison test (MATLAB function multcompare), was used when comparing different age groups.
in the population that received an input from this spatial location. Hence, this represents a spatial map of connection probability (Fig. 1). The connection probability for each stimulation site indicates the fraction of neurons that received an input from this location regardless of the strength of this input. We observed that L1 cells received inputs from within L2/3 but also from other cortical layers (Fig. 1). Excitatory inputs originating from stimulation sites in L1 are likely because of activation of apical dendrites of L2/3 neurons (Meng et al., 2019).

We then computed connection probability maps for the different age groups (Fig. 2a). Qualitative analysis of these maps revealed that the spatial profiles changed with development. To quantify the laminar changes, we identified laminar borders for each cell from the infrared images and calculated the input profile from each layer (Meng et al., 2015, 2017b, 2019). To visualize and quantify the differences between cells, we determined the total area for each cell in each layer where stimulation evoked EPSCs in L1 neurons. Because L2/3 cells can be driven by

Figure 2. Excitatory circuits to L1 neurons rearrange during development. a, Average maps of spatial connection probability of excitatory connections in different age groups. The pseudocolor indicates the connection probability. Left, The short white horizontal bars indicate averaged laminar borders. Scale bar, 200 μm. Shaded area on the right side of each probability map indicates the laminar marginal distributions. Black patches indicate the direct activation area. b, Columnar input map obtained by summation of inputs along laminar direction for each recorded cell across the different age groups. Each column represents one cell. The white circles represent the locations of cell bodies. The short white bars represent layer boundaries. Bottom, The grayscale bar on the bottom indicates mouse age for each recorded cell. Different ages are marked on each segment. c, The mean (solid) and SEM of total area, mean charge, and percentage of inputs L2/3 (including L1, green), L4 (red), L5/6 (blue), and SP/DL 6 (black) to L1 neurons in different age groups. d, The mean (solid) and SEM of the distance of 80% of excitatory inputs to each L1 cell originating from L2/3 (including L1, green), L4 (red), L5/6 (blue), and SP/DL 6 (black). The integration distances of L2/3, L4 inputs begin to increase at early age up to P16 and decrease thereafter. *p < 0.05, **p < 0.01, ***p < 0.001. The dashed line in a marks the time of ear opening.
stimulation from L1, we lumped those stimulation sites into L2/3. We find a temporary increase in intracortical synaptic connectivity over development. From the youngest ages on, L1 cells received input from all layers including supragranular, infragranular, and subplate layers. Inputs from all layers increased after P5–9, peaked at P10–16, and then decreased (Fig. 2bc). The laminar distribution of input area for each recorded cell in Figure 2b shows that the input to L1 cells in adult mice are mainly from intralaminar locations and upper L5. They also receive inputs from subplate or deep Layer 6 (Fig. 2b). Approximately 67% of the recorded neurons in the P5–9 group receive SP input, whereas ~76% of neurons in the P10–16 group receive SP input. To further quantify the balance of intralaminar to interlaminar inputs, we next determined the relative input L1 neurons receive from each layer. This analysis confirmed that the relative L4 input to L1 neurons reaches its peak at P10–16 (Fig. 2c), whereas the total inputs from L2/3, L4, and L5/6 significantly decreased after P16. In contrast, the relative input from deep L6 increased after P16, peaked at P20, and remained constant until adulthood. In addition to the number of inputs, the effective synaptic strength contributes to the functional laminar connectivity. To test whether synaptic strength from each layer also changed over development, we plotted the mean EPSC charge for EPSCs evoked from each layer (Fig. 2c). Although the mean synaptic strength for infragranular inputs to L1 remained constant from P5 to P16, intralaminar and L4 inputs strengthened until P16 (Fig. 2c). Input strength decreased after this period in all layers, with the largest decrease occurring in L4 (P10–16 vs P28–32: 525% for L5/6, 1328% for L4, and 711% for L2/3). These results indicate a temporary expansion and strengthening of interlaminar inputs to L1 at P10–P16, which encompasses the critical period for tonotopy and spectral tuning in rodents (Zhang et al., 2001, 2002; de Villers-Sidani et al., 2007; Bartka et al., 2011).

So far we assessed circuit expansion across the laminar dimension. Circuit integration across the orthogonal axis in topographic maps indicates topographic integration. Our thalamocortical slices of A1 contain the tonotopic axis, therefore the distance at which presynaptic cells are located in the rostrocaudal axis is a proxy for integration along the frequency axis (Meng et al., 2017a, 2019). We thus compared the rostrocaudal spread or integration distance of interlaminar and intralaminar inputs at the different ages (Fig. 2d). We find that inputs from L4 and L5/6 originated from a narrow region at P5–9 and that the integration distance increased thereafter, peaking at P10–16. The integration distance in all layers decreased by P28–32, and decreases were largest in L4 (P10–16 vs P28–32: 209% for L4 vs 191% for Deep L6, 150% for L5/6, and 131% for L2/3). Thus, our results indicate that there is a temporary lamina-dependent period of hyperconnectivity of intracortical excitatory circuits in development, which supports integration across the tonotopic axis.

**Transient hyperconnectivity of inhibitory circuits to L1 cells at P10–16**

Our data show a substantial remodeling of excitatory connections to L1 cells over development. In L2/3, developmental remodeling of excitatory connections is mirrored by remodeling of inhibitory connections (Meng et al., 2019). We thus investigated whether inhibitory connections to L1 also change over development and mapped inhibitory connections by holding cells at 0 mV (Fig. 1cg). Average maps of connection probability across ages appeared different, generally showing a similar developmental trajectory as excitatory connections (Fig. 3a,b). This was confirmed quantitatively; the total area generating inhibitory input for all layer decreased after P16 but with modest increase in L2/3 and L4 from P5 to P9 (Fig. 3c); the total inhibitory area of P10–16 vs P5–9: 209% for L4, 153% for L2/3, 106% for L5/6, 101% for Deep L6), which is different from excitatory inputs (Fig. 2c). The fraction of inhibitory input from L5/6 decreased after P9 and kept constant afterward, whereas the fraction of input from L4 showed modest increase until P16 and then decreased to adulthood. The mean charge of IPSCs originating from within L2/3 and L4 were strongest during P9–P16 and then significantly reduced after that (Fig. 3c). In contrast to excitatory inputs, only inhibitory inputs from L4 but not L2/3 and L5/6 showed decreased integration distance with age, indicating a lack of tonotopic refinement (Fig. 3d). The marginal distribution of inhibition across the tonotopic axis (Fig. 3e) eventually becomes comparable to that of excitation at P28. Thus, together with the changes in excitatory and inhibitory connections, these data show that there is extensive remodeling of excitatory and inhibitory connections to L1 neurons but that the spatial pattern is distinct for each layer. Our results indicate that there is a temporary increase in relative excitatory but not inhibitory inputs from L4 and L5/6 during the critical period.

**The balance of excitatory and inhibitory inputs to L1 cells varies by layer, and inhibition in general dominates excitation at P28**

Given that both excitatory and inhibitory circuits change over development, and because the spatial and temporal patterns of these changes appeared different, we hypothesized that the balance of excitation and inhibition would change over development. Studies in the visual cortex have suggested that maturation of functional inhibition, and thus the balance of excitation and inhibition, is a prime driver of critical period plasticity (Hensch, 2004). To determine the functional and spatial balance of excitation and inhibition, we devised an excitation/inhibition (E/I) index, (E–1)/(E+1), that computes the dominance of either excitatory or inhibitory inputs from each layer based on the area as well as the charge of excitatory and inhibitory inputs (Meng et al., 2015, 2017b; Fig. 3e). The index can vary between −1 and 1, with 1 indicating dominant excitation and −1 indicating dominant inhibition. This analysis showed that excitation dominated inhibition for inputs originating in L4 and remained constant until P28. In contrast, for inputs originating in L2/3, L5/6, and deep L6, the index remained constant during P5–P23 and decreased at P28. Thus, initially excitation is dominant, but this dominance decreases with age so that inhibition is larger than excitation in adults.

**Emergence of circuit diversity to L1 neurons by P20**

L1 neurons form a heterogeneous neuropil in the adult, and we thus explored whether this circuit diversity changes over development. Our prior studies of L2/3 circuits showed that circuits to L2/3 could be diverse (Meng et al., 2017b) and that this diversity emerged over development (Meng et al., 2019). We quantified the emergence of L1 circuit diversity by calculating the pairwise correlation of the LSPS maps of the sampled L1 cells. We find that correlations for both excitatory and inhibitory maps increased from P5–9 to P10–16, and then decreased by P20–23 and remained constant thereafter (Fig. 4a). Thus, L1 neurons form a relatively homogenous population based on intracortical circuit topology during the critical period and circuit diversity emerges after P16.

To gain more detailed insight in how inputs from each layer contributed to the developmentally emerging circuit diversity,
Figure 3. Inhibitory circuits to L1 neurons rearrange during development. 

(a) Average spatial maps of inhibitory connections to L1 interneurons in different age groups. Pseudocolor represents connection probability. The short white horizontal bars indicate averaged laminar borders. Scale bar, 200 μm. Shaded area on the right side of each probability map indicates the laminar marginal distributions.

(b) Columnar input map for inhibitory inputs. Each column represents one cell. The white circles represent the locations of cell bodies. The short white bars

(c) Total Input Area (μm²), Mean Charge (pC), Relative laminar contribution

(d) Laminar integration (μm)

(e) (E-I)/(E-I) area, (E-I)/(E-I) charge

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we computed the variability for each stimulation location in inputs across the population. As a measure of variability we computed the Fano factor ($\sigma^2/\mu$) for each spatial location over the cell populations in each age group (Fig. 4b). We find that the Fano factor was lowest, indicating highest circuit homogeneity, within L2/3 across ages. Interlaminar inputs, especially those from out of column, showed the highest Fano factors, indicating that the increase in circuit diversity is driven by remodeling of interlaminar inputs.

**Morphologic complexity of L1 neurons decreases over development**

Our functional data reveal a decrease in inputs to L1 neurons after P16. Dendritic complexity can be related to the numbers of functional inputs neurons receive (Meng et al., 2019). We thus investigated whether the morphologic complexity of L1 cells in A1 also decreased. We reconstructed 31 L1 cells across different age groups (P5–P9, n = 7; P11–P16, n = 7; P20–P23, n = 5; P28–P32, n = 12; Fig. 5a). We then analyzed the dendrites of the reconstructed neurons. This analysis indicated that L1 cell complexity, expressed as the number of nodes and ends, increased from P5–P9 to ~P10–P16 and then decreased with age (Fig. 5b). Sholl analysis showed that most branch intersections of L1 cells at older age (P20–32) were within ~100 µm from the cell body, whereas branches were present up to ~150 µm at P10–16 and up to ~300 µm at P5–P9 (Fig. 5c). Comparing cells showed that intersections close to the soma (~50 µm) were more prominent at P11–P16. Comparison of cells at P20–23 and P28–32 showed that in the younger group the dendritic branch intersections farther away from the soma (~150–200 µm) were eliminated. Together, these results indicated that L1 cell morphology became more complex between P5–9 and P10–16 and then became simpler during subsequent development, consistent with our functional measures.

**NDNF+ subtypes of L1 neurons show distinct inputs from subplate at young ages**

L1 neurons form a heterogeneous neuropil in the adult and L1 neurons can be grouped by firing patterns and expression of molecular markers such as NDNF, 5HT3, and others (Winer and Larue, 1989; Hestrin and Armstrong, 1996; Soda et al., 2003; Muralidhar et al., 2013; Ma et al., 2014; Schuman et al., 2019). To validate our observations, we also investigated whether the majority subclass of L1 neurons, NDNF neurons, followed a similar trajectory of developmental circuit changes as the general population. We thus crossed NDNF-Cre (catalog #028536, The Jackson Laboratory) with reporter mice (catalog #007909, The Jackson Laboratory; CAG promoter-driven TdTomato), patched TdTomato-expressing cells in mid-L1, and performed LSPS on NDNF+ neurons within L1 (n = 70 neurons; P5–P9, n = 16; P10–P16, n = 18; P20–P23, n = 16; P28–P32, n = 20; Fig. 6a,b). Targeted neurons showed nonpyramidal morphology (Fig. 6a). At P5–P9 NDNF+ neurons receive strong inputs from L5/6 but also subplate, rather than the local inputs from within L2/3 (Fig. 6c,d), and only few inputs from L4. At young ages ~15% of input comes from subplate versus ~4% from L4. At P10–P16 the interlaminar inputs from L2/3, L5/6, and L4 showed significant increase and then decreased afterward (Fig. 6c,d). At P28, the major excitatory inputs to NDNF+ L1 cells are from within L2/3
and L5/6 (Fig. 6c,d). In contrast to the general population, the relative contribution of L5/6 decreased with age. Moreover, NDNF+ L1 cells showed increased circuit heterogeneity after P16 (Fig. 6e). Comparing NDNF+ cells with the overall L1 population (Fig. 6f,g) showed that at young ages NDNF+ cells had fewer inputs from L2/3 and L4, whereas at the oldest ages NDNF+ cells received more inputs from L2/3 than the general L1 population.

Inhibitory inputs to NDNF+ L1 neurons showed a similar pattern. Major inhibitory inputs originate from within L2/3 and L5/6 at P5–P9. At P10–P16 input from L2/3, L4 and L5/6 reached their peaks and then decreased thereafter (Fig. 7a–c). NDNF+ cells showed fewer inputs from L2/3 at young ages than all L1 cells but more inhibitory inputs at P10–P23. These results show that translaminar hyperconnectivity to L1 neurons at P10–16 occurs in NDNF+ neurons but that there are some differences such as lower number of inputs from L2/3 at young ages in NDNF+ cells, suggesting that L1 neurons show some diversity in circuit patterns.

Peripheral activity shapes L1 integration into cortical circuits

L1 neurons in adult V1 can respond to visual stimulation (Mesik et al., 2019), and L1 neurons in S1 can respond to whisker stimulation (Zhu and Zhu, 2004; Che et al., 2018). Given that whisker deprivation can alter the correlation for some L1 neurons (Che et al., 2018), and that we observe changes in L1 connectivity at distinct developmental stages, we investigated the role of peripheral inputs. Hair cell activity is transmitted to the auditory nerve via specialized synapses with obligatory dependence on Otoferlin expression in the hair cell presynapse. Therefore, OTOF-KO mice are profoundly deaf (Roux et al., 2006; Mukherjee et al., 2021). Because L1 cells showed an increase in excitatory and inhibitory connections at the onset of low-threshold hearing at P10, we performed LSPS recordings from L1 neurons in OTOF-KO mice at both P6–P9 and P10–P16. We found that L1 cells from OTOF-KO mice at both ages showed reduced excitatory connections from all cortical layers (Fig. 8a,e,f). Moreover, the strength of excitatory inputs was lower in cells from OTOF-KO mice (Fig. 8c,g). Excitatory inputs to L1 in OTOF-KO mice originated from a narrower region, indicating decreased integration across the tonotopic axis (Fig. 8d,h).

We next investigated the effect of OTOF-KO on inhibitory inputs (Fig. 9). We find that L1 cells from OTOF-KO mice at both ages show reduced inhibitory connections from cortical L4 and L5/6, shifting the balance of inhibitory inputs toward L2/3 (Fig. 9a–c). Moreover, the strength of inhibitory inputs from L2/3 at P6–9 and L4 at P10–16 was lower in cells from OTOF-KO mice (Fig. 9c,e). Inhibitory inputs to L1 from L2/3 in OTOF-KO mice at P6–9 originated from a narrower region, indicating decreased integration across the tonotopic axis (Fig. 9c).

As L1 cells from C57BL/6j animals showed an increase in connectivity from both deep and superficial layers between P5–P9 and P10–16, we next compared the amount of excitatory and inhibitory inputs between OTOF-KO cells from the two age groups (Fig. 10a,b). This comparison showed that although the area of excitatory inputs from L2/3 increased over development similar to wild type (Fig. 2c), inputs from L4, L5/6, and subplate did not (Fig. 10c). Thus, the developmental increase in excitatory inputs from deep layers to L1 is impaired in the absence of...
Figure 6. Excitatory connections to L1 NDNF interneurons decrease after P16. **a**, Left, *in vitro* patch-clamp recording of NDNF interneuron (red, n = 15 mice). Inset, The twice enlarged area around cell body. Scale bar, 50 μm. Right, An example of reconstructed L1 cells at P29. Scale bar, 50 μm. **b**, Pseudocolor maps show EPSC charge and IPSC charge for two NDNF neurons at P12. Black pixels indicate direct responses. **c**, Average spatial maps of excitatory connection to L1 NDNF interneurons in different age groups (P5–9, n = 14 cells; P10–16, n = 16 cells; P20–22, n = 15 cells; P28–32, n = 19 cells). Pseudocolor represents connection probability. White horizontal bars indicate averaged laminar borders and are 100 μm long. **d**, The mean (solid) and SEM (shaded) of total area, mean charge, and percentage of inputs from L2/3 (including L1, green), L4 (red), L5/6 (blue), and SP/DL 6 (black) to L1 NDNF interneurons in different age groups. At P10–16 L1 NDNF interneurons receive excitatory inputs from all layers. However, in the adult, excitatory inputs are mainly from local L1, L2/3, and L5. **e**, The mean (solid) and SEM (shaded) of the pairwise correlations between all excitatory maps to L1 NDNF interneurons. **f**, **g** Comparison of total area and mean charge between NDNF cells and all L1 cells at the different age groups (Fig. 2). *p < 0.05, **p < 0.01, ***p < 0.001.
Otoferlin-mediated transmission. Similarly, the area of inhibitory inputs from L2/3 increased, whereas inputs from L4 and L5/6 remained the same (Fig. 10c). The mean charge of excitatory inputs and relative laminar contribution remained stable across age groups, whereas inhibitory inputs strengthened (Fig. 10d,e). Cells from OTOF-KO mice did show an increase in laminar integration with age (Fig. 10f). Thus, the developmental increase in excitatory

Figure 7. Inhibitory connections to L1 NDNF interneurons decrease after P16. a, Average spatial maps of inhibitory connections to L1 NDNF interneurons in different age groups. Pseudocolor represents connection probability. White horizontal bars indicate averaged laminar borders and are 100 μm long. b, The mean and SEM of total area, mean charge, and percentage of inputs from L2/3 (including L1, green), L4 (red), L5/6 (blue), and SP/DL 6 (black) to L1 NDNF interneurons in different age groups. At P10–16 L1 NDNF interneurons receive inhibitory inputs from all layers. However, in the adult, inhibitory inputs are mainly from local L1 and L2/3. c, The mean and SEM of the pairwise correlations between all inhibitory maps to L1 NDNF interneurons. d, e, Comparison of total area and mean charge between NDNF cells (dashed lines) and all L1 cells (solid lines, data from Fig. 2) in the different age groups. *p < 0.05, **p < 0.01, ***p < 0.001.
Intracortical excitatory connectivity is lower in Otoferlin-KO (OTOF-KO) mice.  

**Figure 8.** Intracortical excitatory connectivity is lower in Otoferlin-KO (OTOF-KO) mice.  

**a.** Average spatial maps of excitatory connections to L1 interneurons at P6–P9 in WT and OTOF-KO mice. Pseudocolor represents connection probability. Left, The short white horizontal bars indicate averaged laminar borders.  

**b.** Columnar input map. Each column represents one cell. The white circles represent the locations of cell bodies. The short white bars represent layer boundaries.  

**c.** Mean and SEM of total area, mean charge, and fraction from each layer in cells from WT and OTOF-KO mice.  

**d.** Mean and SEM of the distance of 80% of the excitatory inputs to each L1 cell originating from each layer.  

**e-h.** Same as **a-d** for WT and OTOF-KO at P10–P16. Scale bar, 200 μm. *p < 0.05, **p < 0.01, ***p < 0.001.

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and inhibitory inputs from deep layers to L1 is impaired in the absence of Otoferlin-mediated transmission.

To compare the relative changes of excitatory and inhibitory inputs for each cell, we calculated E/I balance index for each layer (Fig. 10g). At P6–P9, the E/I index based on either area or charge was positive in L2/3 and decreased at P10–16. The E/I index for the other layers did not change. This comparison showed that in cells from OTOF-KO inputs from L2/3 remodel and that laminar excitatory inputs are decreased more than inhibitory inputs. Together, these results show that not only are there fewer inputs to L2/3 cells in OTOF-KO mice but the increase in L4 and deep layer connections to L1 neurons is also impaired in OTOF-KO mice. Thus, peripheral sensory activity can sculpt the translaminar connections to L1 neurons and, in particular, connections arising from deep layers.

Discussion

In this study we revealed the changing mesoscale inputs to L1 neurons over development. We find that in early development L1 neurons received a large number of excitatory and inhibitory inputs from across all layers of the cortical plate including the subplate. We also find that there is a developmental increase followed by a decrease in intracortical connections from thalamorecipient layers (L4, SP) during the second postnatal week, whereas in adult animals L1 receives inputs from within L2/3 and L5. Our results also show that the developmental increase in connections is absent in Otoferlin-deficient mice (Fig. 10h). These results suggest that L1 neurons are intimately connected with thalamorecipient layers during cortical development including the critical period and that circuits impinging on L1 neurons are influenced by early sensory deprivations. Because L1 neurons signal across the cortical column, changes to L1 neuron circuits may be able to influence cortical plasticity during the developmental period.

Multiple anatomic studies showed that there are axonal projections from deep layers into L1 in development (Kasper et al., 1994; Larsen and Callaway, 2006; Romand et al., 2011; Viswanathan et al., 2017; Abs et al., 2018). Although these axons can potentially target dendrites of many neurons, we here show that these axons target L1 neurons. This is consistent with rabies tracing studies showing that supragranular and L1 interneurons receive subplate inputs (De Marco Garcia et al., 2015; Che et al., 2018).

We find that functional intracortical connectivity to L1 neurons decreases over development concurrent with a decrease in dendritic complexity. This is in contrast to the observation that a decrease in functional inputs is associated with increased dendritic complexity in excitatory neurons in L2/3 over the same age range (McMullen et al., 1988; Meng et al., 2019). These results suggest that dendritic complexity does not necessarily correlate with the number of presynaptic local cortical neurons but likely also depends on distant inputs and also might be differentially regulated between neurons.

We here show that circuits to L1 neurons are altered by lack of Otoferlin in the first postnatal week and that the development of the transient hyperconnectivity, especially from deep layers, is altered. This suggests that young L1 neurons are responsive to sensory stimuli. Otoferlin-deficient mice have hearing loss (Roux et al., 2006) already evident in the first postnatal week (Mukherjee et al., 2021), thus the effects on L1 neurons are likely because of reduced auditory experience. These findings are consistent with studies in S1 showing that developing L1 activity can be modulated by sensory stimuli (Che et al., 2018). How then would sensory activity reach L1? Some sensory activity might reach L1 via direct thalamic synapses present on L1 cells in development (De Marco Garcia et al., 2015; Che et al., 2018). L1 neurons in adult can also respond to sensory stimulation (Zhu and Zhu, 2004; Mesik et al., 2019), suggesting that these neurons take part in sensory processing. The short latency of sensory responses in adult L1 neurons is also consistent with direct thalamic activation. However, in vitro studies have shown that short-latency responses in cortex first emerge in the subplate before being apparent in L4 (Higashi et al., 2002; Barkat et al., 2011). This suggests that thalamic activation of L1 neurons also develops after thalamic inputs to subplate have been present and that most early sensory input might reach L1 via subplate. Ascending thalamic input to the developing cortex first targets subplate neurons (Friauf and Shatz, 1991; Hanganu et al., 2002; Higashi et al., 2005; Zhao et al., 2009; Kanold and Luhmann, 2010), and subplate neurons are the first cortical neurons to respond to sensory stimuli (Wess et al., 2017). Auditory cortex shows sound-evoked responses at the end of the first postnatal week, and circuits to subplate neurons are affected by sensory experience in the first postnatal week (Meng et al., 2021). Thus, because subplate neurons send axonal projections across cortical layers including L1(Friauf et al., 1990; Clancy and Cauller, 1999; Viswanathan et al., 2017; Ghetti et al., 2021) and the deep input to L1 are most affected in OTOF-KO, we speculate that a large fraction of sensory information might be relayed to L1 via subplate neurons, although subplate is not the dominant input to L1. Thus, early peripheral activity even before ear opening has the ability to shape the intracortical organization of multiple layers in auditory cortex.

Lack of Otoferlin not only affects developing L1. Subplate neurons in OTOF-KO mice show altered patterns of intracortical connectivity (Mukherjee et al., 2021). In particular, OTOF-KO mice show increased intracortical inputs, possibly because of homeostatic compensation to decreased ascending input. Thus, early sensory experience can alter circuits across the cortical column.

Recent studies have suggested that L1 contains topographic maps (Takesian et al., 2018), which might aid in shaping maps in L4. Our observation that sensory deprivation alters L1 connections suggests that effects of sensory deprivations on L4 activity might be because of both direct effects of sensory deprivations on thalamocortical circuits as well as circuits associated with L1 neurons. Moreover, as early topographic maps develop in the subplate (Wess et al., 2017), our results here suggest that topographic maps in L1 may result from subplate projections to L1.

We find that L1 neurons also receive large inputs from L5/6 during the critical period. L5/6 contains multiple cell types with diverse connectivity (Games and Winer, 1988; Prieto and Winer, 1999; Winer and Prieto, 2001; Petroff et al., 2012; Kim et al., 2014). Subclasses of L5/6 cells, for example, NTTSR1-positive L6 neurons, are involved in gain control (Olsen et al., 2012; Bortone et al., 2014; Guo et al., 2017). Thus, altered L5/6 circuits to L1 in deafness might contribute to abnormal auditory function in deafness (Chambers et al., 2016; Eggermont and Kral, 2016).

L1 neurons comprise a diverse population of neurons (Jiang et al., 2013; Lee et al., 2015; Tremblay et al., 2016; Schuman et al., 2019). Our results show that the overall circuit changes are replicated in NDNF-positive subtypes of L1 neurons, which encompasses two classes of L1 neurons (Schuman et al., 2019). However, we uncoved subtle differences between NDNF cells and the general L1 population, suggesting slight differences in
Intracortical inhibitory connectivity is lower at P10–P16 in Otoferlin-KO (OTOF-KO) mice. 

**Figure 9.**

- **a**, Average spatial maps of inhibitory connections to L1 interneurons at P5–P9 in WT and OTOF-KO mice. Pseudocolor represents connection probability. Left, The short white horizontal bars indicate averaged laminar borders. 
- **b**, Columnar input map. Each column represents one cell. The white circles represent the locations of cell bodies. The short white bars represent layer boundaries. 
- **c**, Mean and SEM of total area, mean charge, and fraction from each layer in cells from WT and OTOF-KO mice. 
- **d**, Mean and SEM of the distance of 80% of the excitatory inputs to each L1 cell originating from each layer. 

* *p < 0.05, **p < 0.01, ***p < 0.001.
developmental trajectories. Although we here use NDNF as a marker for a specific class of interneurons, NDNF is a neurotrophic factor that potentially could have effects on target cells (Kuang et al., 2010). Even though a secretory role for L1 neurons has not been established, a secretory role has been suggested for a subpopulation of subplate neurons (Kondo et al., 2015), which express connective-tissue growth factor (CTGF; Kondo et al., 1999; Friedrichsen et al., 2003; Heuer et al., 2003; Wang et al.,

Figure 10. Inputs from L2/3 but not deep layers increase with age in Otoferlin-KO (OTOF-KO) mice. a, b, Comparison of average excitatory and inhibitory connection maps of cells from OTOF-KO mice between P6–9 and P10–P16 (from Figs. 8, 9). c–f, Comparison of total area, mean charge, and fraction from each layer and laminar integration in cells from OTOF-KO mice at P5–P9 and P10–P16. Only inputs from L2/3 increase with age. g, Balance of excitatory and inhibitory inputs from each layer based on charge or area of inputs. h, Schematic of observed circuit changes to L1 over development and in OTOF-KO.
References


