Inhibitory circuit gating of auditory critical-period plasticity

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Cortical sensory maps are remodeled during early life to adapt to the surrounding environment. Both sensory and contextual signals are important for induction of this plasticity, but how these signals converge to sculpt developing thalamocortical circuits remains largely unknown. Here we show that layer 1 (L1) of primary auditory cortex (A1) is a key hub where neuromodulatory and topographically organized thalamic inputs meet to tune the cortical layers below. Inhibitory interneurons in L1 send narrowly descending projections to differentially modulate thalamic drive to pyramidal and parvalbumin-expressing (PV) cells in L4, creating brief windows of intracolumnar activation. Silencing of L1 (but not VIP-expressing) cells abolishes map plasticity during the tonotopic critical period. Developmental transitions in nicotinic acetylcholine receptor (nAChR) sensitivity in these cells caused by Lynx1 protein can be overridden to extend critical-period closure. Notably, thalamocortical maps in L1 are themselves stable, and serve as a scaffold for cortical plasticity throughout life.

Individualized perception of the external world is established during windows of early life when sensory circuits adapt to the surrounding environment. During such ‘critical periods’, infants, for example, become rapidly attuned to relevant speech sounds heard repeatedly1–2. Perceptual narrowing may occur in part through a restructuring of sound frequency maps (tonotopy) in the developing auditory cortex3–5. How experience sculpts sensory maps, and why this robust plasticity is restricted to early life, remains poorly understood. The identification of neural targets that define these windows of cortical plasticity would offer both novel insight into neurodevelopmental disorders and potential strategies to promote plasticity in adulthood.

A convergence of sensory and contextual signals is thought to underlie the refinement of functional connectivity in the developing brain6. Where such signals meet to drive plasticity in thalamocortical circuits is unclear. Located directly beneath the pial surface, cortical L1 may be one such integration site for diverse projections7–9, including neuromodulatory axons that target the sparse cells there10,11. Nearly all L1 neurons bear the ionotropic 5-HT₃A receptor (5-HT₃AR), a unique marker for one of three major subpopulations of cortical inhibitory interneurons12–14. Perhaps by punching ‘holes’ in networks of blanket inhibition15, 5-HT₃AR-expressing cells become important controllers of adult cortical activity by targeting other GABAergic cells16–17. However, the effects of L1 cells in the developing brain remain unknown.

Here we show that 5-HT₃AR⁺ cells are key sites of convergence for sensory and neuromodulatory inputs to orchestrate critical-period plasticity in A1. Moreover, these cells form an unexpected topographic map that is engaged early in life by transiently enhanced nAChR function, serving as a scaffold to gate plasticity in the cortical layers below.

Results

Neuromodulatory and sensory signals converge on superficial 5-HT₃AR⁺ interneurons. The canonical model of A1 circuitry dictates that input from the primary auditory thalamus, the ventral medial geniculate body (MGB), terminates in cortical L4. However, several reports suggest that divisions of MGB also send projections to L118–20. We expressed an anterograde viral tracer in MGB cells to visualize their projections to A1, and found robust innervation of cortical L1 (Fig. 1a,b). MGB axons surrounded L1 interneurons, forming putative contacts that colocalized with vGluT2, a marker of thalamocortical boutons11 (Supplementary Fig. 1).

After electrical stimulation of MGB fibers in auditory thalamocortical slices, the average minimum and maximum amplitudes of excitatory postsynaptic potentials (EPSPs) in L1 interneurons were similar in strength to those recorded in L4 pyramidal cells (Fig. 1c–e). L1 interneuron subtypes that fire nonadapting, late-spiking (LS) action potentials and those that fire adapting, non-late-spiking (non-LS) action potentials2 showed similar EPSP amplitudes (Supplementary Fig. 2a,b).

We observed a low average coefficient of variation of the EPSP-onset rise time in both L1 and L4 pyramidal cells, consistent with a monosynaptic connection from the MGB21 (Fig. 1e). Despite slower EPSP-onset latency in L1 interneurons, reflecting delayed axonal conduction from L4 to L1, MGB-evoked EPSPs in both layers had similar rise times and half-widths (Supplementary Fig. 2a,b). Finally, the EPSPs recorded during trains of MGB stimuli in L1 interneurons showed short-term depression as in L4 cells (Supplementary Fig. 2c,d). Together, these results provide evidence for a strong, temporally precise MGB connection to L1 interneurons within A1.

Notably, L1 is also a target of neuromodulatory inputs, such as cholinergic axons21–22 (Fig. 1f). 5-HT₃AR⁺ interneurons showed EPSPs after focal application of both the 5-HT₃-selective agonist m-CPPB and the nAChR-selective agonist nicotine (100µM; Fig. 1g). When we used fluorescence-activated cell sorting (FACS) to examine gene expression in the three subpopulations of interneurons—parvalbumin-expressing, somatostatin-expressing, and 5-HT₃AR-expressing cells23—we found that the genes encoding the major nAChR subunits (α7, α4, and β2) were expressed preferentially in 5-HT₃AR⁺ interneurons (Fig. 1h). Therefore, L1 interneurons are direct sites of convergence for sensory inputs from the MGB and fast neuromodulatory inputs.

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Narrowly descending 5-HT₃AR⁺ cell axons target intracolumnar PV cells. To situate 5-HT₃AR⁺ cell action within the auditory thalamocortical circuit, we next carried out a series of anatomical and optogenetic electrophysiological studies. First, we used adeno-associated virus (AAV)-Brainbow technology⁴¹. This genetic strategy uses Cre–loxP recombination to induce stochastic combinatorial expression of fluorescent proteins to individually label cortical 5-HT₃AR⁺ cell morphology with distinct colors and identify potential postsynaptic targets (Fig. 2a–c). Across all cortical layers in A1, 5-HT₃AR⁺ cells rarely formed boutons on pyramidal cell somata (Fig. 2d), consistent with their inhibition of calcium signals along apical dendrites⁴⁴. In contrast, PV cell bodies in L2/3 and L4 received more putative contacts from 5-HT₃AR⁺ cells (Fig. 2d), arising from a greater number of 5-HT₃AR⁺ cell axons, as distinguished by the different Brainbow colors (Fig. 2e).

To determine the columnar and laminar organization of individual L1 cells targeting these PV cells, we further traced the dendritic and axonal arbors of 5-HT₃AR⁺ cells across the tonotopic axis of A1 (Fig. 2f). Although their dendrites generally remained confined to superficial cortical layers, many 5-HT₃AR⁺ cell axons descended vertically in a narrow cortical column and contacted postsynaptic PV cell targets within a tight span in L4 (average arbor width of 23 μm) along the rostro-caudal tonotopic axis (Fig. 2g).

5-HT₃AR⁺ cells gate a window of thalamocortical disinhibition. We then asked how these 5-HT₃AR⁺ interneuron projections affect the function of their L4 targets. We crossed 5-HT₃AR⁺-Cre mice with floxed Chr2 (A132) mice to generate a strain with selective expression of the light-activated channel rhodopsin-2 (Chr2) (Fig. 3a,b). To identify postsynaptic targets, we recorded from morphologically and electrophysiologically identified PV and pyramidal cells within...
L4 of A1 in acute slices (Fig. 3b). Optogenetic activation of 5-HT\textsubscript{3A}R\textsuperscript{+} interneurons produced fast, bicuculline-sensitive (GABA\textsubscript{A}R) inhibitory postsynaptic potentials (IPSPs) in PV cells, but generally mixed GABA\textsubscript{A}R and slow, SCH-50911-sensitive (GABA\textsubscript{B}R) IPSPs in pyramidal cells (Fig. 3c,d). In fact, 5-HT\textsubscript{3A}R\textsuperscript{+} cell–induced IPSPs recorded in pyramidal cells showed significantly longer half-widths and decays compared with those recorded in PV cells (Fig. 3d).

Prolonged IPSPs in pyramidal cells showed an enhanced late inhibitory phase (200–600 ms; Fig. 3d). Overall, this suggests that activation of 5-HT\textsubscript{3A}R\textsuperscript{+} interneurons produces distinct inhibition of PV and pyramidal cells in L4. The L1 interneuron population consists primarily of LS cells that target both dendritic GABA\textsubscript{A} and GABA\textsubscript{B} receptors, and non-LS cells that target primarily GABA\textsubscript{A}R synapses\textsuperscript{14}. We obtained paired recordings of these 5-HT\textsubscript{3A}R\textsuperscript{+} cell subtypes and PV cells in L4. Only spikes in the non-LS interneurons produced inhibitory postsynaptic currents (IPSCs) in these distant targets (Fig. 3h); non-LS, 2/7 tested connections; LS, 0/8 tested connections). These cells showed irregular spiking and fast after-hyperpolarizations, similarly to an L2/3 5-HT\textsubscript{3A}R\textsuperscript{+} cell population identified previously\textsuperscript{13}. These results reveal a novel connection between L1 interneurons and the PV interneurons below them.

We therefore recorded the functional effects of 5-HT\textsubscript{3A}R\textsuperscript{+} cell axons contacting PV and pyramidal cell somata. (L2/3: PV, 2.59 ± 0.20, n = 22 cells/4 mice; PYR, 1.34 ± 0.12, n = 53 cells/4 mice; two-tailed unpaired t-test, t(72) = 3.84, P = 0.0033. L5: PV, 3.43 ± 0.24, n = 44 cells/4 mice; two-tailed unpaired t-test, t(63) = −3.84, P = 0.0033.) Box plots show the median (center lines), lower and upper quartiles (box edges), minima and maxima (whiskers), and outliers (circles). Mean ± s.e.m. indicated by gray crosses. 

Fig. 2 | 5-HT\textsubscript{3A}R\textsuperscript{+} interneurons target intralaminal PV interneurons in L4. a–c, Brainbow-expressing 5-HT\textsubscript{3A}R\textsuperscript{+} and PV interneurons (white) in primary auditory cortex (A1) within a thalamocortical slice. Scale bars, 500 μm (a,b) or 50 μm (c). Representative image from 1 of 4 mice. d, Left, Brainbow-expressing 5-HT\textsubscript{3A}R\textsuperscript{+} cell axons form numerous putative contacts (white arrowheads) with a PV cell soma, but only sparsely contact a pyramidal cell soma (PYR; blue), in L4 of A1. Representative image from 1 of 4 mice. Scale bar, 10 μm. Right, the number of 5-HT\textsubscript{3A}R\textsuperscript{+} cell puncta forming putative contacts with PV and pyramidal (PYR) cell somata. (L2/3: PV, 5.05 ± 0.54, n = 22 cells/4 mice; PYR, 2.00 ± 0.20, n = 53 cells/4 mice; two-tailed unpaired t-test, t(27) = −5.31, P < 0.001. L4: PV, 5.30 ± 0.40, n = 43 cells/4 mice; PYR, 3.50 ± 0.24, n = 62 cells/4 mice; two-tailed unpaired t-test, t(72) = −3.84, P = 0.0033. L5: PV, 3.43 ± 0.24, n = 44 cells/4 mice; two-tailed unpaired t-test, t(63) = −3.84, P = 0.0033.) e, More 5-HT\textsubscript{3A}R\textsuperscript{+} cell axons target PV cell soma than pyramidal cell soma in cortical L2/3/4. Left, individual Brainbow-expressing 5-HT\textsubscript{3A}R\textsuperscript{+} cell axons forming putative contacts (colored arrowheads) with target cells can be distinguished by Brainbow color. Representative images from 1 of 4 mice. Scale bars, 10 μm. Right, the number of 5-HT\textsubscript{3A}R\textsuperscript{+} cell axons contacting PV and pyramidal cell somata. (L2/3: PV, 2.59 ± 0.20, n = 22 cells/4 mice; PYR, 1.34 ± 0.12, n = 53 cells/4 mice; two-tailed unpaired t-test, t(72) = 3.84, P = 0.0033. L5: PV, 3.43 ± 0.24, n = 44 cells/4 mice; two-tailed unpaired t-test, t(63) = −3.84, P = 0.0033.)
cells of L4 (Fig. 3e,f). Electrical stimulation of the MGB afferents triggers large EPSPs in PV cells that generally result in action potentials. When we coactivated 5-HT3AR+ cells, the amplitudes of these thalamocortical EPSPs were blunted (Supplementary Fig. 3) and PV-cell spiking was reduced (Fig. 3fg) for up to 100 ms, which indicated powerful descending control of feed-forward inhibition. Consistent with this, L4 pyramidal cells responded biphasically to 5-HT3AR cell activation were robustly suppressed (Fig. 3f,g).

To explore how these descending 5-HT3AR cell projections shape network activity across the tonotopic axis of A1, we used voltage-sensitive dye imaging (VSDI) to examine the effects of silencing of these cells on MGB-driven cortical responses. Silencing of 5-HT3AR+ cells decreased responses within L2/3 and L4 of the topographically active columns while laterally increasing responses in neighboring columns (Supplementary Fig. 4). Moreover, these effects occurred on different timescales corresponding to the distinct kinetics of EPSPs evoked in PV and pyramidal cells (Fig. 3a–d): the decrease in the MGB-activated column occurred during the early peak of the response, whereas the enhancement in the neighboring columns occurred during the later phase. Thus, 5-HT3AR+ cell activation orchestrates both temporal and spatial activity patterns in A1, rapidly sharpening activity within frequency columns by inhibiting PV cells directly below, while truncating responses outside the primary column through delayed lateral inhibition of pyramidal cells.

Silencing of 5-HT3AR+ cells prevents critical-period plasticity. A global tonotopic organization across the A1 rostro-caudal axis arises from the topographically organized projections from the MGB27,28. We previously showed that passive sound exposure during a brief critical period (postnatal days 12–15 (P12–15)) does not alter the thalamic tonotopic map, but induces robust shifts in the thalamocortical topography of L4 in mouse A1. We asked whether 5-HT3AR+ interneuron activity is important for such developmental rewiring.

We silenced 5-HT3AR+ cells in vivo during passive tone exposure via a chemogenetic approach28. We injected an AAV carrying an engineered transgene for a G-protein-coupled receptor (hM4DTAGI–mCherry) into A1 of 5-HT3AR-Cre pups and Cre-negative littermate controls at P2 (Fig. 4a). To verify our silencing method, we evaluated at P12–15 the ability of the agonist clozapine N-oxide (CNO) to reduce synaptic transmission from 5-HT3AR+ cells in A1 brain slices also expressing ChR2 (5-HT3AR-Cre×Ai32 mice). Optogenetic activation of 5-HT3AR+ cells evoked IPSCs in nearby L2/3 pyramidal cells as expected. Bath application of CNO (15µM) reduced this IPSC amplitude significantly when 5-HT3AR+ cells also expressed hM4D(Gi) and increased the paired-pulse ratio, thus validating this approach to reduce 5-HT3AR+ cell GABA release28 (Fig. 4b).
To investigate the effects of such 5-HT$_3$R$^+$ cell silencing on tonotopic plasticity, we then examined changes in connectivity between the MGB and A1 in thalamocortical slices. We exposed pups to a pulsed 7-kHz tone concurrent with CNO treatment (1 mg/kg body weight intraperitoneally (i.p.) twice per day) during the typical critical period from P12 to P15 before obtaining slices at P20. We stimulated the MGB at six different sites along the tonotopic lateral-medial axis to mimic tones at varying frequencies, and measured cortical responses by VSDI as described previously (Fig. 4c,d, Supplementary Fig. 5). To quantify thalamocortical topography, we plotted the location of the peak response ($\Delta F/F$) along the tonotopic rostro-caudal axis of cortical L4 as a function of MGB stimulation site (Fig. 4e).

We observed a significant decrease in the slope of the topographic curve that reflected plasticity after exposure to 7-kHz tone pips. Changes in thalamocortical connectivity were assessed by in vitro voltage-sensitive dye imaging at P20. b, hM4D reduces GABA release by 5-HT$_3$AR$^+$ cells. Left, representative 5-HT$_3$AR$^+$ cell-evoked IPSCs recorded in L2/3 pyramidal cells in response to paired pulses of laser light (arrowheads) before (Pre) and after CNO bath application (15 $\mu$M) in slices from a P13 hM4D-expressing mouse and a littermate control. Right, mean (and s.e.m.) normalized (norm.) amplitudes (control, 1.09 ± 0.10, n = 9 cells/2 mice; hM4D, 0.55 ± 0.18, n = 6 cells/3 mice; two-tailed unpaired t-test, t(13) = 2.89, P = 0.013) and paired-pulse ratios (control, 0.19 ± 0.06, n = 9 cells/2 mice; hM4D, 0.44 ± 0.09, n = 6 cells/3 mice; unpaired t-test, t(13) = 2.40, P = 0.032; interstimulus interval, 80, 80, 160, 240, 320, 400 ms). c, A schematic of an auditory thalamocortical slice illustrating the six stimulus positions in the ventral MGB (MGBv) and locations analyzed in L4 of A1. LGN, lateral geniculate nucleus. d, Left, an example of A1 responses ($\Delta F/F$) along the tonotopic latero-medial axis to mimic tones at varying frequencies, and measured cortical responses by VSDI as described previously (Fig. 4c,d, Supplementary Fig. 5). To quantify thalamocortical topography, we plotted the location of the peak response ($\Delta F/F$) along the tonotopic rostro-caudal axis of cortical L4 as a function of MGB stimulation site (Fig. 4e).

Nicotinic recruitment of 5-HT$_3$R$^+$ interneurons controls critical-period timing. The mechanisms underlying critical-period closure in A1 are largely unknown, but semplasticity studies of cholinergic manipulation in adults point to this pivotal neuromodulatory system. Thus, we asked whether the developmental loss of tonotopic plasticity is due to changes in the cholinergic recruitment of 5-HT$_3$R$^+$ cells. We found only modest changes in gene expression of nAChR subunits in FACS-sorted cells from A1 between P11 and P20, when tonotopic map plasticity winds down (Supplementary Fig. 6). Instead, we found a nearly twofold developmental increase in the expression of Lynx1, which encodes an endogenous prototoxin known to be expressed in cortical interneurons (Supplementary Fig. 7) and to inhibit nAChR function (Fig. 5a).

Consistent with the emergence of Lynx1, nAChR sensitivity of 5-HT$_3$R$^+$ cells was attenuated in adulthood. Nicotine (bath-applied; 10 $\mu$M) robustly increased spontaneous spiking of 5-HT$_3$R$^+$ cells in young pups during the critical period (P12–15), but this effect was largely lost 1 week later and beyond (Fig. 5b,c). In contrast, heightened nicotine sensitivity was retained in older Lynx1$^-/$ (Lynx1-knockout) mice. Nicotine further induced a barrage of inhibitory IPSCs in 5-HT$_3$R$^+$ cell–target PV and pyramidal cells in the mutant mice (Supplementary Fig. 8).

These nicotine-induced IPSCs persisted in mice with double deletion of Lynx1 and the gene encoding the a7 nAChR subunit (Chrnα7), as well as during blockade of a7 nAChRs by the selective antagonist methyl-lycaconitine (10 nM, bath-applied;
The ectopic plasticity persisted in mice with double deletion of Lynx1 and the gene encoding α7 nAChR (Chrna7) (Fig. 5e), which suggests, as above, that α7 nAChRs are not involved. Instead, administration of DHβE (1 mg/kg i.p. daily), which also did not alter peripheral hearing thresholds (Supplementary Fig. 9e–h), prevented the extended plasticity in Lynx1−/− mice (Fig. 5e).

Using in situ hybridization, we found that 90% of 5-HT3AR+ interneurons (36/40) coexpressed Chnna4 and Lynx1 mRNAs. To establish whether nAChR activation of 5-HT3AR+ cells is essential for the heightened plasticity in Lynx1−/− mice, we adopted an optogenetic approach. We generated triple-transgenic 5-HT3AR−/−/Chrna4Cre × Ai35 mice expressing an inhibitory opsin in α4-containing nAChR cells only when each tone was played. Photoinhibition of 5-HT3AR+ cells in vivo through a cleared skull cap abolished the sound-evoked thalamocortical plasticity in Lynx1−/− mice (Fig. 5f). Together, these results suggest that active downregulation of α4-nAChR signaling in 5-HT3AR+ cells contributes to the developmental loss of tonotopic plasticity.

The VIP+ 5-HT3AR+ cell subtype does not regulate critical-period plasticity. A subtype of 5-HT3AR+ interneuron that selectively expresses vasoactive intestinal peptide (VIP) sends descending axons to deeper cortical layers and disinhibits pyramidal cells13–14. Thus, we asked whether this subpopulation mediates PV-cell suppression in L4 and tonotopic plasticity.
VIP⁺ interneurons, often found in deeper layers⁴,⁵, received some VGluT2-expressing puncta from the MGB, but received significantly fewer than those on L1 VIP⁻ cells (Fig. 6a,b). Using in situ hybridization, we also found less mRNA encoding α4 nAChR and Lynx1 in VIP⁺ cells compared with the amount in the general 5-HT³A⁺ cell population (Fig. 6c,d). Notably, a subset of the 5-HT³A⁺ cells in A1 were VIP⁺ cells projecting preferentially to PV cells across cortical lamina. These IPSPs were smaller than those observed after activation of the general 5-HT³A⁺ population (Fig. 3d), causing only a rapid, modest suppression of thalamic-driven firing in PV cells, with little effect on EPSPs in pyramidal cells (Fig. 6j,k). Thus, VIP⁺ cells could contribute to intracolumnar PV-cell suppression, but do not act alone to disinhibit L4. Indeed, silencing of the VIP⁺ cell population alone did not significantly affect tonotopic plasticity (Fig. 6l), which highlights a role for non-VIP⁺ cells in orchestrating this developmental critical period.

A cortical L1 map tunes auditory cortex. Finally, we examined the precision of thalamic input to 5-HT³A⁺ cells. An assessment of topographic maps using VSDI showed that L1 activity mirrored that in L4 after MGB stimulation (Fig. 7a–d). This topographic organization had a linear slope in L1 similar to that observed in L4 (Fig. 7c).
To further determine whether MGB innervation of 5-HT<sub>3</sub>AR<sup>+</sup> cells in L1 is topographically organized, we used 5-HT<sub>3</sub>AR-Cre×Ai95 mice to express a calcium indicator (GCaMP6f) in 5-HT<sub>3</sub>AR<sup>+</sup> cells. Stimulation of discrete positions within either the medial or the lateral MGB activated 5-HT<sub>3</sub>AR<sup>+</sup> cells in rostral or caudal L1 of A1, respectively (Supplementary Fig. 10).

We next examined the origin of MGB axons projecting to cortical L1 by injecting fluorescent dyes conjugated to wheat germ agglutinin into L1 and visualizing retrogradely transported dye-labeled vesicles 24 h later (Fig. 7e–h). Focal, superficial injections of fluorescent dyes conjugated to wheat germ agglutinin into rostral and caudal sites within L1 of A1 from 3 mice. Scale bar, 500 µm. f, Normalized (norm.) positions of all MGBv cells containing dye-labeled vesicles after 4 injections in 3 mice (colors correspond to injections shown in e). Crosses indicate mean±s.e.m. caudal-rostral and medial-lateral positions for each injection group. g, A thalamocortical slice from a mouse injected with wheat germ agglutinin in rostral and caudal sites within L1 of A1. Scale bars, 500 µm (top) or 100 µm (bottom). h, Dye-labeled vesicles in caudolateral (top; blue) and rostromedial (bottom; orange) MGBv locations from caudal and rostral A1 injections, respectively. Scale bar, 10 µm.

Discussion

Our studies unveil a new picture of the neuronal network of 5-HT<sub>3</sub>AR<sup>+</sup> cells in cortical L1 (Supplementary Fig. 11). The canonical cortical circuit model relies on MBG axons terminating solely in L4. Our study shows that these inputs extend up into L1 and robustly innervate resident 5-HT<sub>3</sub>AR<sup>+</sup> inhibitory interneurons. This is in agreement with recent reports suggesting that superficially positioned interneurons may receive thalamic input<sup>26,27</sup> and respond to sound in A1<sup>28</sup>. This MGB input to L1 is precise, exhibiting a topographic map across the A1 tonotopic axis that mirrors the one in L4 (Fig. 7). Recent work has revealed that the dorsal lateral geniculate nucleus also sends retinotopically organized projections to superficial layers in the primary visual cortex that convey precise information about visual space<sup>29</sup>, direction, and orientation<sup>30</sup>. It will be interesting to determine whether sensory maps in superficial cortical layers generally modulate L4 activity to guide plasticity across modalities.

Maps in L4 and L1 in our study were independently controlled by early sensory experience: sound exposure robustly restructured L4, leaving L1 intact. This raises the possibility that thalamocortical inputs to L4 and L1 have distinct plasticity mechanisms. The excitation of L1 appears more focal than that observed in L4 (Fig. 7b,d), perhaps because it is restricted spatially by inhibitory interactions between L1 interneurons<sup>31</sup>. The L1 map may thus serve as a potentially stable reference scaffold available to rapidly mold L4 maps during periods of heightened plasticity throughout life. A similar strategy is used in the barn owl tectum, which can acquire multiple coexisting maps of interaural time difference during development to enable rapid adaptive plasticity in adulthood<sup>32</sup>.

Apart from being contacted by thalamic axons, 5-HT<sub>3</sub>AR<sup>+</sup> cells in A1 are activated by cholinergic inputs arising from the basal forebrain<sup>33</sup>. These afferents may preferentially relay phasic signals to 5-HT<sub>3</sub>AR<sup>+</sup> cells via nAChRs<sup>10</sup> (Fig. 1) with remarkable temporal precision in response to ethologically relevant stimuli, such as air puffs and water rewards<sup>34</sup>. Although generally thought to be broadcast across the extent of A1 to depolarize 5-HT<sub>3</sub>AR<sup>+</sup> cells homogeneously, these fast modulatory signals may serve to briefly lower the spiking threshold of L1 cells and switch on portions of the L1 map.
that are coactivated by precise MGB inputs. This circuit organization identifies L1 interneurons as hubs for the integration of diffusely projecting contextual signals with specific sensory stimuli. Thus, 5-HT_{3A}R^+ cells are uniquely poised to translate global changes of neuromodulation into focal plasticity.

The 5-HT_{3A}R^+ cells have been shown to act within various circuits across the cortex\textsuperscript{10-17}. We here demonstrated a direct connection from L1 interneurons to L4 PV interneurons. Through differential targeting of subcellular domains and the use of distinct postsynaptic inhibitory timescales, 5-HT_{3A}R^+ cells differentially affect thalamic drive onto PV and neighboring pyramidal cells in L4. Precisely timed activation of L1 interneurons suppressed thalamic-driven spikes in PV cells, thereby disinhibiting pyramidal cells (Fig. 3). Consequently, L1 interneuron activation generates time windows of enhanced thalamic drive onto L4 pyramidal cells that may support the induction of spike-timing-dependent plasticity, a potential mechanism underlying sound-evoked shifts in A1 tuning\textsuperscript{6}. Thus, the relative timing of L1 and L4 activation may be a key determinant of plasticity.

It is important to note that 5-HT_{3A}R^+ interneurons are a heterogeneous population. They may act in various ways to constrain cortical activity and plasticity in a temporospatially precise manner. Although the two major subtypes, LS and non-LS interneurons, were both robustly activated by MGB inputs (Fig. 1), they may act on different postsynaptic elements. The LS subtype often targets dendritic GABA_A and GABA_B receptors, and thus is likely to produce the prolonged GABA_B-receptor-mediated inhibitory potentials we observed in pyramidal cells. These interneurons send lateral projections and may act to reduce prolonged activity outside the cortical column (Supplementary Fig. 4). Conversely, the non-LS subtype sends narrowly descending projections, targeting GABA_A receptors on PV interneurons within L4 (Figs. 2 and 3).

A subset of these non-LS cells that expresses VIP is also found in deeper cortical layers, and targets both somatostatin and PV interneurons\textsuperscript{18-20}. VIP^+ cells received fewer vGluT2\textsuperscript{+} puncta than VIP^− cells did (Fig. 6), consistent with the weak thalamic innervation of VIP^+ cells observed in recent optogenetic studies\textsuperscript{6}. VIP^+ cells also expressed fewer mRNAs encoding nAChR subunits (Fig. 6), and may instead express more 5-HT_{3A}R_{S}\textsuperscript{+}, which suggests that VIP^+ cells and VIP^− cells are engaged by distinct neuromodulators. Silencing of VIP^+ interneurons, however, did not have a significant effect on critical period plasticity in A1, in agreement with recent observations in visual cortex that indicate no role before P30\textsuperscript{21}. Together, these findings point to a non-LS subtype that lacks VIP as the pivotal player in A1 development. It will be important to identify molecular markers for L1 interneuron subtypes with distinct connectivity patterns in order to determine their separate roles in the regulation of cortical plasticity.

Seminal work in kitten visual cortex indicates a requirement for both noradrenergic and cholinergic fibers in ocular dominance plasticity\textsuperscript{3}. Cholinergic inputs target diverse cortical cell populations to influence network activity through both ionotropic nAChRs and G-protein-coupled muscarinic receptors. Notably, both nicotinic disinhibition and muscarinic enhancement of thalamic glutamate release by suppression of adenosine signaling may act synergistically to induce thalamocortical plasticity in mature A1\textsuperscript{14}. Although all cholinergic actions must be considered, young 5-HT_{3A}R^+ cells in L1 are rapidly and robustly activated through ionotropic nAChRs (Fig. 5). These interneurons express both α4 and α7 nAChRs (Fig. 1)\textsuperscript{21}, but the former may mediate the strong nicotine-induced spiking of these cells (Fig. 5, Supplementary Fig. 8). Whereas α7 nAChRs quickly desensitize and contribute only transiently to the activation of 5-HT_{3A}R^+ interneurons, α4-nAChR signaling was necessary for tonotopic plasticity (Fig. 5e) and may likewise play a key role in the deprivation-induced plasticity of mouse somatosensory cortex\textsuperscript{28}. Notably, cholinesterase inhibitors, which can reinstate A1 plasticity in periods beyond early life (Fig. 5d), selectively augment α4-nAChR responses\textsuperscript{22}. The developmental reduction of α4-nAChR signaling by the appearance of Lynx1 in 5-HT_{3A}R^+ cells (Fig. 5a–c) may then explain how this protein serves as a cortical plasticity ‘brake’\textsuperscript{23,24}.

So far, the effect of 5-HT_{3A}R^+ cells on the developing cortex has largely been ignored. Here we found that they may serve as endogenous regulators of PV cells and thus of critical-period plasticity. This may explain in part how perinatal exposure to serotonin reuptake inhibitors might accelerate plasticity windows for phoneme discrimination in human infants\textsuperscript{1}. Transient periods of reduced synaptic inhibition precede A1 receptive-field plasticity in vivo\textsuperscript{10}, and a rapid reduction of PV-cell spike rates precedes ocular dominance plasticity in the visual cortex during the critical period\textsuperscript{14}. Reversible shifts in PV-cell state correlate with adult hippocampal learning\textsuperscript{11}, whereas pharmacological resetting of PV cells reopens visual plasticity\textsuperscript{16}.

Studies in mature A1 have instead focused on neuromodulatory influences\textsuperscript{11,26,28}. Direct (basal forebrain)\textsuperscript{28} or indirect (vagal nerve) stimulation\textsuperscript{16} of cortical cholinergic release promotes adult auditory plasticity. Behavioral training with rewarding or aversive stimuli, known to rapidly engage cholinergic afferents\textsuperscript{25} and 5-HT_{3A}R^+ cells\textsuperscript{11,13,15}, also drives tone-evoked shifts in adult tonotopic maps\textsuperscript{12,26}. More recently, cholinergic signaling has been found to engage 5-HT_{3A}R^+ cell activity across cortical areas\textsuperscript{14,13} and facilitate adult plasticity in both visual\textsuperscript{12,14} and auditory systems\textsuperscript{11,15} (Fig. 5).

Our findings thus resolve a link between two long-standing models of cortical plasticity (PV-cell function and neuromodulation) through L1 cells. Engagement of contextual signals paired with training is needed to enhance plasticity even during early development. Phonetic learning can occur after brief exposure to structured communication during infancy, but requires social interaction with a tutor in both human adults\textsuperscript{12,14} and songbirds\textsuperscript{15}. By leveraging the L1 map to promote specific plastic change, neuromodulators, as well as inputs from diverse thalamic\textsuperscript{16} or cross-modal cortical areas\textsuperscript{17}, may influence cortical critical periods. Knowledge of how and when the L1 map can be engaged throughout life will provide key insights for educational and therapeutic strategies in which heightened plasticity is desirable.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-017-0064-2.

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Author contributions
A.E.T. and T.K.H. designed the study and wrote the manuscript. A.E.T. conducted the experiments and analyzed the data. J.W.L. and L.J.B. assisted with the Brainbow experiments, imaging and analysis.

Competing interests
The authors declare no competing financial interests.

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Brainbow expression in 5-HT\(_R\)\(^*\) cells and imaging. We injected the Brainbow virus\(^*\) into the primary auditory cortex (A1) of 5-HT\(_R\)^* cells (P41–42; two male and two female). We anesthetized mice with isoflurane and used sterile technique to prepare them for surgery. Using a stereotaxic apparatus, we made a craniotomy above A1, 2.5 mm posterior to bregma and 4.5 mm lateral to the midline. We made three superficial (300 \(\mu\)m) injections in the left hemisphere of A1 (500 nL per injection site) using a 10-\(\mu\)L Hamilton syringe (Hamilton; 76301) with a motorized piston (Narishige; speed: \(\pm 25 \mu\)m/min). To label MGB projections to 5-HT\(_R\)\(^*\) interneurons, we injected one Brainbow virus into A1 of adult 5-HT\(_R\)^* mice (>P60; two male and two female) as described above, and the other Brainbow virus, combined with Cre (1:100), into the MGB (1 \(\mu\)l per injection site: 3.28 mm posterior to bregma, 2.0 mm lateral to the midline, and 3.25 mm below the pial surface). The head wound was sutured, and mice were immediately given an injection of the nonsteroidal anti-inflammatory agent meloxicam. After a period of recovery on a heating pad with accessible food pellets and Hydrogel, the mice were returned to their home cages. The mice were given a second dose of meloxicam the following day.

After 3 weeks of infection, mice were perfused intracardially with saline followed by 4\% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) and then post-fixed overnight at 4 °C. Brains were washed in phosphate-buffered saline (PBS) and sectioned peripherorally (60–100 \(\mu\)m). Sections were blocked in StartingBlock (Thermo Scientific; 95%, with 4\% PBS and 1\% Triton X-100) for 1 h at room temperature (RT), then washed in PBS-T (PBS with 0.25\% Triton X-100) for five nights of incubation at 4 °C in the following primary–antibody cocktail: a base of 0.02\% NaPBS, with 1\% Triton X-100, chicken anti-GFP (Abcam; ab19700; 1:2,000), and three custom-made antibodies from Covance (rabbit anti-mCherry, guinea pig anti-mKate2, and rat anti-mTFP1). Other primary antibodies were goat anti-chicken 647 (A-21449), rabbit anti-VIP (ImmunoStar; 2077; 1:2,000), guinea pig anti-vGlut2 (Synaptic Systems; 135404; 1:500), and goat anti-choline acetyltransferase inhibitor (anti-ChAT; Millipore; AB144P; 1:100). Sections were washed in PBS-T and incubated for two nights at 4 °C in secondary antibodies. Secondary antibodies used were Dylight-conjugated donkey anti-rabbit 405 (Jackson ImmunoResearch; 1:500; 711–475–152), and Alexa dye-conjugated goat anti-chicken 488 (A-10809), goat anti-mouse 546 (A-10100), goat anti-rat 594 (A-10077), goat anti-guinea pig 647 (A-21450), goat anti-mouse 488 (A-10100), goat anti-rabbit 546 (A-11000), goat anti-guinea pig 546 (A-11076), goat anti-chicken 647 (A-21449), donkey anti-goat 546 (A-11056), and donkey anti-rat 594 (A-21209) (Life Technologies; 1:500). Finally, sections were washed in PBS-T, incubated for 30 min at RT in 1:500 Neurotrace 435/455 (Life Technologies) in PBS-T, and mounted with ProLong Gold (Life Technologies).

Images for analysis of Brainbow-labeled 5-HT\(_R\)^* cells were acquired on a Zeiss LSM 710 confocal microscope with a 63x/1.4 NA oil-immersion Plan-Neofluar objective (1.1x zoom), with voxels sized 94\(\mu\)m in XY and with a 250-\(\mu\)m Z-step, and with optical sections of \(-0.6 \mu\)m per channel (12x12 pixel, 16-bit line-averaging). Images for reconstruction of Brainbow-labeled cells were acquired with a 40x/1.3 NA oil-immersion Plan-Neofluar objective (1.1x zoom). Images were corrected for chromatic aberration in three dimensions with a custom ImageJ/Fiji plugin (D. Cai, University of Michigan), and global pixel-shift values were determined from a set of single-antibody, multi-label images acquired with the same settings.

To quantify Brainbow-labeled 5-HT\(_R\)^* cells input to PV cells and pyramidal cell somata, we manually counted boutons apposed to NeuroTrace-labeled somata using ImageJ/Fiji (Version 2.0.0-rc-151/4.99). Boutons were identified morphologically by the axon, and only boutons in projections that lay between the bouton and the cell body profile were considered. All boutons apposed to the cell body throughout its 3D extent were counted, and only cells fully contained within the stack were analyzed. We tracked liked-colored axonal segments back to a branch point to check whether they were part of the same axon (i.e., a terminal fork) and further disambiguated them by toggling the display of the fourth Brainbow channel—usually left unvisualized on three-color RGB displays to further enhance the channel. To quantify the percentage of 5-HT\(_R\)\(^*\) interneurons, we counted vGlut2^+ boutons apposed to VIP^+ or VIP^+ NeuroTrace-labeled somata. All boutons on the single z-plane in which the 5-HT\(_R\)^* somata was largest were counted.

Whole-cell recordings. Mice were anesthetized with isoflurane and decapitated. The brain was quickly removed in chilled oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 25 glucose, 2 NaHCO\(_3\), 2.5 KCl, 2 CaCl\(_2\), and 1 MgCl\(_2\). Thalamocortical slices were sectioned on a vibrating microtome (Leica Microsystems; VT1200S) and incubated at RT until being placed in a recording chamber. Patch pipettes (4–6 \(\mu\)M) were pulled with a DMZ micropipette puller (Dagan Corporation). Cell capacitance was compensated and the initial series resistance was compensated amplified (Axon Instruments); low-pass filtered at 2 kHz, and digitized with an ITC-18 (InstruTech). Recordings were performed at RT (22–24 °C). Custom-designed IGOR (WaveMetrics, Version 6.36) programs were used for data acquisition and analysis.

All electrophysiological recordings were carried out with experiments for the genotypic identification and drug condition of the subjects. To measure EPSPs evoked by pressure application of nicotine in m or CPBG (100\(\mu\)M) in 5-HT\(_R\)\(^*\) cells in cortical L1, we carried out whole-cell current-clamp recordings in adult (>P60) male and female mice. The current-clamp internal solution contained 150 mM KCl, 1.8 mM GTP sodium salt, and 10 phosphocreatine disodium salt (pH adjusted to 7.2 with KOH). Whole-cell current-clamp recordings were also used to measure the effects of nicotine on spontaneous spiking of 5-HT\(_R\)^* cells in cortical L1. For these experiments, the ACSF was modified to contain 3.5 mM KCl and 0.5 mM MgCl\(_2\) to enhance spontaneous activity for a 450 s baseline measurement, nicotine was bath-applied (10 \(\mu\)M) for 900 s, and then washed out for 900 s. The nicotine effect was calculated as the peak spiking response (bins of 100 s) during nicotine application divided by the mean spiking rate across the whole recording period. Only cells that showed spontaneous spiking responses were analyzed. To measure the effects of bath-applied nicotine (10 \(\mu\)M) on spontaneous IPSCs (sIPSCs), we obtained whole-cell recordings from L4 pyramidal cells in 5-HT\(_R\)^* mice by their soma shape under infrared differential interference contrast imaging (IR-DIC). The nicotinic effect was calculated as the peak sIPSC frequency (bins of 100 ms) during nicotine application divided by the baseline sIPSC frequency. In some experiments, MLA (10 \(\mu\)M) and DHβE (500 \(\mu\)M) were continuously bath-applied throughout the experiment, starting 15 min before baseline IPSC measurements were obtained. sIPSCs were analyzed offline with MiniAnalysis Program (Synaptosoft, Version 6.0.7).
To verify our method of opticogenetic 5-HT3AR\(^+\) cell silencing and assess the effects on A1, we carried out VSDI experiments at P12–15 in 5-HT3AR\(^+\) R-Cre \(^{x} \) Ai35 pups. Responses to MGBv stimulation (0.5 mA, 1-ms pulse) were examined across A1 by VSDI during the presence and absence of long laser light pulses (IkeCoo 595-nm laser; 20-µm fiber) directed toward L1. The location within A1 across the A1 rostro-caudal axis showing the maximal response was designated as the activated column. When possible, VSDI acquisition and analysis were performed blind to genotype/condition (5-HT3AR\(^+\) cell-silencing experiments).

For GCaMP imaging, thalamocortical slices were generated from 5-HT3AR\(^+\) R-Cre \(^{x} \) Ai95 mice (P15–22) that expressed the genetically encoded calcium indicator (GCaMP6f) in 5-HT3AR\(^+\) cells. Sites within the lateral or medial MGBw were stimulated (0.5 mA, 1-ms pulse) and imaged as described above, except with a 497-nm excitation/535-nm emission filter. Fluorescent signals were integrated across trials. To control for constant depth and distance (25–150 µm from the pial surface) using the methods described above.

**Sound exposure.** Mice were placed in a sound-attenuating chamber (IAC) and passively exposed to 7-kHz tones (100-ms pulses at 5 Hz for 1 s, followed by 2 s of silence, 78-dB sound pressure level (SPL)). A 7-kHz frequency was chosen because it is the middle of the sound spectrum for mouse pup wriggling calls\(^{29}\) and thus is ethologically relevant for mice of this age. Mice were otherwise reared under standard conditions (12:12-h light-dark cycle; access to water and food ad libitum). Tones were generated by Audacity software (http://audacity.sourceforge.net; Version 2.1.2) and delivered by speakers placed in two corners of the chamber. Mice pups exposed to test tones during the critical period (P12–15) were placed in the sound-attenuating chamber and exposed with their mothers, and then returned to standard housing conditions until P20. Some mice were administered a cholinesterase inhibitor (AChEe; physostigmine, Sigma-Aldrich; 0.1 mg/kg i.p. daily) or an n4-containing nAChR antagonist (DHβE; Tocris Biosciences; 1 mg/kg i.p. daily) during sound exposure.

**Silencing of 5-HT3AR\(^+\) and VIP\(^+\) cells.** For in vivo chemogenetic silencing, P2 5-HT3AR\(^+\) R-Cre or VIP-Cre mouse pups and Cre-negative littermates were cwayneanesthetized and injected with AAV-hSyn-DIO-mH4D(Gi)-mCherry (UNC; 300 nl) in superficial layers of the left primary auditory cortex (A1) using a 10-µl Hamilton syringe (Hamilton; 765301) with a beveled needle and a motorized piston (Narishige; speed, ~50 nl/min). To activate the mH4D(Gi) receptor, we administered CNO (Tocris; 1 mg/kg i.p. twice daily) during sound exposure (P12–15). To verify our method of silencing\(^{29}\), we injected AAV-hSyn-DIO-mH4D(Gi)-mCherry into 5-HT3AR\(^+\) R-Cre \(^{x} \) Ai32 pups and Cre-negative Ai32 littermates controls at P2 and carried out in vitro experiments at P12–15.

For in vivo opticogenetic silencing, the clear skull procedure\(^{89}\) was performed on P20 mice. Mice were anesthetized with isoflurane and prepared for surgery by sterile technique. A stereotactic apparatus was used to make a small incision in the skin above the primary auditory cortex, 2.5 mm posterior to bregma and 4.5 mm lateral to the midline. A thin layer of cyanoacrylate glue (Krazy Glue) was applied to the area. Once dry, this was covered with a thin layer of clear dental cement (Ortho-Jet). The area was then polished and a layer of clear nail polish was applied (Electron Microscopy Sciences; 72180) to reduce light glare. Finally, a plastic sheath was secured with glue (Locite) and dental cement to allow an optical fiber (20µm; Doric) to be mounted above the auditory cortex. Animals were placed in a sound-attenuated chamber, and the optical fiber was connected to a LED using a 1× microscope port (Doric), connected to dual LED, LED2C-B/A (FC), which permitted animals to move freely about the cage. During sound exposure, light pulses were delivered (595 nm; 4 mW measured at skull surface) during the 1-s delivery of tones to silence 5-HT3AR\(^+\) cells in 5-HT3AR\(^+\) R-Cre \(^{x} \) Lynx1-‐knockout \(^{x} \) Ai35 mice. Control experiments were performed in 5-HT3AR\(^+\) R-Cre \(^{x} \) Lynx1-‐knockout \(^{x} \) Ai35 littermates stimulated by blue (473 nm) light or Cre-negative Lynx1-‐knockout \(^{x} \) Ai35 littermates stimulated by 595-nm light.

**RNA in situ hybridization.** Adult male C57 mice (P102) were anesthetized by isoflurane and decapitated. The brains were rapidly removed and frozen in medium (Tissue-Tek) on dry ice. Coronal brain slices (25µm) containing A1 were cut with a cryostat (Leica CM 1900), adhered to SuperFrost Plus slides (VWR), and immediately refrozen on dry ice. In situ hybridization was performed with the protocol provided in the RNAsecope Multiplex Assay manual (Advanced Cell Diagnostics). The following fluorophore-conjugated RNA probes were used: Mmn-Lynx1-c1 (Cat No. 449071), Mmn-Htr3a-c2 (Cat No. 411141), Mmn-VIP-c2 (Cat No. 415961), and Mmn-Chrna4-c3 (Cat No. 429787). Confocal images were acquired with a Zeiss LSM 710 confocal microscope using a 63×/1.4 NA oil immersion PlanAPO objective (1.1x zoom).

**Auditory brainstem responses and distortion-product otoacoustic emissions.** Adult (P>60) mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, and placed in a sound chamber lined with anechoic foam. Three needle electrodes were placed subdermally: (1) behind the right ear pinna (recording electrode), (2) at the dorsal midline between the ears (reference electrode), and (3) at the base of the tail (ground common). We elicited auditory brainstem response
(ABR) potentials by presenting 5-ms tone pips (0.5–ms rise-fall, with a cos² envelope, at 40/s) at frequencies ranging from 5.6 kHz to 32 kHz to the right ear. The response was amplified (10,000×), filtered (0.1–3 kHz), and averaged with an analog-to-digital board in a PC-based acquisition system (EPL Acoustic system, MEEl, EPL Cochlear Function Test Suite, Labview). The SPL was increased in 5-dB steps from 10 to 80 dB. ABR waveforms were averaged from 512 presentations of the tone in alternating phase. The ABR threshold was defined as the minimal SPL that evoked a response by visual inspection.

Distortion-product otoacoustic emissions (DPOAEs) in response to two primary tones of frequency f1 and f2 were recorded at 2f1 – f2, with f2/f1 = 1.2 and the f2 level 10 dB lower than the f1 level. Ear-canal sound pressure was amplified and digitally sampled at 14-μs intervals. Fast Fourier transforms were computed from averaged waveforms of ear canal SPL, and the DPOAE amplitude was extracted at 2f1 – f2. The background level of ambient noise was measured, defined as the average of six points in the fast Fourier transform on either side of the 2f1 – f2 frequency and surrounding noise. Iso-response curves were obtained in 5-dB steps of the f1 level. Threshold was defined as the f1 level required to produce a DPOAE signal at 0-dB SPL.

Stimuli were generated with 24-bit digital I–O cards (National Instruments PXI-4461) in a PXI-1042Q chassis, amplified by an SA-1 speaker driver (Tucker–Davis Technologies, Inc.), and delivered to two electrostatic drivers (CUI CDMDG18008–03A). An electret microphone (Knowles FG-23329-P07) was monitored to monitor ear-canal sound pressure. The Auditory Waveform Analysis software (https://bitbucket.org/bburian/auditory-waveform-analysis; version 1.0.0.6) was used for ABR analysis.

To determine whether DHJHE affects peripheral hearing, we carried out ABR and DPOAE measurements in Lynx1-knockout mice before and 30 min after administration of DHJHE (1 mg/kg i.p.).

Labelling of retrogradely transported vesicles from cortical L1. Fluorescent dyes (Alexa Fluor 488, 555, 594, or 647) conjugated to wheat germ agglutinin were stereotaxically injected into L1 within A1 of adult C57BL/6J mice (>P60; three male and three female). Four superficial (~50 μm below the pial surface) injections were made within the left hemisphere of A1 (100 nl per injection site) along the rostral–caudal tonotopic axis (2.2–3.5 mm posterior to bregma) using glass pipettes pulled with a DMZ micropipette puller attached to a 5-motorized piston (speed, ~25 nL/min). The head wound was sutured, and mice were immediately given an injection of meloxicam and allowed to recover on a heating pad with accessible food pellets and HydroGel. The mice were returned to their home cages, and 24 h later they were perfused intracardially with saline followed by 4% PFA in 0.1 M PB and then post-fixed overnight at 4°C. Brains were washed in PBS, and thalamocortical slices (100 μm) were sectioned on a vibrating microtome (Leica Microsystems, VTT1200S). Sections were incubated for 2 h at RT in 1:200 NeuroTrace 435/455 (Life Technologies) in PBS-T, and mounted using ProLong Gold (Life Technologies). Images were acquired on a Zeiss LSM 710 confocal microscope using a 40x/1.3-NA oil-immersion PlanApo objective (1.1x zoom).

Statistical analyses. Statistical tests were done with JMP statistical software (SAS Institute, Version JMP Pro 12). For two-group comparisons, statistical significance was determined by two-tailed unpaired Student’s t-test for data distributed normally or by nonparametric two-tailed Mann–Whitney U-test for data not distributed normally. For comparison across groups with unequal variance, the unequal variance t-test with an unpoled standard error was performed. Multi-group comparisons were done by one-way ANOVA followed by Dunnett’s corrected test with a control (parametric) or Kruskal–Wallis test followed by Steel’s corrected test (nonparametric) with a control. A multivariate ANOVA was used to test for genotype differences in ABR hearing thresholds across tonal frequencies. A paired t-test was used to evaluate the acute effects of silencing of 5-HT₁A, R² cells on VSDI responses. The Shapiro–Wilk test was used to test for normality, and Levine’s method was used to test for equal variance. P values less than 0.05 were considered statistically significant.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. All data relevant to this work will be made available by the corresponding author upon request.

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Life Sciences Reporting Summary

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 Experimental design

1. Sample size
   Describe how sample size was determined.

   The sample size for each experiment was determined based on literature in the field and our prior experience with the analyses shown. The number of electrophysiological recordings required to obtain reliable measures of synaptic properties (Figs. 1c-e, 3, 4b, 5a-c, 5d-k, 5s, 58) was based on previous studies referenced within the manuscript (Ji et al., 2016; Cruikshank et al., 2013; Bennett et al., 2012; Lee et al, 2010; Takesian et al., 2010; Rose and Metherate, 2005). The number of mice required for reliable measurements of auditory thalamocortical connectivity (Fig. 4c-f, 6i, 7a-d, 54, 55) has also been determined previously (Hackett et al., 2011; Barkat et al., 2011).

2. Data exclusions
   Describe any data exclusions.

   The Methods section explains criteria that excluded data points. These criteria were established prior to data collection. Electrophysiological data were only acquired from cells with a resting potential less than -55 mV, an initial series resistance < 20 MΩ, and overshooting action potentials.

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   For all experiments, the number of repeating experiments is the n described within the figure legends and the manuscript. Given the biological variability of our measures across cells and animals, our studies are designed to compare population data. Our figures include the individual data points when possible. No attempts were made to replicate these population data.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   Some of the controls (Figs. 4, 5f, 6i) were WT mice within mixed litters of both WT and Cre-expressing mice. These controls were identified by genotyping only after completion of the experiment and analysis. For experiments examining the effects of drug administration (Fig. 5d,e), mouse pups were taken from the litter at random and administered either drug or vehicle. For these studies, the weight and sex of the animal was recorded at the time of experiment to ensure that there were no significant differences between groups. For developmental assessments (Fig. 5a-c, 5f, Fig. 56), mouse pups were randomly selected from litters at varying ages.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   In some experiments (Fig. 4, 5f, 6i), the mouse genotype was only determined after acquisition and analysis were performed. The acquisition and/or analyses of all voltage-sensitive dye imaging (VSDI) experiments were performed blind to the age, genotype, or drug condition when possible. All electrophysiological analyses were performed blind to the age, genotype, cell type, or drug condition.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- Confirmed

- □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- □ □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- □ □ A statement indicating how many times each experiment was replicated
- □ □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- □ □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- □ □ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- □ □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- □ □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

The software used to acquire and analyze the data in this study are described in the Methods.

- Image J/Fiji (NIH, http://imagej.net/Welcome, Version: 2.0.0-rc-15/1.49i) was used for image analysis.
- Custom IGOR Pro (WaveMetrics, Version 6.36) and Matlab (MathWorks, Version R2017a) scripts, and MicAM Ultima Software (Brainvision, Version 10.01.20) were used for analysis of voltage-sensitive dye imaging (VSDI) and electrophysiological experiments. These codes will be available upon request. The MiniAnalysis Program (Synaptosoft, Version 6.0.7) was used to analyze spontaneous currents.
- For sound exposure experiments, tones were generated by Audacity software (http://audacity.sourceforge.net/, Version 2.1.2).
- The Auditory Wave Analysis software (Buran, https://bitbucket.org/bburan/auditory-wave-analysis/src, Version 1.0.0.6) was used to analyze auditory brainstem responses (ABRs).
- JMP statistical software (SAS Institute, Version JMP Pro 12) was used for statistical analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All reagents used are commercially available, with the exception of the Lynx1 KO x α7-nACHR KO and PV-GFP mice, which are available from the corresponding author on reasonable request.
9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Details about the antibodies used in this study are provided in the online Methods (Brainbow expression in 5-HT3AR cells and Imaging) and listed below. The validation of these antibodies were provided from the companies that provided the antibodies and listed citations.

Primary Antibodies:
- Chicken anti-GFP (Abcam, ab13970; 1:2000),
- Custom-made antibodies from Covance (rabbit anti-mCherry, guinea pig anti-mKate2, & rat anti-mTFP1.0).
- Mouse anti-Parvalbumin (Swant, 235; 1:300)
- Rabbit anti-vasoactive intestinal protein (Immunostar, 20077; 1:200)
- Guinea pig anti-vesicular glutamate transporter 2 (VGluT2; Synaptic Systems, 135404; 1:500)
- Goat anti-choline acetyltransferase inhibitor (Chat; Millipore, AB144P; 1:100).

Secondary antibodies:
- DyLight-conjugated donkey anti-rabbit 405 (Jackson ImmunoResearch, 1:500 711-475-152), and alexa dye-conjugated goat anti-chicken 488 (A-11039), goat anti-mouse 546 (A-11030), goat anti-rat 594 (A-11007), and goat anti-guinea pig 647 (A-21450), goat anti-mouse 488 (A-11001), goat anti-rabbit 546 (A-11010), goat anti-guinea pig 594 (A-11076), goat anti-chicken 487 (A-21449), donkey anti-goat 546 (A-11056), and donkey anti-rat 594 (A-21209; Life Technologies, 1:500).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. This study did not contain cell lines.
b. Describe the method of cell line authentication used. This study did not contain cell lines.
c. Report whether the cell lines were tested for mycoplasma contamination. This study did not contain cell lines.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. This study did not contain cell lines.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Description of mouse strains, sex, and ages used are stated in Methods (Mice) within the manuscript. A description is repeated below:

Wild-type (C57Bl6/J; JAX 000664), PV-RES-Cre (B6;129P2-Pvalbtm1(cre)Arbr/J; JAX 008069), SOM-RES-Cre (SSTtm2.1(cre)Zjh/J; JAX 013044), Ai35 (B6;129S-Gt(ROSA)26Sortm35.1(CAG-aop3/GFP)Hze/J; JAX 012735), Ai32(Chr2(H134R)-EYFP; JAX 012569), and Ai95 (RCL-GCaMP6F)-D; JAX 024105) breeders were purchased from Jackson Laboratories. S-HT3AR-Cre (Tg(Htr3a-cre)NO152Gsat/Mmucd; MMRRC) breeders were purchased from MMRRC. Lynx1 knockout (KO) breeders were provided by J. Miwa (LeHigh University), and Lynx1 KO x α7-nAChR KO breeders were provided by H. Lester (California Institute of Technology). PV-GFP67 transgenic mouse breeders were provided by H. Monyer (Heidelberg University). Both male and female mice aged postnatal day (P) 1 to 150 were used for experiments.
Policy information about studies involving human research participants

12. Description of human research participants
Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.