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# Asynchronous development of the mouse auditory cortex is driven by hemispheric identity and sex

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Lateralized auditory processing is essential for specialized functions such as speech processing, typically dominated by the Left Auditory Cortex (ACx) in humans. Hemispheric specializations also occur in the adult mouse ACx, but their developmental origins are unclear. Our study finds that the Left and Right ACx in mice reach developmental milestones at different ages. Thalamocortical responses and maturation of synaptic dynamics develop earlier in the Right ACx than the Left. We show that this timing offset predicts hemisphere-dependent differences in sensory-driven plasticity. Juvenile tone exposure at specific times results in imbalanced adult tone frequency representations in the Right ACx plasticity occurs before male Right ACx, and female Left ACx aligns with male Right ACx plasticity. Our findings demonstrate that sex and hemispheric identity drive asynchronous development and contribute to functional differences in sensory cortices.

In humans and mice, a fundamental feature of mature auditory sensory processing is the allocation of specialized cognitive functions to the Left and Right ACx<sup>1-3</sup>. Failure of the ACx to develop lateralized function is a common endophenotype of human cognitive disorders, such as Autism Spectrum Disorders and Schizophrenia<sup>4,5</sup>. In children, the development of functionally lateralized auditory evoked potentials occurs during the first 3 years of life<sup>6,7</sup>, and abnormal auditory experiences during infancy lead to degraded language abilities<sup>8</sup>. Given the significance of lateralized auditory processing in human cognition, having an animal model that captures this fundamental functional feature would be extremely valuable for mechanistic studies. As a result, an increasing number of studies are investigating whether rodents, despite their simpler vocal communication systems, exhibit hemispheric specializations and are affected by hemisphere dysfunction in a similar manner to humans. In rodents, selective deactivation of one of the auditory cortices impairs distinct auditory processing functions9-11. Furthermore, prior work from our group has revealed hemispheric asymmetries in ACx circuit structure, establishing the mouse as a suitable model for mechanistic investigations<sup>2,3,12</sup>.

The foundation for long-term circuit structure is established during sensory critical periods, brief developmental phases when intense cortical plasticity tunes neuronal networks to best represent the sensory information in the current environment<sup>13</sup>. Sound exposure during the auditory critical period selectively shapes ACx representations, whether for experimentally controlled tone pips or for the cadence of a child's household language(s)<sup>14-16</sup>. In animal studies, altering the acoustic environment during postnatal development results in impaired spectral tuning and abnormal circuit patterns<sup>2,17,18</sup>. In addition to mechanisms of experience-dependent cortical plasticity underlying brief critical periods, there is also ongoing cortical maturation during postnatal development. Several hallmarks of mature cortical circuits appear in the first weeks of postnatal development. One significant change is thalamocortical axons migrating from the cortical subplate and neurons in deep cortical layers forming arborizations and synapses in the adult thalamorecipient layers<sup>19-21</sup>. Additionally, as cortical maturation progresses, both excitatory and inhibitory synaptic dynamics go through major transitions<sup>22,23</sup>. Synaptic connectivity, receptor subunit composition, and vesicle

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release dynamics, among many other developmentally regulated processes, have been shown to undergo shifts during cortical maturation<sup>23-27</sup>. Importantly, existing studies of cortical maturation are unilateral (i.e., carried out in one hemisphere) or often do not report the hemisphere studied, so the point at which specializations arise in homotopic regions of cortex is unknown.

Here, we determine when signs of mature cortical circuits emerge in Left and Right ACx during postnatal mouse development. Surprisingly, we find that ACx circuits in the two hemispheres mature asynchronously. We then test whether temporally offset maturation also correlates with temporally offset critical periods, and whether this mechanism contributes to imbalanced sensory representations, thereby driving specialized functions in the adult brain. Importantly, we find that sex also determines the timing of ACx maturation and critical period plasticity. Based on these results, we argue that asynchronous cortical development is a mechanism capable of generating lateralized cortical processing.

## Results

Crucial insights into mesoscale dynamics of developing and mature auditory cortical circuits have been revealed by physiological assays performed in vitro using connected auditory thalamocortical (TC) slice preparations<sup>19-21</sup>. As described, this preparation involves the loss of one hemisphere to achieve the correct compound slice angle. To compare Left and Right ACx circuits in the same animal, we devised a new preparation technique for simultaneous retrieval of connected TC slices from both hemispheres (Fig. 1a, see "Methods" section). Directly comparable to previous studies, we record optical signals (Fig. 1b) in voltage-sensitive-dye (VSD) stained slices (Fig. 1c) to capture the full range of sub- and suprathreshold neuronal depolarizations driven in the ACx by electrical activation of TC axons.

As expected, auditory TC responses in both Left and Right ACx of male mice change significantly across postnatal maturation (Fig. 1d). Consistent with the standing model of immature TC responses initiating in the cortical subplate<sup>20,28-30</sup>, we found that in postnatal (P) ages <P12, the Left ACx TC response arises first in lower layers and shows delayed activation of layers 2/3-4 (Fig. 1d, e). By contrast, at ages older than P16, the thalamocortical response in the Left ACx initiates in laver 4 (L4) and lower L2/3, indicating maturity of the TC projection pattern. We then asked whether this spatial shift in TC response location occurs during the same developmental timeframe in the Right ACx (Fig. 1d, e). Surprisingly, in age- and animal-matched slices, the laminar shift in TC responses is significant in the Right ACx at earlier ages compared to the Left ACx (P14–P16, Fig. 1e, f). Consequently, between hemispheres, there is a significant difference in thalamocortical response location at ages P14-P16, with the Right ACx displaying this sign of maturity and the Left ACx appearing less mature (Fig. 1f).

Previous studies have shown that relative response latency within layers is another metric to indicate the spatial density of direct thalamic input<sup>20,31</sup>. In population-averaged contour plots reporting the spatial distribution of response latencies, the region of earliest response is spatially shifted to upper layers in the mature ACx (Fig. 1g). Again, at ages P14–P16, this shift is apparent in the Right ACx and not yet present in the Left ACx. Thus, across age- and animal-matched animals, both spatial and temporal data indicate that the migration of thalamocortical axons to mature recipient layers occurs earlier in the Right ACx.

If structural changes in TC projection patterns exhibit asynchronous maturation between Left and Right ACx, we reasoned that other mature circuit phenomena might also emerge in the two hemispheres at different ages. We measured spontaneous miniature postsynaptic currents in ACx Layer 4 neurons using whole-cell voltage clamp recordings in bilateral horizontal slice preparations. We analyzed changes in both inhibitory (mIPSCs) and excitatory (mEPSCs) events across ages in pyramidal cells in L4 of age- and animal-matched Left and Right ACx of male mice (Figs. 2 and 3, see "Methods" section).

First, we observed that the frequency of mIPSCs increases significantly at P12–15 in the Right ACx but not the Left ACx (Fig. 2b, c). Moreover, the average frequency of mIPSCs remains stable in the Right ACx with no further changes beyond the P12–15 time window (Fig. 2b–d). Interestingly, the Left ACx undergoes more complex fluctuations in mIPSC frequency during development. At P16–20, there is a brief increase in average frequency and shortened interevent intervals in the Left ACx (IEIs, Fig. 2b–d), consistent with prior findings of transient hyperconnectivity in cortical layers<sup>32</sup>. We did not observe this transient mIPSC frequency increase in the Right ACx, suggesting that absolute differences may exist between the maturation pathways of the two auditory cortices.

After P20, mIPSC average frequencies and IEI distributions in the Left ACx are comparable to the same measures in mature Right ACx (Fig. 2e). Next, we examined the decay time constant of mIPSCs, which has been shown to decrease as intracortical inhibition matures<sup>23,33,34</sup>. As expected, we found that the decay time shortens with age in both auditory cortices (Fig. 2f). However, the Right ACx demonstrated a shift to shorter decay times at P12-15 and no further changes with age (Fig. 2f-h). By contrast, in the Left ACx the age-related decrease was less abrupt and did not reach significance until later ages (Fig. 2g, h). These observations may reflect lateralized changes in GABAA receptor kinetics during development, potentially due to changes in receptor subunit composition<sup>24</sup>. We found mIPSC amplitude to be stable across ages and hemispheres, with no significant trends between age groups (Supplementary Fig. 1a, b). Trends in hemispheric-specific time differences in the maturation of mEPSCs (Fig. 3) are similar to those observed for mIPSCs. A transient surge in the average frequency of mEPSCs is also observed in the Left ACx at P16-20 (Fig. 3c, d). In the Right ACx, mEPSC frequency matures earlier than in the Left ACx (Fig. 3d), similar to the trend observed for mIPSCs. Other mEPSC parameters did not significantly differ between the hemispheres (Supplementary Fig. 1c-f).

Time periods of experience-driven cortical plasticity lead to the formation of finely-tuned representations of the sensory environment in the mature brain<sup>13-16</sup>. To determine if the asynchronous maturational events in the ACx have an impact on experience-dependent plasticity, we tone-reared mouse pups with patterned 7 kHz pure tone pips<sup>18,20</sup> from P12-15. During this time, our in vitro TC maturation results predict that in male mice, the Right ACx is transiently more sensitive to the sensory environment compared to the Left ACx (Fig. 1e, f). After the tone-rearing period, mice were returned to the colony until adulthood. To measure the impact of this juvenile transient tone exposure on mature tonotopic representations, we performed in vivo, anesthetized, bilateral extracellular recordings using multichannel silicon probes in mature (P35-57) male mice. Spike times were determined blind to tone stimuli presentation and were grouped into clusters based on spatiotemporal template matching using Kilosort (see "Methods" section). Spike clusters were included in determining the tone response properties of a given location based on the presence of spikes time-locked to tone presentation (see "Methods" section).

Confirming our hypothesis, in adult male mice briefly exposed to 7 kHz tones as juveniles, the Right ACx is significantly more responsive to tones close to 7 kHz–within a third of an octave–when compared to the Left ACx (Fig. 4a–d). Recording locations in the Right ACx are more likely to have a higher percentage of isolated clusters responsive to 7 kHz tones, and the fraction of locations with more than 50% responsive clusters is also greater in the Right ACx (Fig. 4c). In addition, there is a statistically significant contingency between brain hemisphere and an individual cluster's responsiveness to 7 kHz tones (Fig. 4d, Fisher's exact test, p = 0.0015). There was no trend in the position of recordings within the Left and Right ACx craniotomies, distributed across the anterior to posterior axis, which roughly aligns



**Fig. 1** | **Hemispheric differences in the maturation of thalamocortical input to the ACx. a** Connected bilateral TC slice preparation developed to study the maturation of the Left and Right ACx in the same animal. **b** Experimental set-up for VSD imaging. **c** Picture of a connected TC slice stimulated in the MGBv with ACx labeled where voltage changes were measured. **d** Movie frames (4 ms rate) for Left and Right ACx TC responses; first frame: first movie frame determined to show a significant TC response (see "Methods" section). **e** Cumulative binned depths of responsive locations for pixels in the upper quartile (>75th percentile) of signal magnitude for the first frame. Left ACx: blue, Right ACx: red; mean: solid lines, 95% confidence intervals: dotted lines. Circles indicate 25th, 50th (median), and 75th percentiles of the population average and S.E.M. There was no significant difference in the youngest group <P14 (Left panel, unpaired Welch's *t*-test, Holm–Šídák method of correction for multiple comparisons; *n* = 9 and 7 slices for Left and Right ACx, respectively). A significant difference between the Left and Right ACx was observed at P14-16 (middle panel: 25th percentile *p* = 0.00107, 50th percentile

with the expected tonotopic axis (Fig. 4e). Similar trends are observed in mice recorded at ages later than the reported critical period for sweeps (>P40, Supplementary Fig. 2a-c)<sup>18</sup>. We did not observe an overrepresentation of 7 kHz in either hemisphere in naïve mice, consistent with previous studies (Supplementary Fig. 2d-f)<sup>16,17,20,35-38</sup>. These results demonstrate that tone-rearing during brief periods of asynchronous hemisphere development can create an imbalance in the tone frequency representations found in the Left and Right ACx of adult male mice.

Previous tone-rearing studies have reported experiencedependent plasticity in both the Left and Right  $ACx^{17,18,20}$ . Importantly, these studies were either performed in female animals or were based on data sets for which sexes were combined. To determine whether the sex of the animal was correlated with the

p = 0.000939, 75th percentile p = 0.0041; n = 7 and 6, unpaired Welch's *t*-test, Holm–Šídák correction). There was no significant difference in the oldest group (>P16, right panel, n = 9 and 5; unpaired Welch's *t*-test, Holm–Šídák correction). Within a hemisphere, filled circles indicate a statistically significant change from the youngest age group (empty circles). See Supplementary Table 1 for withinhemisphere statistical test results. **f** Centroid of response calculated for the first frame across age and hemisphere groups. The centroid was not significantly different for ages <P14 nor >P16 (unpaired Welch's *t*-test, Holm–Šídák correction; for <P14 n = 9 and 7; for >P16 n = 9 and 5). The only significant difference in centroid response between the Left and Right ACx occurred at ages P14–16 (p = 0.000747, n = 7 and 6; unpaired Welch's *t*-test, Holm–Šídák correction; See Supplementary Table 1 for between-hemisphere statistical tests for these data). Filled circles and empty circles as in **e. g** population-averaged contour plots reporting the spatial distribution of thalamic input response latency. Data are presented as mean values ± SEM. All statistical tests performed were two-sided.

hemisphere(s) affected by tone rearing, we measured the impact of tone exposure on females in the same time window as males (P12–15). We performed in vivo, anesthetized, bilateral extracellular recordings between P35–57. Surprisingly, in adult female mice tone reared from P12–15, we find significant expansion of 7 kHz representation in the Left ACx compared to the Right ACx (Fig. 4f–j). Thus, adult male and adult female tone frequency maps are imbalanced in opposite directions for the same juvenile tonerearing history. If our hypothesis of asynchronous maturation, with the Right ACx maturing before the Left ACx, applies to both sexes, then the observation that the Left ACx is plastic in females during the same time window as the Right ACx in males suggests an overall temporal offset in maturation between the sexes. Therefore, we predicted that the Right ACx matures earlier in



**Fig. 2** | **Hemispheric differences in the maturation of inhibitory synaptic input in the ACx. a** Slices were collected from both hemispheres for age-matched and within-animal comparison of mIPSCs. **b** Sample traces of mIPSCs recorded at four ages starting from prior to ear canal opening (P10) to the end of the tone critical period (P21). **c** Cumulative histograms of mIPSC interevent interval from the Left and Right ACx at 4 age groups. **d** Quantification of developmental changes in mean mIPSC frequency within each hemisphere. (Comparison to the youngest group within each hemisphere. Left ACx: <P12 (n = 12 cells) vs. P12–15 (n = 7), p = ns; <P12 vs. P16–20 (n = 8), p = <0.0001; <P12 vs. >P20 (n = 5), p = 0.0236. Right ACx: <P12 (n = 15) vs. P12–15 (n = 7), p = 0.0038; <P12 vs. P16–20 (n = 9), p = 0.0015; <P12 vs. >P20 (p = 7), p = 0.0055, Kruskal–Wallis, post hoc Dunn's multiple comparisons test). **e** Comparison of developmental changes in mean mIPSC frequency between the hemispheres (<P12, p = ns, n = 12 and 15 cells, Left and Right ACx, respectively; P12–15,

females than in males. To test for signs of earlier ACx circuit maturation in females, we returned to VSD imaging of connected TC slices. Indeed, in female pups, TC projections appear mature in both hemispheres at P16 (Fig. 5a, b), an age where the Left ACx of males is still immature (Fig. 1e, f; Fig. 5b gray).

These unexpected findings prompted us to investigate whether female Right ACx demonstrates critical period plasticity in response to tone rearing during an even earlier time window. We performed tone exposure in females from P9–12 and measured tone representations across Left and Right ACx in adulthood (Fig. 5c). Consistent with our hypothesis, we find a significant expansion of 7 kHz responsiveness in

*p* = 0.031099, *n* = 7 and 7 cells; P16–20, *p* = 0.032951, *n* = 8 and 9; >P20, *p* = ns, *n* = 5 and 7, Multiple Mann–Whitney test, Holm–Šídák correction). **f** Normalized mIPSCs illustrate developmental changes in the decay time constant. **g** Quantification of mIPSC decay time constant maturation within each hemisphere. (Comparison to the youngest group within each hemisphere. Left ACx: <P12 (*n* = 14) vs. P12–15 (*n* = 5), *p* = ns; <P12 vs. P16–20 (*n* = 8), *p* = ns; <P12 vs. >P20 (*n* = 5), *p* = 0.0109. Right ACx: <P12 (*n* = 18) vs. P12–15 (*n* = 9), *p* = 0.0076; <P12 vs. P16–20 (*p* = 9), *p* = 0.0318; <P12 vs. >P20 (*n* = 6), *p* = 0.0277, Welch's ANOVA test, post hoc Dunnett's *t*-test). **h** Comparison of developmental changes in mIPSC decay time constant between the hemispheres (<P12, *p* = ns, *n* = 14 and 18; P12–15, *p* = 0.04821, *n* = 5 and 9; P16–20, *p* = ns, *n* = 5 and 6 unpaired Welch's *t*-test, Holm–Šídák correction). *N* = 14 mice. Data are presented as mean values ± SEM; the center of the error bars represents the mean. All statistical tests performed were two-sided.

Right ACx compared to Left ACx (Fig. 5d–f), resulting again in significantly imbalanced tone frequency representations between the hemispheres. Finally, we investigated whether tone exposure at later developmental time windows would lead to tonotopic map plasticity in the Left ACx in male mice (Fig. 6a). We first performed juvenile tone exposure at P16–19 and investigated tone-specific map expansion in adulthood. Interestingly, we do not observe an increase in the representation of 7 kHz in the Left ACx compared to the Right ACx of adult male mice (Fig. 6b–d). Next, we performed juvenile tone exposure at P18–21 and again found no significant hemispheric difference in the overrepresentation of 7 kHz in adult tone maps (Fig. 6e–g). These



**Fig. 3** | **Hemispheric differences in the maturation of excitatory synaptic input in the ACx. a** Voltage clamp recordings were performed sequentially from excitatory neurons in L4 of the Left (left traces) and Right (right traces) ACx (order was randomized between animals). Sample traces of mEPSCs recorded at three ages: P15, P19, and P22. b Cumulative histograms of mEPSC interevent interval from the Left and Right ACx at 4 age groups. c Comparison of the ages to half-peak and max mEPSC frequency maturation between the Left (P15.6–19.2) and Right (P13.9–17.1) ACx indicated by green arrows. Solid lines are polynomial fits to the daily averages of the data collected. d Quantification of developmental changes in mean mEPSC frequency within each hemisphere. (Comparison to the youngest group within

results suggest that innate limitations may exist on tone frequency map plasticity in the male Left ACx.

Overall, our findings suggest that both brain hemispheres and sex play significant roles in determining the timing of local developmental events and the timing of plasticity for tone frequency representations in the Left and Right ACx. Determining how these factors contribute to the asynchronous development and plasticity observed in the ACx may be significant for understanding functional specializations in the brain.

## Discussion

We set out to determine when and how Left and Right auditory cortical circuits diverge during development. We identify temporal shifts in maturation trajectory as a potential mechanism for establishing different sensory representations in homotopic cortical regions. We chose both network-level indicators (thalamocortical axon migration) and cellular-level indicators (spontaneous synaptic events) of maturity. These indicators represent conserved developmental changes that take place in many sensory cortical areas<sup>24,39,40</sup>. We show that these signs of maturity arise in the Right ACx days earlier than in the Left ACx, resulting in a time window of hemisphere-specific circuit organization. Signs of structural and functional maturation in the auditory cortex do not, however, indicate whether the circuit mechanisms necessary to produce overrepresentation of a tone frequency are active. Maturation refers to many developmental processes and the stabilization of adult neuronal connectivity patterns, whereas critical period plasticity involves the capacity for change and adaptation to the sensory environment within those circuits<sup>27</sup>. To establish whether tone plasticity time windows are also offset between the hemispheres, we adopted the classic juvenile tone-rearing approach, which has been



each hemisphere. Left ACx: <P12 (n = 5 cells) vs. P12–15 (n = 19), p = ns; <P12 vs. P16–20 (n = 15), p = <0.0002; <P12 vs. >P20 (n = 8), p = ns. Right ACx: <P12 (n = 8 cells) vs. P12–15 (n = 16), p = ns; <P12 vs. P16–20 (n = 16), p = 0.0022; <P12 vs. >P20 (p = 8), p = ns, Kruskal–Wallis, post hoc Dunn's multiple comparisons test). **e** Comparison of developmental changes in mean mEPSC frequency between the hemispheres (<P12, p = ns, n = 5 and 8 cells, Left and Right ACx, respectively; P12–15, p = ns, n = 19 and 16 cells; P16–20, p = 0.014, n = 15 and 16; >P20, p = ns, n = 8 and 8, Multiple Mann–Whitney test, Holm–Šídák correction). N = 17 mice. Data are presented as mean values ± SEM; the center of the error bars represents the mean. All statistical tests performed were two-sided.

shown to modify tone frequency representations in adult auditory cortex<sup>16,17,20</sup>.

Our in vitro and in vivo results together are broadly consistent with previous studies demonstrating that maturation of thalamocortical projections is closely correlated with the critical period for tonotopic map plasticity in the ACx<sup>20</sup>. However, the development of other indicators of cortical maturity, such as spontaneous synaptic current dynamics, may not always align precisely with specific experience-dependent plasticity windows. For instance, previous studies in the ACx have shown that the balanced state of excitatory and inhibitory synapses observed in the adult is not achieved until closer to P30<sup>15,41</sup>. It is therefore possible that the maturational trajectory of PSCs and E-I balance could play a more profound role in separate, later critical periods in the ACx (e.g., sweep sensitivity)<sup>18</sup>. Similarly, other physiological properties determining network functions mature over time, including shifts in the intrinsic excitability of neurons and the repertoire of active currents expressed in neuronal subtypes<sup>27,42</sup>. Future work will be required to fully characterize the relative timing of molecular, cellular, and functional events linking cortical maturation with plasticity windows.

In early postnatal development of the mouse, rapid changes occur both within the animal (e.g., ear canal opening) and in its environment (e.g., littermate vocalizations). As a result, the acoustic inputs shaping circuit plasticity can change dramatically on the timescale of days. It follows that a hemisphere-dependent shift in the timing of molecular events driving experiencedependent circuit maturation has the potential to precipitate the lateralized functionality found in adult cortical circuits and disrupted in various brain disorders<sup>4,5</sup>. Our observation of earlier Right ACx maturation in mice is reminiscent of earlier right









organized along the anterior-posterior axis of the ACx. f Female mouse pups were exposed to 7 kHz tone pips between P12-15. Between P35-57 bilateral extracellular recordings were performed. g Examples of individual neuron tone responses (circled in tessellation maps) for the Left and Right ACx. Raster plots, peristimulus time histograms, and normalized tuning curves are shown for each neuron. The same analyses described above (c-e) were performed for recordings in female mice. h Percentage of putative individual clusters responding to 7 kHz at a given location (Mann–Whitney, p = 0.0129 violin plots), and pie charts showing the fraction of locations with more than 50% of clusters responsive to 7 kHz (Fisher's exact test p < 0.0001). i Fisher's exact test (two-sided) indicates that in females, the Left ACx had more clusters responsive to tones close to 7 kHz compared to the Right, p < 0.0001. Left ACx n = 58 and Right ACx n = 52 total tone-responsive recording locations from 4 mice. j Anatomical positions of all recording locations organized along the anterior-posterior axis of the ACx. Dashed lines through violin plots depict quartiles. Data are presented as mean values ± SEM; the center of the error bars represents the mean.

hemisphere maturation reported in the human auditory system<sup>7,43</sup>. In both humans and rodents, it has been proposed that the protracted maturation of the left hemisphere may render those circuits more vulnerable to injury<sup>44</sup>. On the other hand, delayed maturation of the Left ACx may also facilitate its ability to fine-tune circuits for other acoustic parameters, such as spectrotemporal sensitivity, which is crucial for recognizing the statistical structure of species-specific vocalizations<sup>1,45-47</sup>.

Importantly, we identify sex as a critical factor influencing the timing of cortical plasticity. Sex hormones are known to play a significant role in the timing of cortical maturation<sup>48</sup>. Additionally, neuromodulatory systems such as acetylcholine, noradrenaline, and



**Fig. 5** | **The sex of the animal also influences maturation and plasticity. a** VSD imaging analysis performed in P16 females as described in Fig. 1d. Movie frames (4 ms rate) for Left and Right ACx TC responses; first frame: first movie frame determined to show a significant TC response (see "Methods" section). b (left **panel**), Cumulative binned depths of responsive locations from the Left and Right ACx in P16 females (blue and red solid lines, respectively) are overlaid on top of P16 males (shown in gray, see Fig. 1e). **b (right panel**), Centroid of response calculated for the first frame for P16 females and hemisphere groups (color dots) overlaid on male data (in gray). *N* = 3 mice, *n* = 2 and 2 slices for Left and Right ACx. **c** Female mouse pups were exposed to 7 kHz tone pips between P9–12. Between P35–57, bilateral extracellular recordings from the Left and Right ACx were performed under anesthesia using 32-channel, dual-shank silicon probes.

**d** Percentage of putative individual clusters responding to 7 kHz at a given location (Mann–Whitney, p = 0.0039 violin plots), and pie charts showing the fraction of locations with more than 50% of clusters responsive to 7 kHz (Fisher's exact test p = 0.0281). **e** The relationship between the brain hemisphere and the responsiveness of individual clusters to 7 kHz tones is statistically significant (solid = responsive, empty = not responsive). The Fisher's exact test (one-sided) indicates that the Right ACx had more clusters responsive to tones close to 7 kHz compared to the Left, p < 0.0001. Left ACx n = 42 and Right ACx n = 42 total recording locations, from 3 animals. **f** Anatomical positions of all recording locations organized along the anterior-posterior axis of the ACx. Dashed lines through violin plots depict quartiles. Data are presented as mean values ± SEM; the center of the error bars represents the mean.

oxytocin also contribute to sex-specific cortical developmental processes, including the development of synaptic dynamics, plasticity, and neuronal excitability<sup>49</sup>. While the influence of sex hormones on auditory system function has been documented<sup>50-53</sup>, their impact on development remains largely unexplored. Our results thus offer new insights and introduce a novel model system to study the poorly understood earlier maturation of auditory processing observed in human females<sup>54</sup>, and differences in plasticity between the sexes<sup>55,56</sup>.

While sexual dimorphism and left-right hemispheric asymmetry have been described for hippocampal circuits in animal models<sup>57,58</sup>, far less is known about functional lateralization and sex differences in cortical circuits<sup>59,60</sup>. Studies of lateralized function in auditory cortex are emerging, and our findings contribute to the idea of temporal differences leading to predictable imbalances of sensory maps. One surprising exception was our inability to induce lasting changes to tone frequency representations in the male Left ACx. By contrast, female Left ACx does not appear to have this same limitation. Whether there are processes in males that are actively preventing Left ACx plasticity or favoring Right ACx plasticity will require further mechanistic investigation. Together, our results provide an essential temporal framework for future discovery of circuit mechanisms driving cortical plasticity and emphasize important modifications to classic tone-rearing protocols.

Finally, our results argue against experimental designs in which corresponding brain areas are assumed to be identical across hemispheres and therefore suitable for combining or for providing control data for unilateral manipulations. Similarly, the sex-specific developmental trajectories we report here underscore the importance of separating female and male experimental groups and, in some cases, making sex-specific modifications to experimental hypotheses. Further studies are needed to test whether the divergent maturational trajectories we observe here causally lead to sex-specific hemispheric specializations in healthy circuit structure and function.



**Fig. 6** | **The male Left ACx is not responsive to juvenile tone-rearing history. a** Male mouse pups were exposed to 7 kHz tone pips between P16–19 and P18–21. Between P35–57 bilateral extracellular recordings from the Left and Right ACx were performed under anesthesia using 32-channel, dual-shank silicon probes. **b**–**d** (P16–19 exposure), Percentage of putative individual clusters responding to 7 kHz at a given location (Mann–Whitney, *p* = ns violin plots), and pie charts showing the fraction of locations with more than 50% of clusters responsive to 7 kHz (Fisher's exact test *p* = ns). **c** The relationship between the brain hemisphere and the responsiveness of individual clusters to 7 kHz tones is not statistically significant (solid = responsive, empty = not responsive). The Fisher's exact test (one-sided) indicates that the Left ACx did not have more clusters responsive to tones close to 7 kHz compared to the Right, *p* = ns. Left ACx *n* = 106 and Right ACx *n* = 86 total recording locations, from 7 animals. **d** Anatomical positions of all recording locations are organized along the anterior-posterior axis of the ACx. **e**–**g** Male mouse pups were exposed to 7 kHz tone pips between P18–21. Between

## Methods

## Acute slice preparation and voltage-sensitive dye imaging

Experiments were performed using male and female CBA/J mice in strict accordance with the National Institutes of Health guidelines, as approved by The City College of New York and WashU Institutional Animal Care and Use Committee. Mice were kept in the vivarium under a 12-h light/dark cycle and maintained at 23 °C and 40% relative humidity. For in vitro studies, male mice aged P8–P25 (and female mice aged P16) were anesthetized with 4% isoflurane and then decapitated. Brains were removed and placed into chilled carbogen-bubbled cutting solution composed of (in mM): 110 choline chloride, 25 NaHCO3, 25 d-glucose, 11.6 sodium ascorbate, 7 MgCl<sub>2</sub>, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, and 0.5 CaCl<sub>2</sub>. In experiments where thalamocortical connectivity was not required, slices were cut along the horizontal plane on a Leica vibratome, using standard in vitro slice preparation



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P18

P21

P35–57 bilateral extracellular recordings from the Left and Right ACx were performed under anesthesia using 32-channel, dual-shank silicon probes. **e** Percentage of putative individual clusters responding to 7 kHz at a given location (Mann–Whitney, p = ns violin plots), and pie charts showing the fraction of locations with more than 50% of clusters responsive to 7 kHz (Fisher's exact test p = ns). **f** The relationship between the brain hemisphere and the responsiveness of individual clusters to 7 kHz tones is not statistically significant (solid = responsive, empty = not responsive). The Fisher's exact test (one-sided) indicates that the Left ACx did not have more clusters responsive to tones close to 7 kHz compared to the Right, p = ns. Left ACx n = 32 and Right ACx n = 30 total recording locations, from 2 animals. **g** Anatomical positions of all recording locations are organized along the anterior-posterior axis of the ACx. Dashed lines through violin plots depict quartiles. Data are presented as mean values ± SEM; the center of the error bars represents the mean.

procedures with cyanoacrylate glue and a flat stage. The tissue blocking approach to retrieve connected thalamocortical slices from both hemispheres is depicted in Fig.1b. First, the brain was hemisected along the midline to expose the surface of each hemisphere along the sagittal plane. The cut surfaces were then affixed with cyanoacrylate glue to a rectangular block of -3% low-melting agarose as shown. The agar block and two brain hemispheres were glued to a 15-degree wedge, which was printed out of Nylon 12 by Shapeways (Livonia, MI, USA).

Once slices were obtained, they were transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 127 NaCl, 25 NaHCO<sub>3</sub>, 25 Dglucose, 2.5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 1.25 NaH<sub>2</sub>PO<sub>4</sub> and continuously bubbled with carbogen. Slices were incubated in a recirculating chamber filled with ACSF warmed to 32 °C for one hour and then held at room temperature for the duration of the experiment. For voltagesensitive dye (VSD) imaging preparations, slices were individually stained for 40–90 min in a miniaturized recirculating bath chamber filled with 15 mL total of room temperature, carbogen-bubbled ACSF with the addition of 15  $\mu$ L of 5 mg/mL Di-4-ANNEPS (Thermofisher #D1199) in high purity ethyl alcohol (EtOh), so that the final concentration of dye in the chamber was 5  $\mu$ g/mL and the final concentration of EtOH was 0.1% by volume. The stained slice was then placed in a large volume (-300 mL) recirculating, carbogen-bubbled, room temperature ACSF incubation chamber for a minimum of 20 min to remove excess unbound dye and any particulate accumulation before imaging.

For VSD optical signal acquisition, individual slices were transferred to a room temperature submersion-style recording chamber mounted on a modified upright microscope (BX51-WI; Olympus). Optical recordings were obtained as a single trial, 4 ms frame rate, 1028 ms total length, movies using a CCD camera (MiCam02, Brain-Vision) and corresponding supporting hardware and software from BrainVision. Illumination was delivered using a halogen lamp (MHAB-150W, Moritex), and a dichroic filter cube was custom designed to maximize the excitation, collection, and rejection of the appropriate optical spectra (excitation Edmund #86-354, emission Edmund #84-745, dichroic Semrock FF560-Di01-25×36). Light was delivered and collected via a 4× objective (NA, 0.28; Olympus) and passed through a 0.25× demagnification step (U-TVO.25XC; Olympus) before reaching the camera, resulting in measured pixel dimensions of approximately  $31 \,\mu\text{m} \times 36 \,\mu\text{m}$ . The timing of the lamp shutter and electrical stimulus delivery was precisely controlled by the Brainvision camera system. Electrical stimuli consisted of single 100 µs pulses of constant current delivered to the thalamocortical axon bundle, medial in the slice with respect to the rostral tip of the hippocampus, using an AMPI stimulus isolation unit triggered by an FHC Pulse-01 and single pole tungsten electrodes modified to be 50-200 kOhm in resistance.

### VSD movie analysis

For VSD image processing, we combined and adapted procedures reported in previous studies<sup>20,31,61</sup>. Between 2 and 6 trials at a given stimulus electrode location were collected as time-series movies, averaged frame-by-frame, and then smoothed with a 3×3-pixel flat filter. To align slices across experiments and account for non-standard camera pixel arrangement, movie frames were linearly interpolated to a grid of locations spaced evenly 25 µm in each direction and rotated so that the vertical axis of the grid was perpendicular to the layered organization of the cortex in the region of the ACx. Frames were then individually smoothed with a 3 × 3-pixel Gaussian filter and cropped to a rectangle of 1400 µm in the vertical dimension (across cortical layers) and 800 µm in the horizontal (anterior-posterior). There was no signal conditioning in time. In voltage-sensitive dye signals of this nature, an increase in cellular membrane voltage is observed as a decrease in raw signal amplitude. Therefore, we invert the signal polarity such that membrane depolarization is reflected as an increase in the optical signal. We present the stimulation-elicited change in fluorescence (dF/F) in terms of z-score, or number of standard deviations above the baseline subtracted mean, to account for differences in technical and biological variability in the slice preparations.

To describe and compare the spatiotemporal dynamics of the cortical response in a movie, we first determined the movie frame demonstrating the earliest indication of a significant response after the stimulus (termed "first frame" in Fig. 1d). To make the determination of the first frame, a threshold was applied to the image ( $60 \times 80$  pixels) and the number of non-contiguous pixels (each  $25 \,\mu\text{m} \times 25 \,\mu\text{m}$ ) with a value higher than that threshold were counted. A table was generated for each movie wherein the first frame was calculated for a range of threshold values (z-score 2–7.5 in steps of 0.5) and area values (4–84 pixels in steps of 4 pixels). The most commonly occurring value (mode) in each table was selected to be the first frame for that movie.

To assess the spatial location of the initial response over the cortical layers, we binarized the first frame for each movie with a z-score threshold set to the 75% signal level and binned the depths of the above-threshold pixel locations into cumulative histograms starting at the cortical surface. This cumulative depth histogram approach (Fig. 1e) aids both interpretation and statistical testing of the distribution of the response across the population, since the cortical depths containing specific percentiles of the total responding areas are represented. Across the age and hemisphere groups, the average depth histograms are reported along with 95% confidence intervals (Fig. 1e solid and dashed lines, respectively). In addition, we indicate mean and S.E.M. of the 25th, 50th (median), and 75th percentiles in Fig. 1e (circles).

For a single value to quantify the mean response location across cortical layers, we calculated the vertical location of the centroid of the first frame image using the equation:

$$C_{y} = \frac{\Sigma_{i} C_{iy} A_{i}}{\Sigma_{i} A_{i}}$$
(1)

We further sought to quantify the response across time as an indication of direct functional connections to a given location. To account for biological variability and preparation-related differences in absolute latency, we report latency across the population as time elapsed from the first frame time, determined as described above. To generate average latency contour plots for age and hemisphere groups, contour plots were first calculated for individual movies, smoothed with a 5-pixel sliding box average across only the horizontal dimension (to aid alignment over the anterior-posterior axis), and averaged (Fig. 1g).

### Whole-cell voltage clamp recording analysis

For intracellular recording of spontaneous miniature postsynaptic currents, excitatory neurons located in layer 4, approximately 350-450 µm in depth from the cortical surface and 50-80 µm below the cut surface of the slice, were visualized using infrared gradient contrast optics and patched with glass electrodes (6-7 MOhm) containing the following intracellular solution (in mM) 128 K-methylsulfate, 4 MgCl, 10 HEPES, 1 EGTA, 4 NaATP, 0.4 NaGTP, 10 Naphosphocreatine, and 0.01 QX-314 (pH 7.25 and 300 mOsm). The ACSF bath contained 1 µM TTX and 50 µM D-AP5. Additionally, for mIPSC recordings, the ACSF bath also contained DNQX (10 µM), and for mEPSC recordings Gabazine (10 µM). Recordings were made in wholecell voltage clamp mode using a Multiclamp 700B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, California, USA). We measured inhibitory currents at a holding potential of 0 mV. We measured excitatory currents at a holding potential of -70 mV. Recordings were performed sequentially from excitatory neurons in L4 of the Left and Right ACx (order was randomized between animals). We used the custom software package ephus (http://www.ephus.org) for instrument control and acquisition written in Matlab (MathWorks, Natick, MA, USA).

### Software and statistical analyses

The investigators were aware of the experimental preparation conditions, but the probe tone-rearing data analyses were performed blind to sample identity. The sample size was determined based on findings in related literature, rather than on a predetermined calculation. For the detection of minis, we used synaptosoft http://bluecell.co.kr/ theme/theme05/product/product\_02\_01.php. For statistical tests, we used Graphpad Prism version 9.5.1 (GraphPad Software, San Diego, California, USA). All software implementation was executed on 64-bit Windows. All error bars shown in the figures are the standard error of the mean, and the center of the error bars represents the mean of the dataset.

For statistical analyses across both VSD and mPSC metrics, we first tested whether each group of measurements in the population was determined to be normally distributed by the Shapiro-Wilk test. When all groups were determined to be normally distributed, we performed Welch's ANOVA test followed by Dunnett's multiple comparisons between the age groups. To test differences across hemispheres, we performed an unpaired *t*-test with Welch's correction followed by Holm-Šídák multiple comparisons. For nonnormally distributed data, we used the non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons test between age groups. For hemisphere comparisons in non-parametric data, we applied the Mann–Whitney U-test followed by Holm–Šídák multiple comparisons. Within hemispheres, planned comparisons were made only between the youngest group and all other groups. Within-group outliers were removed as determined by the MATLAB function isoutlier, which identifies values more than 3 scaled median absolute deviations (MAD) away from the median value. We used a quartic polynomial regression to fit the daily averages of the mEPSC data collected.

## Extracellular recordings to map tone frequency representations

In vivo extracellular electrophysiological recordings were performed in mice aged P33-P57. We administered 75 mg/kg ketamine and 0.5 mg/kg medetomidine for anesthetized recordings. Anesthesia was supplemented during surgery and throughout the recordings as needed. Following anesthesia, mice were kept on a heating pad at 36-38 °C and placed in a stereotaxic instrument equipped with headfixed orbital bars and a bite bar. We made a craniotomy  $(2 \times 2 \text{ mm}^2)$  and durotomy over the ACx, centered around 1.5 mm anterior and 4 mm lateral to lambda. The exposed cortex was kept moist with cortex buffer (in mM) 125 NaCl, 5 KCl, 10 Glucose, 10 HEPES, 2 CaCl<sub>2</sub>, 2 MgSO<sub>4</sub> throughout the recording session. A 32-channel, 2-shank silicone probe (P1. Cambridge Neurotech) was inserted into the auditory cortex at a depth of  $0.6 \text{ mm} \pm (0.1 \text{ mm})$  from the tip of the probe. The probe's recording sites spanned 250 µm, covering mainly the granular layer, but also supra- and/or sub-granular layers. The probe was lowered at a speed of ~100 µm every 5 min. Recordings were obtained using Cheetah software (Neuralynx), with all channels sampled in continuous mode at 30.3 kHz. All recordings were done in a soundattenuated chamber, using a custom-built real-time Linux system (200 kHz sampling rate) driving a Lynx-22 audio card (Lynx Studio Technology, Newport Beach, California, USA) with an ED1 electrostatic speaker (Tucker-Davis Technologies, Alachua, Florida, USA) in a freefield configuration (speaker located 6 inches lateral to, and facing the contralateral ear). The stimuli were created with custom MATLAB scripts to compute tuning curves. We used a set of pure tones (16 frequencies, 3 amplitudes - 20, 40, 60 dB) that lasted 100 ms, with an inter-stimulus interval of 1 s.

For analysis of in vivo extracellular neuronal activity, we used Kilosort (https://github.com/cortex-lab/KiloSort) to extract spike times and determine putative single neuron spike clusters (referred to simply as "clusters" throughout the manuscript), followed by custom routines in MATLAB to determine the tone response properties of each spike cluster. A cluster was first determined to be responsive to tone presentations and included in further analysis based on the approach described by Shimazaki and Shinomoto, <sup>62</sup>, which determines the optimal kernel bandwidth of each peristimulus time histogram (PSTH, https://www.mathworks.com/matlabcentral/fileexchange/24913-

histogram-binwidth-optimization)<sup>62</sup>. Tone frequency tuning curves for each tone-responsive cluster were then computed by counting the total number of spikes occurring for each frequency. The tuning curve of each spike cluster was calculated and considered responsive to 7 kHz if the rate for any frequency within 1/3 octave of 7 kHz exceeded 2/3 of the maximum observed for that cluster. The percentage of 7 kHz responsive clusters was calculated for each recording location and associated with a particular spatial coordinate reflecting the probe shank position within the craniotomy. The sampling locations along the anterior-posterior axis are reported in Figs. 3 and 4. For each experimental group, both location percentages and cluster counts were compared between hemispheres using Mann–Whitney rank sum tests and Fisher's exact tests, respectively, as described in the figure legends.

## **Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

All the data values utilized for statistical comparisons, along with their corresponding statistics, are included in the Source Data file. The raw data from this study are available upon request from the corresponding author (H.V.O.), due to the dataset's size.

## **Code availability**

The code used to analyze VSDi and probe data is available in the Source Data of this paper.

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# Author contributions

A.P.R. performed and analyzed the VSDi experiments, D.N. and R.L. performed minis experiments, D.N. and A.P.R. performed in vivo experiments, C.P. and A.P.R. analyzed in vivo and minis data, and H.V.O. and A.P.R. prepared figures and wrote the manuscript with input from all co-authors. H.V.O. supervised all experiments and data analyses.

## **Competing interests**

The authors declare no competing interests.

## Additional information

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