ARTICLE

Silencing cortical activity during sound-localization training impairs auditory perceptual learning

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The brain has a remarkable capacity to adapt to changes in sensory inputs and to learn from experience. However, the neural circuits responsible for this flexible processing remain poorly understood. Using optogenetic silencing of ArchT-expressing neurons in adult ferrets, we show that within-trial activity in primary auditory cortex (A1) is required for training-dependent recovery in sound-localization accuracy following monaural deprivation. Because localization accuracy under normal-hearing conditions was unaffected, this highlights a specific role for cortical activity in learning. A1-dependent plasticity appears to leave a memory trace that can be retrieved, facilitating adaptation during a second period of monaural deprivation. However, in ferrets in which learning was initially disrupted by perturbing A1 activity, subsequent optogenetic suppression during training no longer affected localization accuracy when one ear was occluded. After the initial learning phase, the reweighting of spatial cues that primarily underpins this plasticity may therefore occur in A1 target neurons.

[https://doi.org/10.1038/s41467-019-10770-4]
Training can improve sensory skills in a range of tasks\textsuperscript{1}, and is useful for treating sensory disorders such as amblyopia\textsuperscript{2} or hearing loss\textsuperscript{3}, as well for reversing age-related deficits in sensory processing\textsuperscript{4}. Although plasticity of cortical function is generally considered to underpin perceptual learning, the processing stages involved remain controversial, with most of the evidence based on observation of physiological changes that correlate with improvements in performance\textsuperscript{5–8}. Fewer attempts have been made to investigate the causal contribution of individual brain regions to perceptual learning. This is challenging to do because manipulating neural activity in sensory areas may affect task performance, making it difficult to identify a specific role for those areas in learning.

An ability to localize sounds accurately and rapidly is of great importance for the way in which humans and other species perceive and interact with their environment. Localization in the horizontal plane relies principally on a comparison of sound reaching the two ears and is therefore impaired immediately following hearing loss in one ear\textsuperscript{6–8}. However, the auditory system can compensate for changes in the balance of inputs between the two ears, both during development\textsuperscript{9,10} and in later life\textsuperscript{6,8,12,13}, and maintain accurate sound localization despite the presence of a hearing loss of up to 40–50 dB in one ear.

In mammals, adaptation to hearing loss in one ear is based principally on a reweighting of sound localization cues, with increased dependence on the monaural spectral cues provided by the normal-hearing ear\textsuperscript{6,8,10,13}, although compensatory adjustments in binaural sensitivity have also been observed\textsuperscript{11,13–15}. Studies in adults have shown that adaptation depends on training\textsuperscript{6,8,13} and is disrupted in animals in which the primary auditory cortex (A1)\textsuperscript{16}, and specifically layer V corticocollateral projection neurons\textsuperscript{17}, are lesioned. Although indicating that early auditory cortex is required for adaptation to an imbalance in inputs between the two ears, these approaches lack the temporal specificity necessary to determine at what stage in the learning process A1 is involved or the contribution of different types of cortical neurons. In particular, it is not known whether cortical activity following sound presentation during training is sufficient for adaptation to take place or whether A1 is also required for learning retrieval when abnormal spatial cues are experienced again.

In this study, we used reversible optogenetic silencing to demonstrate a causal link between A1 activity and adaptation to monaural deprivation by plugging one ear. We were able to dissociate the contribution of A1 to normal localization behavior and learning, and show that activity in its high-frequency region, where spatially informative spectral features are represented, is required when sounds are presented during training for adaptation to take place. Finally, our findings indicate that retrieval of the adaptive changes induced during learning can occur independently of A1, suggesting that these are likely to be consolidated in neural circuits to which this cortical area projects.

Results

Optogenetic silencing of neurons in the auditory cortex. We trained adult ferrets to localize single bursts of noise that were presented from one of 12 loudspeakers positioned at 30° intervals in the horizontal plane (Fig. 1a). To examine the effects of perturbing A1 activity on the performance of the animals in this task, Archarcheodopsin T (ArchT) was expressed in A1 unilaterally by injecting AAV8/CAG-ArchT-GFP or AAV8/CaMKII-ArchT-GFP constructs in the dorsal part of the left middle ectosylvian gyrus (high-frequency A1) (Fig. 1b). We targeted the cortical region representing high sound frequencies (>8 kHz) because it is at these frequencies where direction-dependent spectral cues are most prominent in ferrets and where cue reweighting has been shown to occur in monaurally occluded animals\textsuperscript{10,13}.

ArchT expression was visualized by co-expressed green fluorescent protein (GFP), with the proportion of GFP neurons in the injected A1 (Fig. 2a, Fig. 3a, c) and presence of GFP axons and terminal fields in the contralateral auditory cortex (Fig. 2a, c) and in the auditory thalamus and midbrain (Fig. 2a, b, d, Fig. 3a, b, e, f) used to confirm the location and effectiveness of viral transfection. ArchT expressing cells were silenced by pairing pulses of green light (λ = 532 nm) generated by a compact diode-pumped solid-state (DPSS) laser, delivered via an optical fiber implanted over the location of viral injections in A1, with sound stimulus presentation (Fig. 1a, b, Supplementary Fig. 1).

Suppression of cortical activity during light delivery was demonstrated electrophysiologically, both in vivo in ferrets with each viral construct (Fig. 2e–h, Supplementary Fig. 2) and in vitro in mice (Supplementary Fig. 3). Multiunit responses to auditory stimuli were recorded at 99 locations in the high-frequency region of ferret A1. Acoustically evoked activity was significantly reduced for units recorded within the region where viral injections had been made and where the fiber-optic cannula was within ~800 μm of the recording probe. No changes in the firing rate of cortical cells were found at distances greater than this or when the recording probe and/or the fiber-optic cannula were not located within the viral vector injection site. Activity suppression had a rapid onset and a slower recovery in vitro (up to 2 s after turning off the laser, Supplementary Fig. 3) than in vivo (Fig. 2 and Supplementary Fig. 2), where baseline activity was typically restored within 0.5 s, resembling the temporal dynamics of ArchT-mediated inactivation described in monkey cortex\textsuperscript{18}.

Sound localization under normal hearing conditions. As expected, the animals localized broadband noise more accurately than narrowband noise bursts, as shown by the higher proportion of correct responses (Fig. 1c; Wald $\chi^2_{df = 1} = 1444.3$, $P < 0.0001$) and the lower proportion of both front–back (Fig. 1d; Wald $\chi^2_{df = 1} = 393.4$, $P < 0.0001$) and left–right errors (Fig. 1e; Wald $\chi^2_{df = 1} = 454.7$, $P < 0.0001$). In keeping with previous studies\textsuperscript{19}, localization performance declined for both types of stimuli as the duration was reduced, resulting in fewer correct responses (Wald $\chi^2_{df = 6} = 8241.3$, $P < 0.0001$) and more front–back (Wald $\chi^2_{df = 6} = 1042.3$, $P < 0.0001$) and left–right errors (Wald $\chi^2_{df = 6} = 403.6$, $P < 0.0001$). During these sessions, green laser light pulses were delivered to the implant simultaneously with the auditory stimulus on 50% of randomly interleaved trials. No differences in sound localization accuracy (Wald $\chi^2_{df = 1} = 0.176$, $P = 0.675$), front–back errors (Wald $\chi^2_{df = 1} = 0.468$, $P = 0.494$), or left–right errors (Wald $\chi^2_{df = 1} = 0.003$, $P = 0.953$) were found when A1 activity was suppressed unilaterally (Fig. 1c–e), regardless of the location or duration of the sound stimulus. Response times were faster on correct trials (mean ± SD, 1.75 ± 0.37 s) than incorrect trials (1.96 ± 0.61 s) (analysis of the variance (ANOVA), $F_{1,46054} = 402.902$, $P < 0.0001$), and were not affected by optogenetic suppression of A1 (ANOVA, $F_{1,46054} = 0.181$, $P = 0.309$). Thus, disrupting activity in the high-frequency region of A1 during stimulus presentation had no apparent effect on auditory localization behavior under normal hearing conditions. The intertrial interval was long enough (2–4.5 s) to ensure that any last lingering light-evoked change in neural activity (Fig. 2e–h) was over before the next stimulus presentation.

Sound localization following monaural occlusion. To investigate the role of A1 in adaptation to altered spatial cues, we compared the capacity of a group of control ferrets and the animals in which cortical activity was suppressed optogenetically...
to recover sound localization accuracy during continuous monaural occlusion for 10 days. Although adaptation to unilateral hearing loss is seen at a range of stimulus durations, we used 1000-ms noise bursts for the earplugging experiments to be consistent with previous studies of cortical manipulations. Importantly, those studies demonstrated very similar effects of monaural occlusion on the accuracy of both approach-to-target and head-orienting responses, which have a latency of ∼200 ms, suggesting that the spatial cues provided by the onset of the target sound are used to drive the ferrets’ adaptive localization behavior.

Consistent with the data shown in Fig. 1, the control group and the animals in which each stimulus presentation was paired with optogenetic silencing of A1 neurons localized the BBNs equally accurately in the session carried out prior to monaural occlusion (Fig. 4a, b; preplug scores 0.94 ± 0.09 vs. 0.90 ± 0.06, respectively, t test $t_{24} = 1.149; P = 0.262$). As soon as an earplug was inserted in the right ear, the proportion of correct responses fell to ∼0.2 in all animals (Fig. 4b, c; controls 0.26 ± 0.09; ArchT 0.17 ± 0.05), confirming the importance of binaural cues for this task (Supplementary Fig. 4, Supplementary Fig. 5).

Daily sound localization training with the earplug in place throughout had a different effect on the localization accuracy of the two groups of animals. Control ferrets adapted with daily training, showing an almost complete recovery in the accuracy with which they localized BBNs over 10 days of continuous monaural deprivation (Fig. 4b–d black symbols and lines, Supplementary Fig. 4). This was manifest as an improvement in localization accuracy (slope significantly different from 0;
ANOVA, $F_{1,128} = 204.534, P < 0.0001$) and a reduction in error magnitude on incorrect trials (slope significantly different from 0; ANOVA, $F_{1,128} = 10.014, P = 0.002$) with training.

A different result was found during optogenetic suppression of A1. Although they localized normally when intact binaural cues were available (Fig. 4a), suppression of cortical activity impaired the ability of these animals to adapt to a unilateral earplug (Fig. 4b–d, green symbols and lines, Supplementary Fig. 5; see Table 1 for detailed differences between groups). The proportion of correct responses increased (slope significantly different from 0; ANOVA, $F_{1,128} = 36.282, P < 0.0001$) (Fig. 4b, c) and the error magnitude on incorrect trials declined (slope significantly different from 0; ANOVA, $F_{1,128} = 15.636, P < 0.001$) (Fig. 4d) over the 10 days of monaural occlusion in the ArchT animals. However, while the errors decreased in size at the same rate in both groups (comparison of slopes: ANOVA, $F_{1,256} = 0.307; P = 0.580$), they were significantly larger throughout the period of monaural occlusion in the ArchT group (difference in error intercepts: ANOVA, $F_{1,257} = 173.886; P < 0.0001$) (Fig. 4d) and the localization accuracy of these animals recovered at a slower rate than the controls (ANOVA, $F_{4,580} = 5.863, P < 0.0001$) (Fig. 4b, c).

We can rule out the possibility of a nonspecific impairment because the control group included animals that received injections of the same viral construct, but without the gene for ArchT, or were fitted with the same cranial implant with laser illumination but without a viral vector injection (Supplementary Table 1). Impaired adaptation during optogenetic suppression of A1 was observed with both CAG and CaMKII promoters, indicating that the activity of excitatory cortical pyramidal neurons is essential for learning to occur, without ruling out the possibility that the inhibitory neurons also play a role (Supplementary Fig. 5; adaptation slopes for CAG and CaMKII were not statistically different: ANOVA, $F_{1,126} = 1.354, P = 0.247$). In both control and experimental groups, the adaptation observed was based at least in part on the improvements in performance within each session as well as on the retention or consolidation of such learning between sessions. This is illustrated by the significant contribution of both the order of the trials within each testing session and the change in performance across training days to the general linear model (GLM) (Table 2).

All ferrets in which high-frequency A1 neurons were silenced unilaterally during stimulus presentation showed an immediate improvement in localization accuracy when the earplug was removed, indicating a dependence on binaural cues (Fig. 4b, Fig. 5, and Supplementary Fig. 5). However, the performance of these animals in the first post-plug session was much more
variable than that of the control group (Supplementary Fig. 6; equality of variance test $F_{12,12} = 9.803$, $P < 0.0001$), with some ferrets achieving their pre-plug scores while others performed at a much lower level (post-plug range 0.42–0.93 in the ArchT-laser-on group vs. 0.75–0.97 in the control group). Localization accuracy on the last day of monaural occlusion was a good predictor of the second period of monaural occlusion. Consequently, they achieved higher scores (Table 1) and the slope of the adaptation function was flatter (ANOVA $F_{4,54} = 1.959; P = 0.114$) than when they were first trained with one ear occluded.

We also retested the ArchT animals that had previously shown impaired adaptation when cortical activity was suppressed. This time, however, they were tested without delivering light to the cortical implant during each stimulus presentation (Fig. 6b). Despite the restoration of cortical activity, these animals adapted at the same rate as that observed during the first period of monaural deprivation when the high-frequency region of A1 was inactivated, and significantly more slowly than the control animals during their first period of monaural occlusion (slope 0.028 vs. 0.057; ANOVA $F_{4,580} = 5.863$, $P < 0.0001$, post hoc test $P = 0.013$) than when they were first trained with one ear occluded.

When we again tested the effects of optogenetic suppression on the ability of the ferrets expressing ArchT in high-frequency A1...
**Fig. 4** Optogenetic suppression of A1 impairs auditory spatial learning. **a** Sound localization in the session before the right ear was plugged shown by plotting the mean proportion of correct scores (and 95% confidence intervals) for 1000 ms bursts of broadband noise at each of 12 loudspeakers positioned at equal intervals in the horizontal plane (0° is directly in front). Data are from ferrets in which left A1 activity was suppressed by illumination of ArchT during each stimulus presentation (green, n = 13) and from a control group (black, n = 13). **b** Proportion of correct responses (averaged across all speaker locations) achieved by each animal in both groups in the Pre-plug session, on each of the 10 days over which the plug was worn (days 1–10), and in the session following its removal (Post-plug). **c** The difference in adaptation rate between these groups is shown by plotting the proportion of correct responses for the first 2 days and the last 2 days of monaural occlusion (ANOVA, F_{3,100} = 62.531, P < 0.0001, post hoc Scheffé test, ** indicates P ≤ 0.01). **d** Magnitude of the localization errors on incorrect trials before, during and after this period of monaural occlusion. Symbols in (b) and (d) represent data from individual animals, and the lines and shaded areas are the best linear fits and 95% confidence intervals of these fits, respectively, over the 10 days of monaural occlusion. Source data are provided as a Source Data file.

### Table 1 P values and odd ratios from the generalized linear model (GLM)

|                | Control 1 | CAG Laser on 1 | CAG Laser off | CaMKII Laser on 1 | CaMKII Laser off | Control 2 | CAG Laser on 2 | CaMKII Laser on 2 | CaMKII Laser off |
|----------------|-----------|----------------|---------------|-------------------|------------------|-----------|----------------|-------------------|------------------|
| Control 1      | *0.563    | *0.463         | 1.506         | 1.403             | 3.537            | 1.381     | 1.155          |                   |                  |
| CAG Laser on 1 | *0.022    | 0.824          | *2.676        | *2.493            | *6.285           | *2.454    | 2.052          |                   |                  |
| CAG Laser off  | 0.103     | 0.000          | *3.249        | *3.028            | 7.631            | 2.980     | 2.451          |                   |                  |
| CaMKII Laser on 1 | *0.004 | 0.517          | *3.028        | 0.932             | *2.349           | 0.917     | 0.767          |                   |                  |
| CaMKII Laser off | 0.198  | *0.002         | *2.520        | 0.984             | 0.392            | 0.326     | 0.823          |                   |                  |
| Control 2      | *0.000    | *0.000         | *0.000        | *0.001            | *0.000           | *0.000    | 0.326          |                   |                  |
| CAG Laser on 2 | 0.199     | *0.000         | *0.000        | 0.388             | 0.958            | *0.000    | 0.836          |                   |                  |
| CAG Laser on 2 | 0.586     | *0.016         | *0.000        | 0.375             | 0.062            | *0.000    | 0.550          |                   |                  |

**P values**

GLM via PQL (penalized Quasi-Likelihood procedure) was performed using the R function glmmPQL. Significant values are shown by asterisks (*P < 0.05). Control 1 and 2: control group data for the first and second periods of monaural occlusion, respectively; CAG and CaMKII: different promoters used in the ArchT group. Laser on 1: first period of monaural occlusion with the laser on; laser on 2: second period of monaural occlusion with the laser on.
to adapt to an earplug, we found that their performance changed in precisely the same fashion as in their previous training block without cortical inactivation (Fig. 6c) (slopes 0.030 vs. 0.028; ANOVA, $F_{4,380} = 5.863$, $P < 0.0001$; post hoc test $P = 1$; initial scores $0.361 \pm 0.116$ vs. $0.376 \pm 0.136$, final scores $0.616 \pm 0.196$ vs. $0.603 \pm 0.140$). Thus, in contrast to the first earplugging run with the laser on, the limited capacity of these animals to adapt to further periods of monaural occlusion no longer appears to be dependent on activity in high-frequency A1.

**Discussion**

Our results show that transient perturbation of A1 activity initiated at the onset of the sound presentation during a localization training task impairs the ability of adult ferrets to adapt to altered spatial cues induced by reversible occlusion of one ear. Critically, auditory localization accuracy under normal-hearing conditions was unaffected during optogenetic suppression of cortical activity, demonstrating a specific role for A1 in learning. Further training of these animals without delivering

| Table 2 Odds ratios for between-session and within-session trial order |
|---------------------------------------------------------------|
| **Odds ratio** | **Day of monaural occlusion** | **Trial order** |
| Control 1       | $1.2596$                        | $1.0010$        |
| CAG, Laser on 1 | $1.1527$                        | $1.0031$        |
| CaMKII, Laser on 1 | $1.1665$                     | $1.0051$        |
| CAG, Laser off  | $1.1466$                        | $1.0036$        |
| CaMKII, Laser off | $1.1223$                     | $1.0021$        |
| Control 2       | $1.1769$                        | $0.9991$        |
| CAG, Laser on 2 | $1.1325$                        | $1.0038$        |
| CaMKII, Laser on 2 | $1.1586$                     | $1.0054$        |

Asterisks indicate when the factors day of monaural occlusion or trial order within each session had a significant ($P < 0.05$) effect on localization performance. Generalized linear model via PQL (penalized Quasi-Likelihood procedure) performed using the R function glmmPQL. See Table 1 for definition of animal groups.

**Fig. 5** Difference between localization accuracy before and after monaural occlusion. The difference between the proportion of correct responses is shown for individual animals prior to plugging one ear and following removal of the earplug. The control group of ferrets underwent two periods of training (Control 1, $n = 13$, and Control 2, $n = 9$), whereas the animals in which ArchT was expressed in high-frequency A1 received three blocks of training whilst wearing an earplug, with laser light delivered to the implanted optical fiber on each trial during the first (ArchT laser on 1, $n = 13$) and third block (ArchT laser on 2, $n = 12$), but not during the second block (ArchT laser off, $n = 13$). The gray symbols represent data from individual animals, and the tick marks and error bars indicate the mean ± SD. Source data are provided as a Source Data file.

**Fig. 6** Learning retrieval during successive periods of monaural occlusion. Proportion of correct responses (averaged across all speaker locations) is shown in the session before the right ear was plugged (Pre-plug), on each of the 10 days over which the plug was worn (days 1-10), and in the session following its removal (Post-plug). **a** Data from the control group ($n = 13$) are shown for two 10-day periods of monaural occlusion: lines and shaded areas correspond to the best linear fits and 95% confidence intervals, respectively, while the symbols represent individual performance during the second period of monaural deprivation (individual animal data for the first period are shown in Fig. 4b). **b** Data from the ferrets in which ArchT was expressed in high-frequency A1 ($n = 13$) are shown for the first two 10-day periods of monaural occlusion, with light delivered to the implanted optical fiber to suppress cortical activity on every trial during the first block (laser on 1, green), but not during the second block (laser off, gray). Colored symbols represent individual performance under each condition. **c** Data from the ferrets in which ArchT was expressed in high-frequency A1 for the second and third 10-day periods of monaural occlusion; the laser was off during the second block (gray, as in b, $n = 13$), but on during each stimulus presentation in the third block (laser on 2, green, $n = 12$). Note that perturbing cortical activity no longer has any effect on the localization accuracy of the animals. Source data are provided as a Source Data file.
light to the cranial implant produced less adaptation than in an equivalently trained control group and this limited improvement was unaffected by subsequent cortical inactivation. These findings suggest that although A1 is required for spatial learning when abnormal inputs are first experienced, the resulting auditory memory may reside elsewhere in the brain and that the extent of the adaptive plasticity achieved during the initial learning phase determines how much adaptation is possible with further training.

Previous studies in ferrets and other species have shown that unilateral lesions or inactivation of the auditory cortex produce contralateral localization deficits. This is the case, at least for brief sounds, even when A1 is specifically targeted using temporary inactivation methods. Because a change in sound localization performance would complicate the interpretation of any effect on learning, we focussed on high-frequency A1 and showed that unilateral photostimulation of cortical neurons expressing the proton pump ArchT reduced spiking activity within ~800 μm of the fiber-optic cannula and across much of the depth of the cortex. In contrast to the more extensive inactivation used in previous studies, this did not affect the accuracy with which ferrets localized either broadband or narrowband noise bursts at any of the sound durations tested, presumably because acoustically driven activity was still present in much of A1.

For the broadband stimuli used in the ear-plugging experiments, adaptation to monaural occlusion results principally from learning to up-weight the spectral localization cues available at the nonoccluded ear. Ferrets raised with one ear occluded develop greater sensitivity to high-frequency spectral features that contain most of the directional information in the head-related transfer function. Because adaptation in adult animals was greatly reduced by optogenetic suppression of activity in the region of A1 representing those frequencies, it is likely that the impairment in learning primarily reflects a reduced ability to reweight the different cues used for localizing sound. This reweighting has been shown to occur as soon as human listeners experience an earplug in one ear, which could therefore explain why our ArchT ferrets made larger errors than the controls from the first day of monaural occlusion onwards.

Nevertheless, removal of the earplug normally results in a small and transient aftereffect, indicating that adaptive changes in binaural sensitivity also take place. We found that optogenetic suppression of A1 during monaural occlusion not only reduced the capacity of ferrets to learn to accommodate the altered cues, but also resulted in much greater variation in response accuracy immediately following earplug removal than in the control group. This suggests that perturbing A1 activity led to both incomplete and abnormal changes in cue processing, which recovered following further exposure to normal inputs.

In these experiments, the ferrets wore an earplug continuously for each 10-day period of monaural occlusion and therefore experienced asymmetric hearing loss both during the twice-daily training sessions and in their home cages. Improvements in performance occurred during the training sessions and were maintained between sessions. This is consistent with our previous observation that the rate and extent of adaptation increase with the frequency of training sessions. However, in humans, adaptation to monaural occlusion does not occur if the training is compressed into one day, indicating that off-line memory consolidation is also required. By taking advantage of the transient nature of optogenetic suppression with ArchT, we were able to show that activity in A1 during the sound-localization task is critical for learning, further highlighting the importance of behavioral training in promoting the recovery of performance accuracy in the presence of hearing loss in one ear.

Previous studies have shown that training-dependent improvements in sound detection or discrimination are associated with plasticity in the response properties of A1 neurons, which is likely driven by top-down inputs. Furthermore, direct activation of sensory cortical areas can induce temporary behavioral improvements in the absence of training. Cortical stimulation or activation of neuromodulatory inputs can also enhance perceptual learning, while suppression of A1 activity disrupts learning. Of particular relevance to our results, auditory training has been shown to compensate for deleterious effects of altered experience during infancy on A1 response properties and behavioral sound detection thresholds. Our findings provide further evidence for the role of training in restoring auditory function and for a critical role for A1 in learning. More specifically, they demonstrate that for normal learning to take place, cortical activity is specifically required when auditory stimuli are presented during training and that intact cortical activity between training sessions is not sufficient. It has been argued that transient alterations in the balance of cortical excitation and inhibition contribute to learning and changes in cortical response properties. We have shown that activity in auditory neurons is required for adaptation to asymmetric hearing loss, but plasticity of inhibitory synapses is also likely to be involved, with these changes triggered by neuromodulatory inputs that likely transmit reinforcement signals to auditory cortex.

Although the plasticity in cue processing that underpins adaptation to asymmetric hearing loss is context-dependent and rapidly reversible, the behavioral consequences of a second period of monaural deprivation suggest that the previous learning leaves a memory trace that can be retrieved when the same abnormal hearing conditions are experienced again. Thus, in the control group, a much smaller initial deficit was induced by occluding the same ear for a second time, with correspondingly less adaptation required to approach pre-plug levels of localization accuracy. Similarly, the more limited adaptation observed during optogenetic suppression of high-frequency A1 seems to determine the extent of the plasticity induced by subsequent training with the laser off. This reduced capacity to accommodate abnormal inputs with further training was unaffected when cortical activity was again perturbed by turning the laser on, suggesting that retrieval of previous learning may not be dependent on A1. This could then explain why both control and ArchT animals show a comparable rate of adaptation, with the much higher scores achieved by the controls reflecting the greater learning that occurs during the first earplugging run with normal A1 activity.

Learning-related changes in the functional organization of A1 disappear with further training over time, even though improvements in behavioral performance are retained. While they may still be manifest in A1 in other ways, such as via changes in dendritic spine dynamics, it is also possible that the neural substrate for learned perceptual abilities, or in the case of our results a previously learned ability to reweight different spatial cues, lies elsewhere in the brain. For instance, selective strengthening of auditory corticostriatal synapses has been demonstrated over the course of learning an auditory discrimination task, while higher-order auditory cortical areas have been implicated in the consolidation of fearful memories. Other studies also suggest that distinct brain regions may contribute to initial and subsequent stages of learning.

An additional possibility is that A1 is required for learning and that the processing underpinning sound localization following the initial adaptation to monaural occlusion occurs at subcortical levels. This resembles the effects of lesions of the motor cortex, which prevent learning but not the execution of certain previously acquired motor skills that are instead thought to be mediated by...
downstream circuits. Indeed, there is growing evidence that behavioral training can trigger plasticity at subcortical sites within the auditory pathways19−24, and that this may be enabled via descending projections17,57−63. Our results may, therefore, be consistent with the reverse hierarchical theory of perceptual learning, in which activity-dependent changes proceed in a top-down fashion from higher to lower levels of processing, where the relevant stimulus features may be represented more precisely61−63.

Methods

Animals and viral vectors. Thirty-one adult female ferrets (>6 months of age) and five C57BL/6 mice (3 months of age) were used in this study (Supplementary Table 1). All sample sizes were chosen based on previous behavioral and neurophysiological studies. Animals were divided to different experimental groups by technicians who were blind to the purposes of the study. Portions of the data were also collected by individuals who were unaware of either the project aims or the experimental group. However, group allocation was necessarily known to the lead investigators throughout the study. The experiments were performed under license from the UK Home Office in accordance with the Animal (Scientific Procedures) Act (1986, amended in 2012). Ferrets were housed in small groups (<8) within environmentally enriched laboratory cages. Mice were housed in standard laboratory cages.

Replication deficient AAV8/CAG-ArchT-GFP, AAV8/CaMKII-ArchT-GFP, or AAV8/CAG-GFP control viral constructs were obtained from the Synthetic Neurobiology Group, Media Lab, MIT, Cambridge, MA (ArchT plasmid and map available at Addgene 29777), and viral vectors were produced by the Vector Core Facility at the University of North Carolina at Chapel Hill, with titles of 1012–1013. In ferrets, the virus was injected at 4 sites across the high frequency region of A1 (Figs. 1b, 2a, 3a, Supplementary Fig. 2a) at depths of 1.0 and 0.5 mm below the pial surface. In one case (F1804) both constructs AAV8/CAG-ArchT-GFP and AAV8/CaMKII-ArchT-GFP were injected at different locations (four per construct) in A1 (Supplementary Fig. 2a).

The injections were performed using a pressure injector (Nanojector). Drummond Instruments). The volume of each injection was 50.6 nL, made using glass micropipettes with a 15−20 µm tip (F1217 and F1411, and the first 19 cases in Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). In all cases, injection sites and sizes were very similar, with comparable GFP-positive (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). In all cases, injection sites and sizes were very similar, with comparable GFP-positive (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). In all cases, injection sites and sizes were very similar, with comparable GFP-positive (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). In all cases, injection sites and sizes were very similar, with comparable GFP-positive (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). In all cases, injection sites and sizes were very similar, with comparable GFP-positive (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1). The pipette was left in place at least for 5 min following injection. The post-injection survival time was up to 30 months in the ferrets used for behavior, and 4 weeks in the animals used for recordings and/or anatomy (F1115, F1431, F1003, F1110, F1210, and F1804; Supplementary Table 1).

Behavioral testing. Ferrets were trained by positive-operant conditioning in a horizontal circular arena (Ø 140 cm) surrounded by 12 loudspeakers, spaced 30° apart, and a soundproof chamber. A compact DPSS laser (Shanghai Laser & Optics Century Co.) was used to generate green light (λ = 532 nm) with an intensity of 10 mW measured (Vector H410, Laser Physics UK) at the tip of the final optical fiber (irradiance ~3 mW/mm²), which is sufficient to induce ArchT activation (~400 pA photocurrent) up to ~800−1000 µm from the fiber tip46,53. Modifications to the behavioral testing chamber enabled us to connect the laser to the implanted optic fiber without affecting the animals’ ability to perform the behavioral task (Fig. 1a). The intensity at the tip of the final optical fiber was again measured after the animals were perfused and the brain was extracted from the skull (Supplementary Fig. 1) to check the permeability of the fiber and also its positioning.

Laser and sound presentation triggering were controlled by the same TDT hardware to ensure precise temporal congruency between them. Laser illumination was randomized within testing sessions with a probability of 0.5 during normal sound localization testing. However, for the earplugging experiments, laser illumination was either paired with sound delivery or not within each 14 days period. The exposure of the earplugging experiments. During these periods, they were tested twice a day and the number of trials animals performed ranged from 50 to 100 trials per session. Each animal performed a minimum of 300 trials per sound duration under normal hearing conditions and a minimum of 150 trials per day when wearing an earplug. In a separate block of trials (~35% of the first 19 cases) the concha of the external ear was covered using Otoform-K2 silicone impression material (Dreve Otoplastik, Unna, Germany). The health of the ear was checked before earplug insertion and following its removal by otoscopic examination and tympanometry (Kampyle KLT25 Audiometer, P.C. Werth). Acoustical measurements indicated that the earplugs produced 40−50 dB attenuation at frequencies >3.5 kHz, which rolled off gradually at lower frequencies.

Surgery. Ferrets were prepared for surgery 12 h before the procedure by pre-treatment with the corticosteroid Solu-Medrone (methylprednisolone sodium succinate, 10 mg kg⁻¹, i.m., Pfizer) to prevent cerebral edema. Anesthesia was induced with Domitor (medetomidine hydrochloride, 0.022 mg/kg, i.m., Orion Pharma) and Ketaset (ketamine hydrochloride, 5 mg/kg, i.m., Fort Dodge Animal Health) in saline and maintained with IsoFlo (isoflurane, 1−3% with 100% oxygen as the carrier gas). At the beginning of surgery, the animals were treated with an additional dose of Solu-Medrone, Antisend (atipamezole hydrochloride, 0.06 mg kg⁻¹, s.c., Pfizer) to reverse the effects of Domitor, the antibiotic Synulox (clavulanate-potentiated amoxicillin, 0.1 mg kg⁻¹, i.m., s.c., Pfizer) to prevent infection, and atropine sulphate (0.006 mg kg⁻¹, s.c., Hamecn Pharmaceuticals) to reduce the effects of the anesthetic. A plug was placed over the ear and also its tail, aseptically ventilated after tracheal intubation, and respiratory rate, heart rate and CO₂ levels were monitored continuously. A rectal probe monitored the temperature of the animal, which was maintained using a Lauda waterbath device at 38−39 °C. A solution of glucose serum (0.9% sodium chloride, pH 7.2−7.4, plus 5% glucose) was delivered throughout the surgery at a rate of 3.0−5.0 mL h⁻¹ via a cannula inserted into a forelimb vein. During the surgery, the scalp was incised, the temporal muscles were resected and partially cut away above the area of the skull overlying the temporal lobe, a craniotomy was performed over the left auditory cortex, and the dura mater was punctured to allow free passage of the recording electrode and/or the glass micropipette into the neural tissue.

In the first implanted animals (n = 2), recordings were made at a number of sites in the auditory cortex to determine the location and borders of the A1 and its neighboring structures. After verifying injection sites, the implant was constructed by placing an optical fiber (multimode MM fiber, 200 µm diameter, Shanghai Laser & Optics Century Co.) in the left auditory cortex. The implant was positioned in the dorsal region of the ectosylvian gyrus, which corresponds to the high-frequency region of A1 (Fig. 1b, Supplementary Fig. 2a); the locations of the recordings made during the injection surgeries and the landmarks of the ferret auditory cortex (sulci

Nature Communications | https://doi.org/10.1038/s41467-019-10770-4 | www.nature.com/naturecommunications
and gyri positions) were used to guide the positioning of the implant. A plastic cap was placed around the portion of the implant extending above the skull and secured in place with bone cement. The scalp was then sutured around the implant. The implant was always recovered at the end of the behavior and after perfusion to test the optical permeability of the optical fiber by measuring the light intensity (Supplementary Fig. 1).

**Recordings in the ferret auditory cortex.** Four weeks after the initial surgery to inject the viral constructs, animals used for acute terminal recordings and those in which electrophysiology was concluded (Supplementary Table 1) were perfused for surgery as stated before, except that anesthesia was maintained with an i.v. infusion of Domitor and Ketaset in saline throughout and not with Isoflurane. The head was fixed in place by a metal plate cemented to the skull.

We recorded multi-unit activity using single-shank silicon probes (Neuronexus, Ann Arbor, MI), with 16 electrodes per shank spaced 50 µm intervals (F1421 and F1115), 2 shanks separated by 200 µm with recording sites spaced at 50 µm in a case (F1804; Supplementary Fig. 2), and four shanks separated by 500 µm with 32 recording sites spaced at 50 µm in four cases (Supplementary Table 1). Acoustic stimuli were generated using TDtensor 3 system hardware and presented via Panitary Earphone (Auditory Technologies) mounted on the scalp of the ferret using a 10 µm flattened fiber optic cable. We used multi-unit recordings with an 8 frequency sweeps with a sweep rate of 10 Hz and absolute number steps. Tones were 200 ms in duration (5 ms cosine ramp) and intensity levels were varied between 10 and 80 dB SPL in 10 dB increments. BBNs (40 Hz–30 kHz bandwidth and cosine ramped with a 10 ms rise/fall time; 30–80 dB SPL) of 200 ms or 1000 ms in duration were used.

Recordings were made perpendicular to the cortical surface starting at the corner of the ectosylvian sulcus in close vicinity to the optical fiber, which was placed in the same position as it was in the chronic animals and connected to the same 532 nm DPSS laser activated using the same parameters (10 mW measured at the tip of the optical fiber; Supplementary Fig. 2a). Laser illumination was paired with 50% of the sound stimulus presentations, beginning at the same time. We then varied the positions of the recording probe and optical fiber, so that the distance between the two covered the same range as in previous studies.

**Histology.** Ferrets used exclusively for anatomy were perfused 4 weeks after making viral injections in the auditory cortex, while those used for acute electrophysiology (again 4 weeks after the injections) were perfused as soon as the recovery was complete. Implanted animals used for behavior were perfused 30 months after the viral vector injections were performed, with a subset of 4 used for recordings in the auditory cortex before perfusion (see Supplementary Table 1). Animals were sedated with Domitor, overdosed with Euthatal (400 mg of pentobarbital sodium, i.p., Merial Animal Health), and perfused transcardially with 0.9% saline (weight/volume) and 4% paraformaldehyde (weight/volume) in 0.1 M phosphate buffer, pH 7.4. The brains were removed, embedded in sucrose, cut on a freezing microtome at a thickness of 45 µm in 1 case (F1804; Supplementary Fig. 2), and four shanks separated by 500 µm with 32 recording sites spaced at 50 µm in four cases (Supplementary Table 1). Acoustic stimuli were generated using TDT system 3 hardware and presented to the animal using a standard-wall borosilicate capillary (1.5 mm OD × 0.86 mm ID, Harvard Apparatus) and filled with intracellular solution (140 mM cesium gluconate, 1 mM KCl, 10 mM Hepes, 4 mM potassium phosphate, 4 mM ATP-Mg, and 0.4 mM GTP, and 3 mM/3 µM GTP; pH adjusted to 7.3 with KOH; osmolality, ~300 mosmol–1). Electrophysiological data were analyzed using custom-written routines in MATLAB. Membrane potential and interspike intervals were measured in 1-s time windows and compared to pre-illumination (baseline) values (ANOVA); the latency for recovery to baseline conditions was established when statistical differences were no longer detected. At the conclusion of the recordings, ArchT-transfected cells and biocytin-filled cells were identified using fluorescein-labeled secondary antibodies (Streptavidine, Alexa Fluor647 conjugate (S-21374) and Alexa Fluor488 goat anti-chicken antibody (A-11039)). Confocal images were captured using similar parameters of laser power, gain, pinhole and wavelengths with two channels assigned as the emission color; z-stacks were taken individually for each channel and then collapsed (Supplementary Fig. 3).

**Statistical methods.** Source localization accuracy (proportion of correct scores) plus the incidence of front–back and left–right errors were quantified by submitting individual trials to univariate generalized linear models (bimodal distribution with probit link functions) for each parameter. Rate of adaptation during periods of monaural occlusion was quantified by fitting regression lines for individual animals to the proportion of correct responses on each day. 95% confidence intervals around the mean are shown by shaded areas in the figures. Comparison of the mean scores obtained at the start and end of each period of monaural occlusion and of the regression line slopes were made using ANOVA after the data were checked for normality by inspection of Q–Q plots and application of the Shapiro–Wilks test, with homoscedasticity tested by Levene’s test of equality of variances.

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

**Data availability.** The source data underlying Figs. 1c–e, 4–6 and Supplementary Figs. 3–6 are provided as a Source Data File. All relevant data are available on request from the authors.

Received: 2 May 2018 Accepted: 28 May 2019

Published online: 12 July 2019

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-10770-4 | www.nature.com/naturecommunications
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Acknowledgements

We thank the late Daniel Lunn from the Statistics Department for his advice and help on the implementation of the GLM, Amy Hammond-Kenny, Josh Gold, and Sue Spires for their help with behavioral testing, Louise Upton, Antonio Langefelder and Pantelis Antonoudiou for their contribution to the in vitro patching experiments, Fernando García-Moreno and Alan Wainman for help with the confocal imaging, Kit Reynolds for assistance with the anatomical experiments, Lily Goldblatt for her contribution to the recording analysis, and David Banerjee for comments on the manuscript. This work was supported by the Wellcome Trust (WT108369/Z/2015/Z Principal Research Fellowship to A.J.K.) and by Action on Hearing Loss (S72 Bajo to V.M.B. and A.J.K.).

Author contributions

V.M.B., F.R.N., and A.J.K. conceived and designed the project; V.M.B. and F.R.N. performed research and analyzed all of the data; C.K. and A.O.C. contributed to the behavioral studies; E.S.B provided viral constructs and optogenetic support; E.O.M. performed in vitro recordings; V.M.B., F.R.N., and A.J.K. wrote the paper, with the assistance of E.O.M.; V.M.B. and A.J.K. supervised the work.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-10770-4.

Competing interests: The authors declare no competing interests.

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Peer review information: Nature Communications thanks David McAlpine, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Silencing cortical activity during sound-localization training impairs auditory perceptual learning

Bajo et al.

Supplementary Figures 1 to 6

Supplementary Table 1
Supplementary Figure 1: Optical permeability of the fiber-optic cannulae 30 months post-implantation.

Once the behavioral testing had been completed and the animals perfused for histological confirmation of transfection levels, each chronic implant containing a fiber-optic cannula was dissected along with the temporal part of the skull to ensure that there was no bone growth over the end of the cannula. After inspection under a microscope, the optical fiber was reconnected to the laser source to confirm that the beam was not obstructed and the light intensity remained unchanged. (a) Inner view at the level of the temporal skull after the brain had been removed, showing the tips of the two screws used to anchor the implant, the craniotomy (arrow) where the ectosylvian sulcus was located and the fiber-optic cannula at the top right corner of the craniotomy (asterisk). (b) Different view of the same implant connected to the laser to illustrate the permeability of the optical fiber (asterisk, green light can be seen beneath the skull). (c) Dorsal view of the external part of the same implant, which included a protective circular enclosure, within which the zirconia ferrule of the optical fiber can be observed (open arrow). Pictures were taken from animal F1111 and are representative of every ferret implanted (n = 15, Supplementary Table 1, asterisks).
Supplementary Figure 2: Optogenetic suppression of sound-driven activity in A1.

(a) Histological section of a flattened left auditory cortex (ferret F1804) showing GFP immunofluorescence associated with ArchT expression using two different promoters. The inset shows a photograph of the cortical surface of this animal on which the locations of the recording sites and optical fiber placements in the dorsal high-frequency region of A1 have been marked. (b) Examples of unit responses recorded at location #7 (AAV8/CAG-ArchT-GFP injection) at 5 different cortical depths, which are indicated by the solid black circles on the schematic of the recording probe (distance between adjacent recording sites, 50 µm). For each unit, dot rasters show the responses to 100 presentations of broadband noise (BBN, 1000 ms duration), grouped into laser-off (top half, gray) and laser-on presentations (bottom half, green) in the same fashion as during behavioral testing. Each sweep lasted 5 s (comprising 750 ms of spontaneous activity preceding the 1,000-ms stimuli followed by 3,250 ms after laser offset) so that the time-course of optogenetic suppression could be measured. The next two columns show the corresponding peristimulus time histograms (20 ms bins) for BBN only (left) and BBN plus laser illumination (right). All 5 units exhibited a significant suppression of acoustically-driven activity (comparison of activity during a time window from 760-1,760 ms for BBN laser-off vs BBN laser-on presentation, t-test, P < 0.05 for each unit). During sound presentation, the firing rate of these example units was reduced by laser illumination to different extents (85%-25%), but in each case the prominent offset response observed in the absence of laser illumination was eliminated when the laser was turned on (comparison of pre-stimulus spontaneous activity vs offset response during a time window from 1,800-2,000 ms; laser-off, t-test, P < 0.05; laser-on, P > 0.165). The activity of all the units recovered soon after the laser was turned off (comparison of pre-stimulus spontaneous activity vs activity 0.5 s after the laser was turned off, P > 0.502 for each unit). (c) Histological sections at the level of the ipsilateral medial geniculate body and inferior colliculus showing the terminal fields of the axons of descending cortical projection neurons expressing GFP. Calibration bars in a and c, 1 mm. D, dorsal; M, medial; P, posterior; pss, pseudosylvian sulcus; sss, suprasylvian sulcus.
Supplementary Figure 3: *In vitro* whole-cell recordings to validate the ArchT constructs.

(a) Effect of laser light pulses (green rectangles) of different magnitudes on the activity of a pyramidal neuron in mouse motor cortex. This neuron was filled with biocytin for identification (b) and expressed ArchT-GFP under the CAG promoter (c). (d) Merged image (colocalization of both chromophores is shown in yellow/orange). (e) Linear relationship ($R^2 = 0.982$) between the magnitude of the hyperpolarizing response and the intensity of the light pulse. The black line indicates the mean, with the standard deviation in gray ($n = 6$ cells). (f) Effect of light pulses (green rectangles) of different magnitudes on the activity of a second pyramidal neuron in mouse motor cortex. This neuron was filled with biocytin (g) and expressed ArchT-GFP under the CaMKII promoter (h). (i) Merged image (colocalization of both chromophores is shown in yellow/orange). Calibration bar in b represents 100 μm and applies to c, d and g-i. The light pulses hyperpolarized the cells and completely suppressed action potential firing regardless of the promoter used (hyperpolarization/LEDv = 10.7 ± 6 mV/V). Although the resting membrane potential recovered rapidly (<0.5 s) after laser offset, spiking activity (measured by the duration of the interspike intervals) took longer to return to baseline levels. When the CAG promoter was used to target every cell type at the injection site, the hyperpolarization and cessation of firing caused by the laser pulse were normally followed by a single rebound period of higher firing, which then returned to pre-laser activity levels within 1-3 s after laser offset (a). The firing pattern observed post-illumination was usually more complex when excitatory pyramidal neurons were targeted by expressing ArchT under the CaMKII promoter, which included a combination of rebound firing and a further period of silence, and took longer to return to the pre-light activity. The duration of the post-light suppression of firing (1-4 s) depended on light intensity (f). This difference cannot be attributed to differences in the type of neurons from which these recordings were obtained, which all had a pyramidal morphology, as shown by filling them with biocytin, or to variation in ArchT expression levels, but more likely reflects the overall effect of illumination on the cortical networks in which different populations of neurons expressed ArchT according to the promotor used.
Supplementary Figure 4: Effects of monaural occlusion on auditory localization in control ferrets.

Stimulus-response plots showing the performance of each control animal before (Preplug), during (Plug Day 1, 2, 9, 10) and after (Postplug) the first period of monaural occlusion. Each column shows data from an individual animal, with the ferret number at the top of the column (see Supplementary Table S1). Symbols identify these animals in Figures 4 and 6. The first and final rows show data from the two days before and after monaural occlusion, respectively. The middle four rows show data from the first two and last two days of monaural occlusion, respectively. Negative and positive angles indicate sound locations in the left and right hemifields, respectively. The gray rectangle indicates that the right ear was plugged.
Supplementary Figure 5: Effects of monaural occlusion on auditory localization in ArchT ferrets.

Stimulus-response plots showing the performance of each ‘ArchT-laser on’ animal before (Preplug), during (Plug Day 1, 2, 9, 10) and after (Postplug) the first period of monaural occlusion. Each column shows data from an individual animal, with the ferret number at the top of the column. Symbols identify these animals in Figures 4 and 6. In the first seven cases, the CAG promoter was used in the viral construct (AAV8/CAG-ArchT-GFP) and in the last six cases the CaMKII promoter was used (AAV8/CaMKII-ArchT-GFP; See Supplementary Table 1). The first and final rows show data from the two days before and after monaural occlusion, respectively. The middle four rows show data from the first two and last two days of monaural occlusion, respectively. Negative and positive angles indicate sound locations in the left and right hemifields, respectively. The gray rectangle indicates that the right ear was plugged.
Supplementary Figure 6: Performance after removal of the earplug.

(a) Probability of correct responses (asterisks) and mean error magnitude on incorrect trials (open circles) for the different groups (C1 and C2, control groups during first and second period of monaural occlusion; L1, Loff and L2 ArchT groups during the different periods of monaural occlusion and laser activation). Each symbol represents a different animal and symbols with error bars correspond to mean values. (b-e) Stimulus-response plots based on all the trials across all animals in each group. In each panel, the mutual information (MI in bits) between target and response locations is included. A value of 3.58 bits corresponds to perfect performance. The performance of the control animals was more accurate and less variable than that of the ferrets in which ArchT was expressed in A1, indicating a delayed return to normal localization following monaural occlusion. Sample sizes: n = 26 (a), n = 13 (b, d), n = 12 (f), n = 13 (e), n = 9 (e).
Supplementary Table 1: List of animals used in this study.

| Animal identity | Behavior       | Recordings | Anatomy   | Promoter/control | Figures                        |
|-----------------|----------------|------------|-----------|------------------|--------------------------------|
| Ferret 1127*    | SL & 3 MO      | FL + ABC   | CAG       |                  | Figs. 1c-e, 3 Quant., 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1218*    | SL & 2 MO      | FL + ABC   | CAG       |                  | Figs. 1c-e, 3 Quant., 4, 5, 6b-c, S5 & S6a, b, d. |
| Ferret 1402*    | SL & 3 MO      | FL         | CAG       |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1432*    | SL & 3 MO      | 4 shanks   | FL        | CAG              | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1512*    | SL & 3 MO      | 4 shanks   | FL        | CAG              | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1608*    | SL & 3 MO      | 4 shanks   | FL        | CAG              | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1610*    | SL & 3 MO      | 4 shanks   | FL        | CAG              | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1111*    | SL & 3 MO      | FL + ABC   | CaMKII    |                  | Figs. 1c-e, 3 Quant., 4, 5, 6b-c, S1, S5 & S6a, b, d, f. |
| Ferret 1304*    | SL & 3 MO      | FL         | CaMKII    |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1403*    | SL & 3 MO      | FL         | CaMKII    |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1511*    | SL & 3 MO      | FL         | CaMKII    |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1607*    | SL & 3 MO      | FL         | CaMKII    |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1609*    | SL & 3 MO      | FL         | CaMKII    |                  | Figs. 1c-e, 4, 5, 6b-c, S5 & S6a, b, d, f. |
| Ferret 1115     | 1 shank        | FL + ABC   | CAG       |                  | Fig. 3 & Quant. |
| Ferret 1431     | 1 shank        | FL         | CaMKII    |                  | Fig. 2a-d. |
| Ferret 1003     |                | FL         | CaMKII    |                  |                                  |
| Ferret 1110     |                | FL + ABC   | CaMKII    |                  | Fig. 3 Quant. |
| Ferret 1120     |                | FL         | CAG       |                  |                                  |
| Ferret 1804     | SL & 2 MO      | 2 shanks   | FL        | Control (earplug)| Figs. 2e-h, 4b-d, 5, 6a, S2, S4 & S6a, c, e. |
| Ferret 0227§    | SL & 1 MO      |            | Control (earplug)|                  | Fig. 4b-c, 5, 6a, S4 & S6a, c. |
| Ferret 0249§    | SL & 1 MO      |            | Control (earplug)|                  | Fig. 4b-c, 5, 6a, S4 & S6a, c. |
| Ferret 0543§    | SL & 1 MO      |            | Control (earplug)|                  | Fig. 4b-c, 5, 6a, S4 & S6a, c. |
| Ferret 0702§    | SL & 1 MO      |            | Control (earplug)|                  | Fig. 4b-c, 5, 6a, S4 & S6a,c. |
| Ferret 0509     | SL & 2 MO      |            | Control (earplug)|                  | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 0538     | SL & 2 MO      |            | Control (earplug)|                  | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Animal    | Procedure                  | Fluorescence | Promoter | Control | Figures       |
|-----------|----------------------------|--------------|----------|---------|--------------|
| Ferret 1217* | SL & 2 MO                  | FL + ABC    | Control (light) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 1411* | SL & 2 MO                  | FL          | Control (no ArchT) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 1802 | SL & 2 MO                  | Control (no ArchT) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 1803 | SL & 2 MO                  | Control (no ArchT) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 1805 | SL & 2 MO                  | Control (no ArchT) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Ferret 1806 | SL & 2 MO                  | Control (no ArchT) | Figs. 4b-d, 5, 6a, S4 & S6a, c, e. |
| Mouse M031114 | in vitro               | FL          | CAG      | Figs. S3a-e. |
| Mouse M051114 | in vitro               | FL          | CaMKII   | Figs. S3e-i. |
| Mouse M010882 | FL                      | CAG         |          |              |
| Mouse M029884 | FL                      | CaMKII     |          |              |
| Mouse M049881 | FL                      | CaMKII     |          |              |

Thirty-one ferrets comprising 18 experimental animals (blue) and 13 controls (black), and five mice were used. Different colors in the table cells indicate different procedures: behavior (green), electrophysiological recordings (orange), and anatomy (yellow). An asterisk after the animal name indicates that it was implanted and § indicates when the full set of behavior was not completed. The last column shows which animals contribute data to specific figure panels. Gaps in the table separate animals used for different combinations of procedures: behavior with ArchT using GAG promoter; behavior with ArchT using CaMKII promoter; electrophysiological recording with ArchT; anatomy with ArchT; ferret controls; in vitro mouse recordings with ArchT; in vitro mouse anatomy with ArchT. One of the control behavioral animals (F1804) was later used to record neural activity after ArchT was expressed in the auditory cortex (see Supplementary Fig. 2). It was the only case where both constructs, AAV8/CAG-ArchT-GFP and AAV8/CaMKII-ArchT-GFP, were injected in the same animal, at specific locations in the primary auditory cortex.

Viral construct injections were made in every animal where FL (fluorescence) is indicated in the Anatomy column. In each case, the location of the injection sites in the primary auditory cortex and the presence of GFP-labeled axons and terminals in the contralateral cortex, ipsilateral MGB and bilaterally in the IC was examined using confocal microscopy. ABC in the anatomy column indicates that one set of sections was used for permanent labeling of NeuN- and GFP-positive cells with the avidin biotin complex system; those cases were used for stereological quantification. F1217 was excluded from the quantification due to the absence of GFP-positive neurons, indicating no viral transfection; this animal was therefore treated as a control for the effect of the green light delivery to the auditory cortex during training.

Abbreviations: ABC, avidin biotin complex; CAG, synthetic promoter containing cytomegalovirus early enhancer, the 1st exon and intron of chicken beta-actin gene as promoter, and the splice acceptor of the rabbit beta-globin gene; CamKII, Ca²⁺-calmodulin-dependent protein kinase II; FL, fluorescence; MO, 10-day periods of monaural occlusion; SL, sound localization behavior.