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Original Article

ORIGINAL ARTICLE

Blocking c-Fos Expression Reveals the Role of Auditory Cortex Plasticity in Sound Frequency Discrimination Learning

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Abstract

The behavioral changes that comprise operant learning are associated with plasticity in early sensory cortices as well as with modulation of gene expression, but the connection between the behavioral, electrophysiological, and molecular changes is only partially understood. We specifically manipulated c-Fos expression, a hallmark of learning-induced synaptic plasticity, in auditory cortex of adult mice using a novel approach based on RNA interference. Locally blocking c-Fos expression caused a specific behavioral deficit in a sound discrimination task, in parallel with decreased cortical experience-dependent plasticity, without affecting baseline excitability or basic auditory processing. Thus, c-Fos-dependent experience-dependent cortical plasticity is necessary for frequency discrimination in an operant behavioral task. Our results connect behavioral, molecular and physiological changes and demonstrate a role of c-Fos in experience-dependent plasticity and learning.

Key words: baseline excitability, c-fos blocking, neuronal activity, RNAi, sound-evoked, tuning curves

Introduction

During operant learning, neurons in sensory cortices change their firing patterns as the animal's perceptual abilities improve or its discriminative power increases (Weinberger and Diamond 1987; Recanzone et al. 1992; Schoups et al. 2001). It is not clear, however, whether these plastic changes drive behavioral responses. In fact, in the auditory domain, the circumstances under which cortical plasticity is necessary for behavioral changes are not well understood. While some studies have suggested that discrimination learning depends on higher cognitive structures (Zohary et al. 1994; Yang and Maunsell 2004), acute or permanent inactivation limited to auditory cortex have provided mixed results (Romanski and LeDoux 1992; Boatman and Kim 2006; Ono et al. 2006; Nodal et al. 2010; Porter et al. 2011; Gimenez et al. 2015; Kawai et al. 2015).

Elevated expression of c-Fos, an immediate early gene protein product, is a hallmark of neurons undergoing learning-induced synaptic plasticity (Kaczmarek et al. 2002). Recent studies have shown that c-Fos-expressing neurons are involved in the formation of memory engrams (Gore et al. 2015; Tonegawa et al. 2015). Here, we manipulated c-Fos expression in auditory cortex to elucidate the interaction between cortical plasticity and sound frequency discrimination. Using a novel approach based on RNA interference (RNAi), we blocked c-Fos expression in the auditory cortex of mice and studied the behavioral and electrophysiological consequences of the manipulation in a sound discrimination task.

Results

Sound frequency discrimination was assessed in the Audiobox (de Hoz and Nelken 2014), a fully automated apparatus that allows continuous monitoring of mouse behavior for several days while the mice live in groups and behave freely. The Audiobox is divided into 2 sections, a homecage where food, but not water, is available ad libitum; and a sound-attenuated box with a "conditioning corner" where water is available. A corridor connects the 2 sections. To get water, mice have to enter the corner and nose-poke into a port in the conditioning corner. When they enter the water corner, a train of tone pips of one of 2 frequencies is presented for the duration of the visit. Mice learned to discriminate between the 2 frequencies: a "safe" frequency, which signals safe access to water, and a "withhold" frequency, which signals delivery of an aversive air-puff upon nose-poke. In the experiment, initially, following an adaptation phase, only safe tones were presented. After 4 days "withhold" tones were introduced in a small percentage of the visits (Fig. 1*a*). Mice had to learn to avoid nose-poking when the "withhold" tones were present. Performance became better over time (Fig. 1*b*), as shown by decreasing rate of incorrect operant responses (nose-pokes during "withhold" visits; comparison between the first and last sessions of discrimination training, Kolomogorov–Smirnov test: $D = 0.75$, df = 14, $P = 0.019$). As expected, sound frequency discrimination learning resulted in a clear increase in c-Fos expression in auditory cortex, [one-way ANOVA: $F(3,24) = 8.1$, $P = 0.00067$ followed by Fisher's LSD tests, Fig. 1c].

Next, we reduced behaviorally-induced c-Fos expression using a novel RNAi-based approach, that is, the delivery of a short-hairpin (sh) RNA in a lentiviral vector (LV_sh_c-fos) into auditory cortex (Fig. 1*d*,*e*). The extent of the transfection site (GFP-positive neurons), was 420 ± 100 um (mean ± standard error of the mean (SEM)). In several studies that have mapped the extent of different regions of the auditory cortex in mice, the estimate for the rostrocaudal extent of A1 is about 1 mm (Stiebler et al. 1997; Linden et al. 2003; Joachimsthaler et al. 2014). Thus, the transfection affected almost 50% of the rostrocaudal axis of A1. The effect was pronounced in layers 5 and 6 and progressively less in the superficial layers (Figs 1*e* and 2*a*).

A month after the injection, sound-induced c-Fos expression was reduced by over 40% in the cortex of injected mice (LV_sh_c-fos) as compared with controls (LV_sh_luc; Figs 1*f*,*g*), demonstrating the efficiency of the manipulation (*t*-test: $t = 3.06$, df = 4, $P = 0.039$).

We then tested the effect of c-Fos manipulations on frequency discrimination. Another group of mice was injected with either LV_sh_c-fos or LV_sh_luc (Fig. 2*a*). The mice were then trained in the Audiobox (Fig. 2*b*). Following 4 days in which only safe visits occurred, the "withhold" visits were introduced gradually.

While the number of visits was similar in both groups throughout the training (Kolomogorov–Smirnov test for the subsequent phases of the training: $D = 0.25, 0.46, 0.54, 0.54, 0.54, 0.50, 0.25, 0.25$, df = 18, $P > 0.05$ in all cases, Fig. 2*c*), the rate of incorrect operant responses was significantly higher in the LV_sh_c-fos compared with the LV_sh_luc mice [one-way ANOVA (group × session), the effects of group: $F(1,18) = 9.9$, $P = 0.0057$, session: $F(6\,108) = 71.1$, $P = 3.1 \times 10^{-35}$, and the group × session interaction: $F(6\,108) = 3.0$, $P = 0.0095$; followed by one-way ANOVAs, 17% session: $F(1,18) = 8.2$, $P = 0.010$, 50% session: $F(1,18) = 5.0$, $P = 0.038$, and extD1 session: $F(1,18) = 15.6$, $P = 0.00094$]. Significant group × session interaction reflects faster learning of the LV_sh_luc mice throughout the training. Importantly, the differences between the LV_sh_c-fos and the LV_sh_luc mice became only apparent when the rate of "withhold" visits was high (Fig. 2*d*). Initially, as conditioned visits were introduced at a low rate (5%), the percentage of incorrect responses was similar in both groups; however, when the rate of "withhold" visits was increased to 17% and then to 50%, a clear deficit developed in the experimental group. The number of incorrect responses was significantly higher in the LV_sh_c-fos animals, which also showed faster extinction. During the extinction, when the previous "withhold" visits are no longer associated with an air-puff, LV_sh_c-fos animals made a significantly higher number of operant responses during the "withhold" visits, suggesting that the original memory trace was less well consolidated than in the the LV_sh_luc mice.

To determine the role of c-Fos in experience-dependent plasticity, we first assessed the effect of reducing c-Fos expression on baseline cellular excitability. Since previous attempts to block c-Fos expression by c-*fos* knock-out resulted in electrophysiological changes that were observed well before the induction of plastic changes (the time point which we wanted to study), one can assume that this manipulation interfered with basal neuronal functioning (Fleischmann et al. 2003). Our approach, however, in which the interference with c-Fos induction occurred over a relatively short period of time only, preserved neuronal excitability. Whole-cell current clamp electrophysiological recordings from neurons located in layer 3 of auditory cortex transduced with LV_sh_luc or LV_sh_c-fos showed no significant changes between the number of action potentials (APs) elicited by each depolarizing step current, reaching the maximum of 8.2 ± 0.6 APs in LV_sh_luc group versus 7.8 ± 0.5 in LV_sh_c-fos infected cells (Kolmogorov–Smirnov test: $D = 0.16$, df = 14, $P = 0.27$). The resting membrane potential of LV_sh_c-fos cells was not significantly different from the resting membrane potential of LV_sh_luc cells: −67.5 ± 1.0 mV versus −70.3 ± 0.9 mV (*t*-test: $t = 1.82$, df = 14,

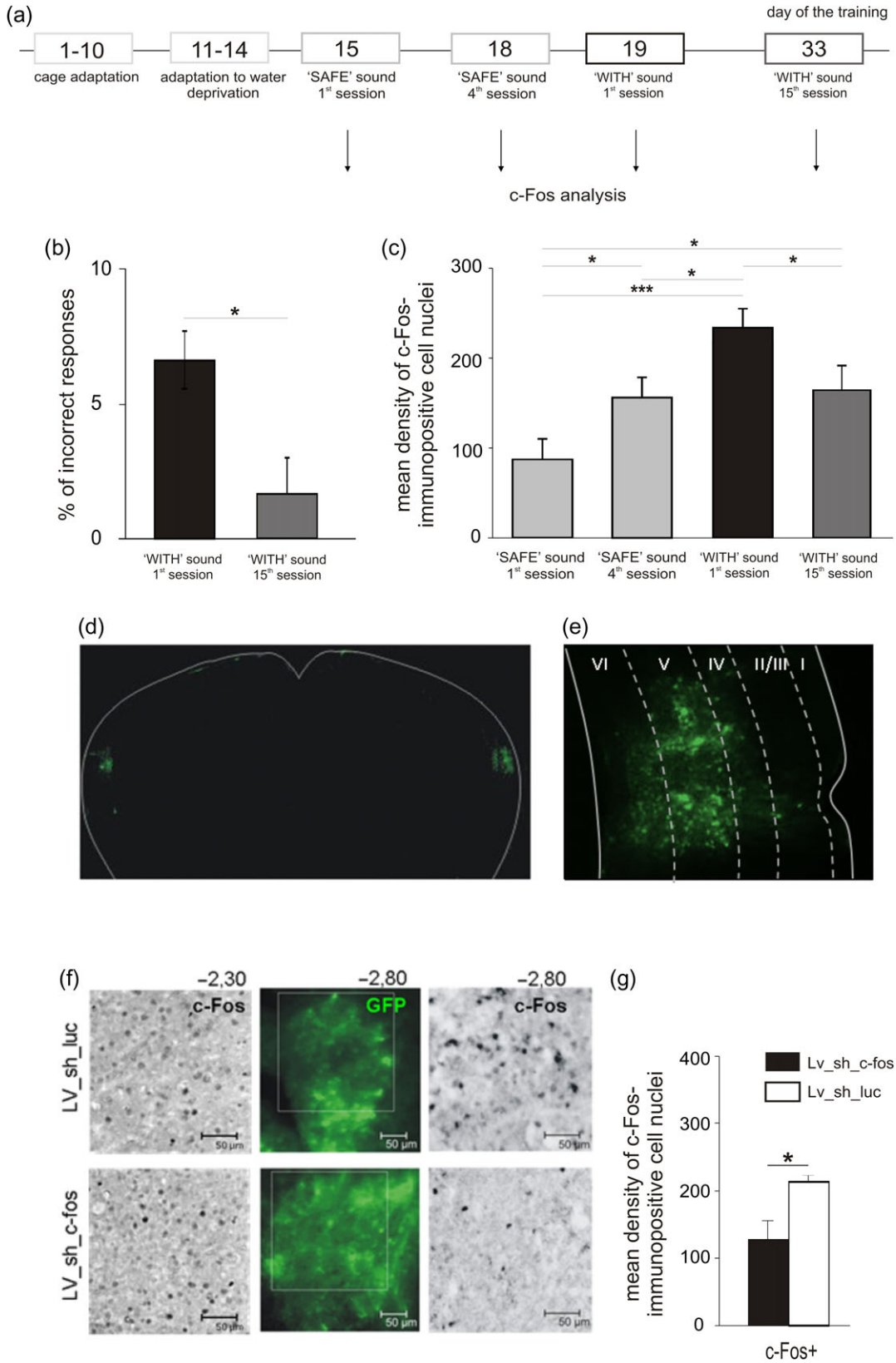

Figure 1. c-Fos expression increase in auditory cortex following sound discrimination training is blocked by lentivirally delivered shRNA against c-fos. (*a*) Sound discrimination training. After adaptation, a "safe" sound (6.7 kHz) accompanied every visit for 4 consecutive sessions. Then, an aversively "withhold" tone (13.4 kHz) was introduced in up to 17% of visits. (*b*) Rate of incorrect operant responses (nose-pokes during "withhold" visits) during the first and last sessions of discrimination training. (*c*) c-Fos expression in auditory cortex during sound discrimination learning. (*d*) GFP signal in the lentivector transduced area. (*e*) higher magnification of the lentivector transduced area showing the cortical layers affected. (*f,g*) Sound-evoked c-Fos expression measured through c-Fos-positive cells within and outside the infusion site, $^{*}P < 0.05$, $^{***}P < 0.001$; ±SEM.

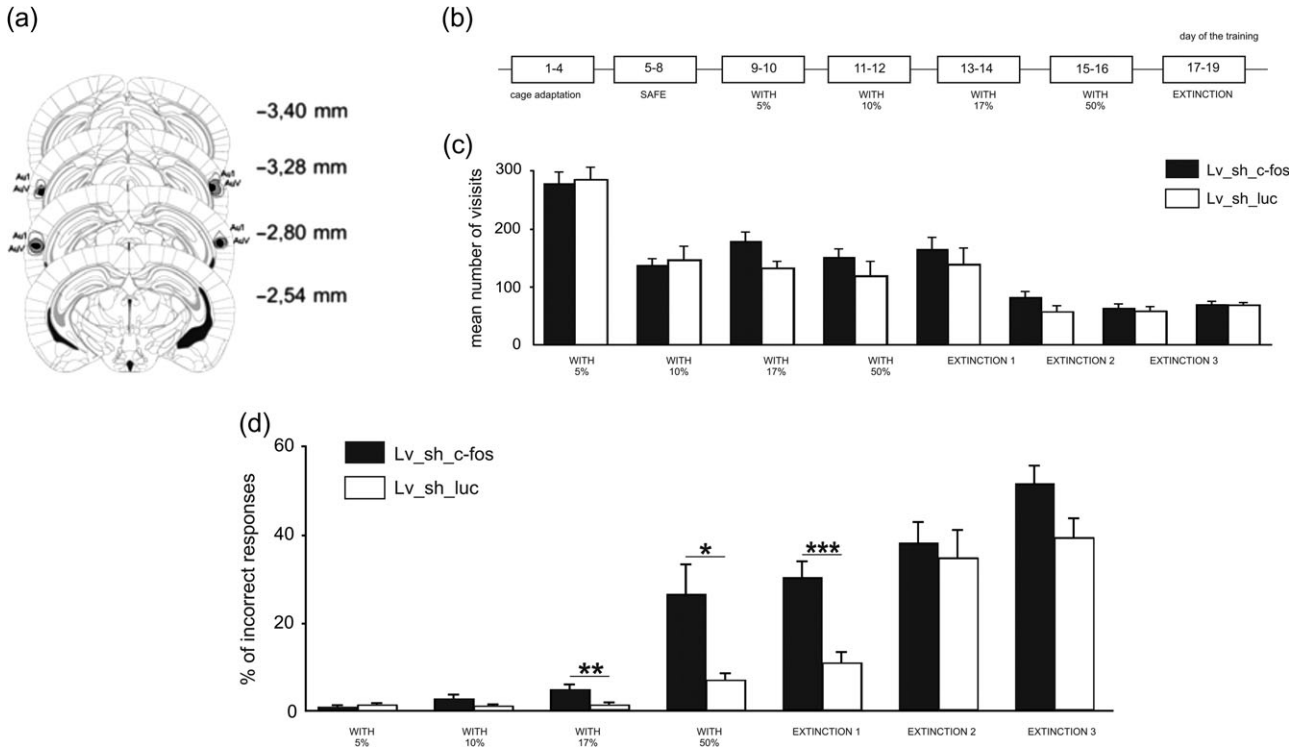

Figure 2. Blocking c-Fos expression in auditory cortex impairs sound discrimination learning. (*a*) Infusion sites of lentivirally delivered shRNA. To inhibit c-Fos expression in auditory cortex, the mice were injected with anti-c-fos shRNA (LV_sh_c-fos group). The LV_sh_luc animals were injected with anti-luc shRNA. The largest (white), smallest (black) and average (gray) infusion sites for the animals included in the analysis are shown. (*b*) Discrimination training scheme. "Safe" and "withhold" phases were followed by 3 extinction sessions, during which nose-poke responses were not punished. (*c*) Number of visits in the conditioning unit. (*d*) Incorrect responses during discrimination learning, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ±SEM.

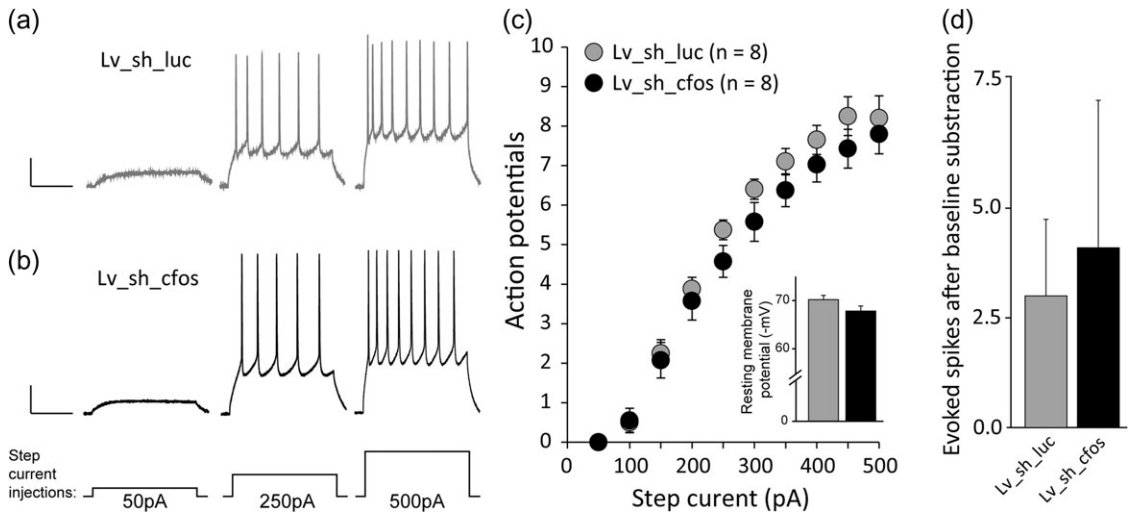

Figure 3. Basal neuronal excitability is intact after blocking of c-Fos expression. Example traces of action potential series in response to depolarizing steps of 50, 250 and 500 pA currents in the LV_sh_luc (*a*) and LV_sh_c-fos (*b*) infected neurons. (*c*) Summary graph of numbers of action potentials evoked by step current injections. (*d*) Bar graph representing mean evoked activity, and SEM, across individual animals in the naive groups (untrained; LV_sh_c-fos in black and LV_sh_luc in gray) in response to a frequency sweep.

$P = 0.085$, Fig. 3*a*–*c*). Figure 3*d* shows the average multiunit responses to sounds recorded in vivo at 350–450 microns depth (layer 3, occasionally 4, Lefort et al. 2009) in naïve mice from the 2 groups. We fitted a linear mixed effects model (fixed effects: tone frequency, sound level, and group – LV_sh_c-fos/LV_sh_luc; random mouse-dependent slopes for sound level). Neither the main effect of group nor the interaction between group and frequency were significant [$\chi^2(22) = 22.92$, $P = 0.41$; and $\chi^2(22) = 22.91$, $P = 0.35$, respectively]. Overall, these results suggest that over the few weeks in which it was partially blocked, the loss of c-Fos expression did not affect auditory cortex function in naïve animals. Layer 3 receives inputs from layers 4 and 5 as well as from the more superficial layer 2 (Lefort et al. 2009). Layer 3 itself projects to layer 5 and to the contralateral cortex, as well as to higher

brain areas. Given the interconnectivity between the layers (Harris and Mrsic-Flogel 2013), the finding that both baseline and evoked activity were normal in this layer in the naïve mouse is strongly suggestive of a normally functioning cortical column.

Having determined that at baseline, manipulation of c-Fos expression did not have an effect on neuronal excitability and sound responses, we next assessed experience-dependent plasticity by in vivo acute electrophysiology in animals that had been trained in the Audiobox as before (trained group) or had spent the same amount of time in their home cage (naïve group). The trained animals were taken out of the Audiobox for electrophysiology during the phase where 50% of the visits had the withhold sound. The data were analyzed by a linear mixed effects model as before, with training (trained/naïve) as an additional fixed factor.

Electrode insertion tracts were levelled with the virus injection tract in the anterior–posterior axis (Fig. 4*a*). The average best frequency of the recording locations in the trained mice was 11 ± 5 kHz for the LV_sh_luc group and 12 ± 8 kHz for the LV_sh_cfos group (mean ± ste at 50/60 dB SPL). Since the middle point between the 2 behavioral frequencies (9.9 kHz) falls within these ranges, most recordings were performed in areas that responded well to both frequencies at 70 dB (Fig. 4*b*). Evoked responses were typically restricted to the onset of the sound and had latencies below 20 ms (Fig. 4*c* and *e*), as expected from responses in A1 (Joachimsthaler et al. 2014). This, in combination with the location of the injection, and the position of the electrodes upon visualization of the vessel pattern after craniotomy, strongly suggest that all recordings were performed in A1.

We found that experience-dependent plasticity was impaired in trained LV_sh_c-fos mice (Fig. 4*b-d*). All fixed effects and interactions were highly significant. The significant main effects of sound level ($F(35\,893) = 4.7$, $P = 0.0029$) and frequency [$F(215\,893) = 42.9$, $P = 3.9 \times 10^{-164}$] were expected, since all mice were trained with the same "safe" and "withhold" frequencies. There was also a significant main effect of training [$F(15\,893) = 9.2$, $P = 0.0024$], due to the general increase in responses in trained animals (Fig. 4*b*, for both groups of animals trained responses were larger than naïve responses). Thus, the operant conditioning paradigm

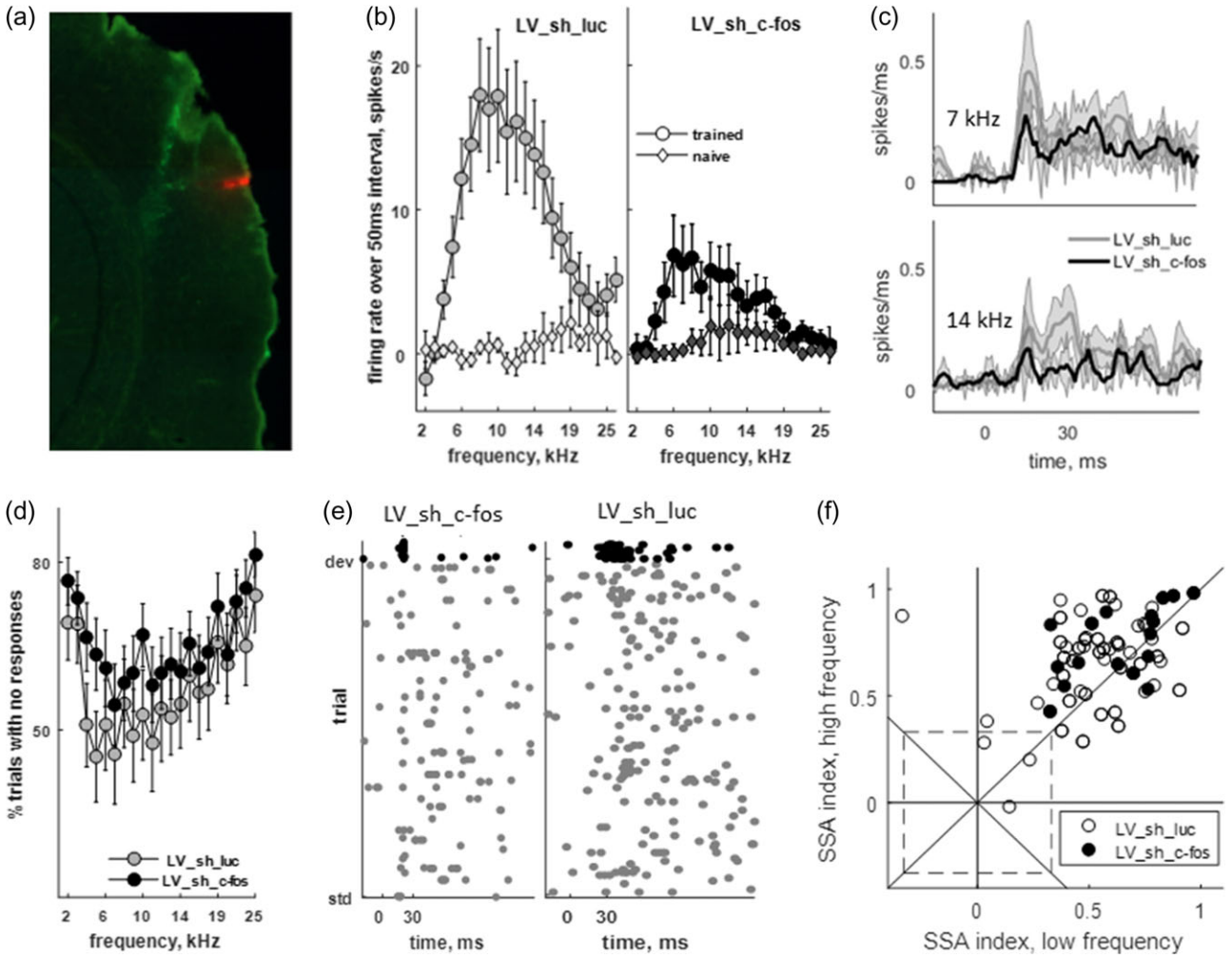

Figure 4. Blocking c-Fos expression in auditory cortex impairs experience-dependent plasticity. (*a*) Example, DiY (red) and GFP signals marking electrode tract and virus injection site, respectively. (*b*) Onset response (firing rate 1–50 ms after stimulus onset at 70 dB) across tone frequencies in trained (circles) and naïve (diamonds) mice from LV_sh_c-fos (right) and LV_sh_luc (left) groups. (*c*) Mean peri-stimulus time histograms evoked by the "safe" (top) and "withhold" (bottom) tone at 70 dB in trained LV_sh_c-fos (black) and LV_sh_luc (gray) mice. (*d*) Mean number of failures (trials without spikes) in evoked responses across tones for trained LV_sh_c-fos (black) and LV_sh_luc (gray) mice. (*e*) Example responses to "withhold" tone when standard (gray) and when rare (black) in a LV_sh_c-fos (left) and LV_sh_luc (right) mouse. (*f*) SSA indices for frequency-pairs presented in the oddball paradigm in trained LV_sh_c-fos (black) and LV_sh_luc (white) mice.

used in the AudioBox significantly modified the neuronal responses in auditory cortex. Importantly, there was a significant group effect [$F(15\,893) = 14.9$, $P = 0.00010$] as well as an interaction of group and training [$F(15\,893) = 6.4$, $P = 0.012$]. Clearly, following training the increase in the responses in the LV_sh_c-fos group (with suppressed c-Fos expression) was substantially smaller than that in the LV_sh_luc group. The responses in naïve animals did not differ between the 2 groups, though (see above, Fig. 3*d*). Finally, all the effects showed frequency dependence (interaction between training and frequency: $F(215\ 893) = 11.1$, $P = 5.6 \times 10^{-37}$; group and frequency: [$F(215\,893) = 11.3$, $P = 1.2 \times 10^{-37}$; group, training and frequency: $F(215\ 893) = 4.5$, $P = 2.9 \times 10^{-11}$]. In particular, onset responses (0–50 ms from stimulus onset) to "safe" and "withhold" tones were weaker in the trained LV_sh_c-fos mice than in trained LV_sh_luc animals [Fig. 4c; 7 kHz: $F(14\,177) = 4.9$, $P = 0.025$; 14 kHz: $F(14\,177) = 12.3$, $P = 4.5 \times 10^{-4}$]. The responses at frequencies much below or above this range were smaller (Fig. 4*b*).

The differences in onset tuning were partly due to the increased number of failures (trials that elicited no spikes) in LV_sh_c-fos mice (Fig. 4*d*). We fitted a linear mixed effects model to the number of failures in the trained animals (fixed effects: tone frequency, sound level, and group – LV_sh_c-fos/LV_sh_luc; random mouse-dependent slopes for sound level; main effects only, since interactions were not significant). The model showed a significant main effect of group on response failures [$F(13\,105) = 28.4$, $P = 1.0 \times 10^{-7}$].

Thus, training led to the expected increase in sound-evoked neuronal activity in auditory cortex of LV_sh_luc mice, an increase that was not matched in magnitude by the LV_sh_c-fos mice.

In addition to the sensory responses, we also tested contextual dependence of the neuronal responses by quantifying stimulus-specific adaptation (SSA), a reduction in neural activity caused by repeated presentation of a stimulus that does not generalize to other stimuli (Ulanovsky et al. 2003; Taaseh et al. 2011). SSA was quantified using the SSA index, the contrast in the responses to the same tone when rare (5% of the trials) and when common (95% of the trials; see Methods; Fig. 4*e* for example response to 14 kHz as rare and common). SSA indices calculated from the peak onset response were in fact significantly higher in the trained LV_sh_c-fos group compared with the trained LV_sh_luc mice (Fig. 4*f*). This is the result of a wider distribution of values in the LV_sh_luc group compared with the LV_sh_cfos group, which had consistently high values. We performed a linear mixed effects analysis on the relationship between the SSA indices of both groups with group (trained LV_sh_luc and LV_sh_cfos), protocol (low or high frequency tone as rare sound), and ΔF as fixed effects, and with mouse identity as a random effect (with by-mouse random slopes for the effect of protocol and ΔF). We found no significant effect of the random intercepts [$\chi^2(1) = 1.653$, $P = 0.20$], nor a main effect of ΔF [$\chi^2(2) = 2.891$, $P = 0.23$]. To check for group effects, we therefore fitted a main-effect model with group and protocol as fixed effects. In this model, there was a significant main effect of group [$F(1\,203) = 12.91$, $P = 0.00041$]. This confirmed that sensitivity to rare tones was not only intact but on average larger in cortical neurons of LV_sh_c-fos mice than in those of LV_sh_luc mice.

Finally, we assessed the specificity of the behavioral deficit both within and outside the auditory domain. Place avoidance was used as a measure of non-auditory learning and was tested in the IntelliCage system. No difference was observed between the LV_sh_c-fos and the LV_sh_luc mice in this task, suggesting no underlying non-specific learning deficits [percentage of incorrect responses, two-way ANOVA (group × session), only the effect of session was significant: $F(1,14) = 279.9$, $P = 1.2 \times 10^{-10}$, followed by one-way ANOVAs (pre-learning vs. learning), sh_c-fos: $F(1,18) = 367$, $P = 2.0 \times 10^{-13}$, sh_luc: $F(1,10) = 53.6$, $P = 0.00025$; activity level measured as number of nose-pokes in all conditioning units, two-way ANOVA (group × session), only the effect of session was significant: $F(1,14) = 24.9$, $P = 0.00020$, the effect of group: $F(1,14) = 0.1$, $P = 0.74$, the group × session interaction: $F(1,14) = 1.0$, $P = 0.33$; Fig. 5*a*]. The stimuli we used lead to amygdala-dependent classical cue-fear conditioning that does not require auditory cortex (Romanski and LeDoux 1992, but see Boatman and Kim 2006).

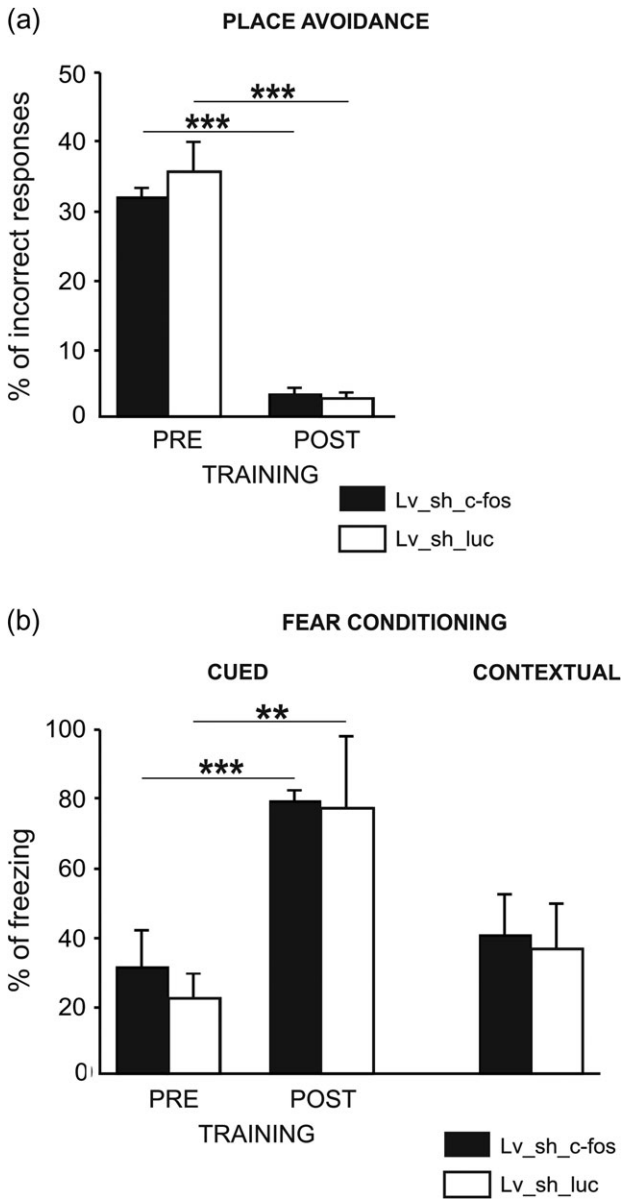

Figure 5. Blocking c-Fos expression in auditory cortex does not affect learning in non-auditory operant task and classical fear conditioning. (*a*) The place avoidance learning in the IntelliCage system. The mice were subjected to 2-day adaptation period and then, 2-day learning session during which the operant responses (nose-pokes) in one of the 4 corners of the cage were punished by air-puffs. Percentage of nose-pokes in the conditioning unit associated with air-puffs and the level of activity measured as number of responses in all 4 conditioning units. (*b*) The level of freezing in the LV_sh_c-fos and LV_sh_luc groups to the tone (measured after 24 h), and to the conditioning context (measured after 48 h); ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$.

In agreement, we found that cue-conditioning was unaffected by blocking the expression of c-Fos and its subsequent effect in cortical plasticity, and the percentage of time that both groups spent freezing after cue presentation was comparable [two-way ANOVA (group × session), only the effect of session was significant: $F(1,12) = 38.3$, $P = 0.000047$, the effect of group: $F(1,12) = 1.7$, $P = 0.21$, the group × session interaction: $F(1,12) = 0.01$, $P = 0.93$, Fig. 5*b*]. Hippocampal-dependent contextual fear conditioning was also unaffected (*t*-test: $t = 0.21$, df = 6, $P = 0.95$, Fig. 5*b*). We conclude that c-Fos expression dependent plasticity in auditory cortex is not necessary for simple classical conditioning. This result refines further the finding that classical conditioning is not dependent on auditory cortex (Romanski and LeDoux 1992). The behavioral deficit induced by c-Fos suppression was, therefore, specific to operant auditory discrimination learning.

Discussion

In the present study we found that mice were impaired in tone frequency discrimination learning following blockade of c-Fos-dependent plasticity in auditory cortex. Our results link inhibition of c-Fos expression to changes in neuronal responses to sounds as well as to the acquisition of behavioral responses.

The hypothesis that c-Fos was involved in learning and memory stemmed from the fact that its expression patterns mirrored different types of behavioral training (Kaczmarek 1993). However, since enhanced c-Fos expression is associated with many different neuronal activation paradigms, potentially involving different molecular and cellular mechanisms, it has been difficult to pin down its role to a particular downstream function, such as the induction of synaptic plasticity. Recently, several studies have concluded that the memory engram localizes to those cells that showed behaviorally-driven c-Fos expression (Tonegawa et al. 2015). These results strongly support the hypothesis that there is a link between c-Fos and memory (Tonegawa et al. 2015). Here we show a causal relationship between c-Fos and learning: when its experience-dependent expression was blocked, corresponding changes in both behavioral and neuronal plasticity were observed.

We found that auditory-driven c-Fos expression blockade affected neither baseline excitability nor auditory evoked activity in naïve animals. There was also no deficit in SSA, again suggesting that basal neuronal processing is intact. The deficit was specific to experience-driven plasticity and operant frequency discrimination. The lack of difference in baseline activity in response to sound is essential to interpret the findings in terms of learning and memory processes. In this regard, our approach has an important advantage over conditional knockouts, a manipulation which clearly interfered with basal neuronal functioning as the electrophysiological changes were observed well before the induction of plastic changes (Fleischmann et al. 2003). Our approach, however, in which the interference with c-Fos induction occurred over a relatively short period of time only, preserved neuronal excitability. Thus, our findings suggest that c-Fos expression is involved in long-term memory formation through synaptic plasticity. This is in striking contrast to the role of CREB transcriptional regulator (Lopez de Armentia et al. 2007; Yiu et al. 2014), whose deficiency leads to reduced excitability and, consequently, to learning and synaptic plasticity deficits (Jancic et al. 2009; Benito and Barco 2015).

In our study, the deficits in mice with blocked c-Fos expression in auditory cortex were limited to tasks in which mice had to discriminate between different sound frequencies. We found no effect of c-Fos blocking on learning per se or, more specifically, on auditory fear conditioning. The latter is in agreement with results of others (Romanski and LeDoux 1992) who have found that auditory cortex is not necessary for the generation of associations between pure tones and foot shock during fear conditioning.

Since SSA refers to the ubiquitous reduction of the responses to a commonly presented tone (Ulanovsky et al. 2003; Taaseh et al. 2011), the results which showed that SSA is intact in the experimental animals seem to be consistent with the results of behavioral training. When the rate of "withhold" visits was low, the deficit in recognition of the "withhold" tone was smaller than when the rate was higher. We hypothesize that the contrast in the responses evoked by the "safe" and "withhold" stimuli due to SSA was the signal through which the experimental animals could discriminate between the 2 tones.

In summary, we link for the first time a specific form of experience-driven c-Fos-dependent plasticity in auditory cortex with a specific behavior. Our findings therefore strongly support the view that c-Fos expression is involved in learning and memory.

Methods

Lentiviral Vectors Production

HEK293T cell line at 70% confluency was transfected with structural plasmid, packaging plasmid and construct pTrip_eGFP bearing shRNA coding sequences sh_luc (5′ aaa agg ttt ttc tga cgc gga ata ctt cga gag gaa tcg aag tat tcc gcg tca g 3′) or sh_c-fos (5′ aag gtt ttt gct tcc ctt gcc tta ttc tag aga act tag aat aag gca agg gaa gcc 3′) sequence (si sequence is underlined). Chemical transfection with PEI (MW ~ 25 000; POCH) was used. After 2 days of incubation culture medium was collected, cetrifuged (150 × *g*, 10 min, RT) and treated with DNAse (1:1000 of collected medium volume 1 M MgCl2, 1 U/µl DNAse, Sigma) for 15 min in 37°C. Vector particles were further purified and concentrated by ultracentrifugation (120 000 × *g*, 1.5 h, 4°C). Pelet was incubated in PBS (2.5 h, 4°C), resuspended, aliquoted and stored in −80°C. Concentration of vector particles was established by qPCR method (Scherr et al. 2001) with empty construct pTrip_eGFP for lentiviral vectors production as a standard, using the following primers: F 5′ agc ttg cct tga gtg ctt ca 3′, R 5′ tga cta aaa ggg tct gag gga 3′.

Subjects

Ethics statement: Experiments were performed in the Nencki Institute of Experimental Biology in Warsaw and in the Max Planck Institute of Experimental Medicine in Göttingen. The animals were treated in accordance with the ethical standards of European regulations (directive no. 86/609/EEC) and, respectively, Polish Local Ethics Committee and the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, project license number 33.14-42 502-04-10/0288. C57BL/6 mice obtained from the commercial suppliers were group-housed and maintained on a 12/12 h light/dark cycle with water and food provided ad libitum. Before experiments all mice were subcutaneously implanted with a sterile transponder (DATAMARS T-IS 8010 FDX-B, 13 mm long, 2 mm in diameter, 0.1 g in weight).

Surgeries and Vector Injection

Two month old C57BL/6 females were kept under isoflurane anesthesia for the duration of surgery. After induction of anesthesia, butomidor (10 mg/ml) was injected subcutaneously

(0.02 ml). Skin on the skull was cut and pulled aside to expose the skull surface for drilling. Injection coordinates were established (AP = −0.25 cm, L = ±0.44 cm, DV = −0.32 cm) from bregma and drilling was performed to enable access to the brain. Lentiviral vectors were bilaterally injected to auditory cortex (4.5×10^7 vector particles per site, rate of injection 100 nl/min) using Hamilton syringe (65 RN with RN NDL 30/2/3 S needle) attached to the infusion pump. LV_sh_luc was injected in auditory cortex of the LV_sh_luc mice and LV_sh_c-fos in auditory cortex of the experimental mice. Before and after injection the needle was kept in the position for 5 min. When the needle was removed, skin was stitched and Triderm was applied around the wound. Animals received Tolfedine (0.08%, 0.1 ml, intramuscular) for 2–4 days after surgery (depending on their condition). Animals were allowed a one month recovery before behavioral experiments began.

Behavior in the Audiobox

Apparatus

Sound frequency discrimination training was carried out in an Audiobox (New Behaviour/TSE, Germany), a device developed for auditory research and based on the IntelliCage (NewBehavior, Switzerland). The model used in Warsaw was a prototype, while the one used in Göttingen is commercially available (TSE). The Audiobox serves both as living quarters for the mice and as their testing arena. The mice are kept in groups of 8–10 animals. Each animal is individually identifiable through the use of the implanted transponder, and the behavior of each mouse is automatically detected by 2 means: reading of the unique transponder carried by each mouse by an antenna at the entrance to the drinking corner (see below), and detection of specific behaviors (nose-poking and licking) through other sensors. The design of the Audiobox restricts access to the drinking corner for a single mouse only. Handling of the animals by the experimenter is reduced to the weekly cleaning of the cages and apparatus.

The Audiobox was kept in a dedicated and temperature regulated room in a 12/12 h dark/light cycle. The Audiobox consists of 2 compartments connected by a long corridor. One compartment, a normal mouse cage, serves as the home cage, where the animals have access to food ad libitum. Water is delivered in the second compartment of the Audiobox, the "corner," which is positioned inside a sound-attenuated box. Entrance into the corner, a "visit," is detected by an antenna located at its opening that reads the implanted transponder. The beginning of the visit is defined by both the detection of a transponder by the antenna and the activation of a temperature sensor within the corner. The end of the visit occurs when the same transponder is not detected anymore by the antenna and the temperature sensor is no longer activated. Thus, the Audiobox identifies the specific mouse that enters the corner, and can therefore select the stimulus to be presented accordingly. All behavioral data is logged for each mouse individually. Once in the corner, the mouse can access water by nose-poking into either of 2 ports, one at each side of the corner. The doors to the ports can be opened or closed depending on the demands of the experiment. A loudspeaker is positioned directly behind the corner, or above it, for the presentation of the stimuli.

Sounds were generated using Matlab (Mathworks) at a sampling rate of 96 or 48 kHz and written into computer files. Output was calibrated using either a Brüel and Kjaer (4939 ¼″free field) or a GRAS (1/4″ 40BE) microphone. The microphone was placed at different positions within the corner, as well as outside the corner. Relevant sounds were played at the nominal intensities used in the study. Microphone signals were sampled at 96 kHz and analyzed in Matlab. Tones between 3 and 19 kHz did not show any significant harmonic distortion. In the rare occasions when harmonics were present, they were at least 40 dB below the main signal. There was a linear correspondence between the nominal sound level and the sound level measured by the microphone.

While sounds played inside the corner were significantly attenuated outside of the attenuated box (>20 dB), there was little attenuation between the corner and the corridor directly leading to it (about 10 dB). In consequence, mice in the corridor could hear the sound presented to the mouse inside the corner.

Stimuli consisted of 30 ms pure tone pips, with 5 ms rise/fall linear slopes, repeated at a rate of 3 Hz. Tones were presented in the corner throughout the visit. All tone pips presented within a given visit had the same frequency.

Throughout the duration of the experiment, one frequency (i.e., 6 670 Hz) was always "safe": when this frequency was presented during a visit, the mice could access the ports and drink water without an associated negative outcome. At some point within the training, a different tone frequency (13 340 Hz) was associated with an air-puff. The purpose of the training was to get the mice to learn this association and stop nose-poking when the visit was accompanied by the "withhold" tone.

Sound Frequency Discrimination Training Protocol Used to Test c-Fos Expression Levels

The aim of this experiment was to test the effect of sound exposure and discrimination learning on c-Fos expression levels. Since the increase in c-Fos expression is known to be transient, the usual protocol (see next section) was modified such that mice had access to water only for 2 h a day (at the same time of the active phase every day), for the remaining 22 h the doors blocking access to water in the drinking corner were closed. This temporal water deprivation evoked intense consummatory activity during a limited time span.

The detailed protocol was as follows (Fig. 1*a*):

Adaptation to cage (days 1–10): 48 h after the transponder implantation, mice were placed into the Audiobox. During this phase the doors giving access to the water within the corner remained constantly open and no sound was presented during the visits.

Adaptation to water restriction (days 11–14): During this phase mice had access to water in the Audiobox corner only for 2 h per day.

"Safe"-only phase (days 15–18): Once the mice had learned to access the water and were drinking freely, the doors were closed and only opened when the mouse nose-poked into the port. At the same time, every visit to the corner was coupled, for the duration of the visit, with the presentation of the "safe" sound (6.7 kHz tone pips).

Conditioning (days 19–33): A "withhold" tone (13.4 kHz) was introduced in a small (see below) percentage of the visits. As in the "safe"-only phase, a train of tone pips was presented for the duration of the visit. The presence of the "withhold" tone had a behavioral significance: a nose-poke during the presentation of the tone resulted in the delivery of an aversive air-puff, through a tube located on the ceiling of the corner. In addition, the doors that gave access to water did not open. The remaining visits were as during the safe-only phase: the

"safe" tone was presented and access to water was granted after a nose-poke.

To eliminate anxiety-evoked suppression of visiting the conditioning unit, the "withhold" (13.4 kHz) tone was introduced gradually over 3 2-day sessions such that in the first 2 days only 5% of visits were conditioned, then 10% and finally, and until the end, 17% of visits were conditioned.

Different mice were removed from the Audiobox at different time points during the training (after the first "safe"-only session $n = 8$ and after fourth "safe"-only session $n = 8$; after the first "conditioned" session $n = 8$ and after 15th "conditioned" session $n = 8$; Fig. 1*a*). Mice were removed always at the end of a 2-h period with water access. Brain tissue was then used for c-Fos immunohistochemistry.

To assess reduction of sound-evoked c-Fos expression in auditory cortex of LV_sh_c-fos injected mice the level of c-Fos expression was assessed in mice exposed for 90 min to the "safe" and "withhold" sounds played alternately in their home cages (LV_sh_c-fos group n=5; LV_sh_luc group $n = 4$).

Sound Frequency Discrimination Training Protocol Used to Assess the Effect of c-Fos Expression Blockade on Both Sound-Evoked Auditory Cortex Responses and Behavior

The aim of this experiment was to test the effect of c-Fos expression blockade on discrimination learning. A new set of mice was used in the experiment (LV_sh_c-fos, $n = 12$; LV_sh_luc, $n = 8$ for the purely behavioral tests).

The detailed protocol was similar to the protocol established in the immunomapping studies with the following modifications (Fig. 3a)—mice had access to water 24 h a day instead of 2 h, and the training was extended by the additional session with 50% of the "withhold" sound and 3 extinction sessions:

Extinction (days 17–19): During the extinction phase the 13.4 kHz tone was no longer conditioned. Thus, half of the visits were "safe" and the other half was accompanied by the previously "withhold" tone but nose-pokes during both types of visits triggered opening of the doors and access to water. No air-puffs were delivered.

c-Fos Immunohistochemistry

Mice were anesthetized with an overdose of sodium pentobarbital and perfused intracardially with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and stored in the same fixative for 24 h at 4°C, and subsequently immersed in 30% sucrose at 4°C. The brains were then slowly and gradually frozen and sectioned at 40 μm on a cryostat. The coronal brain sections containing auditory cortex were collected. The immunohistochemical staining was performed on free-floating sections. The sections were washed 3 times in phosphate-buffered saline (pH 7.4), incubated for 10 min in 0.03% H_2O_2 in PBS, washed twice in PBS, and incubated with a polyclonal antibody (anti-c-Fos, 1:1000; Santa Cruz Biotechnology no. sc-52) in PBS and normal goat serum (3%; Vector) for 48 h at 4°C. The sections were then washed 3 times in PBS with 0.3% Triton X-100 (Sigma), incubated with goat anti-rabbit biotinylated secondary antibody (1:500; Vector) in PBS/Triton and normal goat serum (3%) for 2 h at room temperature, washed 3 times in PBS/Triton, incubated with avidin–biotin complex (1:1000 in PBS/Triton; Vector ABC kit) for 1 h at room temperature, and washed 3 times in PBS. The immunostaining reaction was developed using the oxidase-diaminobenzidine-nickel method. The sections were incubated in distilled water with diaminobenzidine (DAB; Sigma), 0.5 M nickel chloride and peroxidase (Sigma) for 5 min. The staining reaction was stopped by 3 washes with PBS. The reaction resulted in a dark-brown stain within the nuclei of c-Fos immunoreactive neurons. The sections were mounted on slides, air dried, dehydrated in ethanol solutions and xylene, and cover slipped with Permount (Fisher Chemicals). The measure of c-Fos immunopositivity was expressed as density, determined in the following manner: for each brain section, the number of c-Fos immunopositive nuclei in auditory cortex was counted and divided by the area occupied by the structure (in millimeters squared). Image analysis was done with the aid of an image analysis computer program (Image J) on 6 sections per animal brains.

Whole-Cell Current Clamp Electrophysiological Recordings

LV_sh_c-fos and LV_sh_luc mice were anesthetized with isoflurane and decapitated. Coronal brain slices, 250 μm thick, were prepared using Leica VTS1000 vibratome in ice-cold NMDG cutting solution (135 mM NMDG, 1 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $MgCl_2{\cdot}6\,H_2O$, 0.5 mM $CaCl_2{\cdot}2\,H_2O$, 20 mM choline bicarbonate, 10 mM D-glucose, bubbled with carbogene – 5% CO_2, 95% O_2). Slices containing auditory cortex were collected, transferred to ACSF solution (119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM D-glucose) and incubated for at least 30 min at room temperature. The recording chamber was perfused with an ACSF solution, heated up to 29–31°C, constantly bubbled with carbogene. Cortical neurons located in layer 3 were identified visually and patched with a borosilicate glass pipette of 3–5 MΩ resistance. The patch pipette was filled with internal solution: 120 mM K gluconate, 10 mM HEPES, 2 mM $MgCl_2$, 0.4 mM EGTA, 0.1 mM $CaCl_2$, 2.5 mM Na_2ATP, 0.25 mM NaGTP. pH was adjusted to 7.0–7.3 using KOH and osmolarity to 290–295 mOsm with KCl. Recordings were carried out in whole-cell current clamp configuration, filtered at 2 kHz.

In Vivo Electrophysiology

Electrophysiology was performed in anaesthetized mice. The "trained" group was composed of mice that were in the Audiobox, in the conditioned phase with 50% probability of a conditioned visit. These mice (LV_sh_c-fos, $n = 11$ mice and 22 recording locations; LV_sh_luc, $n = 12$ and 18 recording locations) were taken out of the Audiobox and anaesthetized immediately. The "naïve" group (LV_sh_c-fos, $n = 4$ mice and 12 recording locations; LV_sh_luc, $n = 3$ mice and 8 recording locations) was composed of mice that had been sitting in their homecage for at least 7 weeks following viral injection. Mice were anaesthetized with Avertin (tribromoethanol, 250 mg/kg body mass, i.p.) and placed in a stereotaxic frame. The animal was fixed to the stereotaxic frame without ear bars. For this purpose a metal lamina was glued to the top of the skull with hystoacryl and super glue. The lamina was then fixed to the stereotaxic frame. A metal screw was inserted into the skull and used as ground. A craniotomy was performed over the left auditory cortex prior detachment of the muscle temporalis. The craniotomy was about 4 mm long (anteriorposterior axis) and 2 mm wide (dorsoventral axis) and was made following the contour delimited rostrally and ventrally by the temporal fissures, dorsally by the temporal ridge, and caudally by the caudal ridge. Electrodes were glass coated and made of either

tungsten (Alphaomega, 900 kOhm) or a platinum/tungsten mixture (Thomas recording, 1.5–2 MOhm). They were inserted 350–450 microns deep from the surface of the dura and both spikes and local field potentials were recorded during sound presentation.

Electrophysiology Setup

Voltage signals were sampled at 32 kHz through a unity-gain preamplifier (HS36, Neuralynx, Bozeman, MT, USA), amplified (500–1500 times) and band pass filtered (Digital Lynx SX system, Neuralynx, Bozeman, MT, USA). For spike detection, voltage traces were band-passed (500–6000 Hz) offline and negative peaks larger than a threshold of 7 times the median absolute deviation of the filtered trace were considered spikes. Threshold was determined after careful examination of the traces. Spike time was defined as the time of the peak of the spike.

Acoustic Stimulation During the Electrophysiology

Acoustic stimuli were generated using custom-written Matlab routines and converted into analog waveforms by a sound card (Octa-capture, Roland, Nauheim, Germany) and presented via an ultrasonic speaker (Vifa, Avisoft Bioacoustics, Berlin, Germany). Two types of stimuli were presented: broad band noise and pure tones. Broad-band noise (BBN) stimuli at different intensities were used to determine presence and threshold of responses. BBN pulses were 70 ms in length with 5 ms rise/fall linear slopes and were presented at a rate of 2 Hz. Pure tones of different frequencies (2–24 kHz), 30 ms in length and with 5 ms rise/fall linear slopes were presented at a rate of 3 Hz and at intensities ranging between 50 and 80 dB SPL (RMS). Each sound was presented at least 5 times, sometimes 10, and responses to stimulus repetitions were averaged. Tuning curves were constructed from the averaged evoked spike rate. This rate was calculated for each recording position and each pure tone presented based on the sum of spikes elicited over several tone repetitions during an interval of 50 ms from stimulus onset. Different recording locations within a mouse were averaged together. Tuning curves were smoothed with a moving average filter with a span of 3. Recordings from a given mouse were averaged together. Failures were defined as trials in which no evoked spikes were elicited over the 200 ms following stimulus onset.

Oddball paradigms comprising 2 pure tones (ΔF=1.1, 1.2 or 1.44%) were used to determine SSA. Tones were 30 ms in duration with 5 ms rise/fall linear slopes and were presented at a rate of 3 Hz. Each pair of tones was used in 2 oddball protocols. In one, the lower frequency was presented in 95% of the trials (standard or common) and the higher tone in 5% (deviant or rare). In the other protocol the lower tone became the rare tone and the higher tone, the standard. The total number of trials was always such that the rare tone was presented in at least 25 trials. SSA indices were calculated by comparing the peak response, over the 200 ms following stimulus onset, elicited by a given tone when standard and when deviant and normalized to the summed response (deviant-standard/[deviant + standard]).

Place Avoidance in IntelliCage

Place avoidance training was performed in the IntelliCage (NewBehavior, Switzerland, (Puścian et al. 2014)). The LV_sh_c-fos and LV_sh_luc mice were subjected to 2-day adaptation period when they had access to water by nose-poking, and then, 2-day learning session during which the operant responses (nosepokes) in one of the 4 corners of the cage were punished by airpuffs (1 bar). Numbers of visits and nosepokes in all 4 operant learning chambers were quantified.

Fear Conditioning

For fear conditioning (Knapska and Maren 2009), the LV_sh_c-fos and LV_sh_luc mice were placed in the conditioning chambers (MedAssociates) in context A, allowed 2 min free exploration, and then received 3 tone (CS, 20 s; 80 dB; 5 kHz)-footshock (US, 1 s; 0.7 mA) pairings (CS + US) with 40-s intertrial intervals (ITIs). 40 s after the final shock animals were returned to their home cages. Mice were tested in both contexts, B (24 h after the training) and A (48 h after the training). Testing consisted of one 20-s tone CS presentation beginning in the second minute (80 dB; 5 kHz) or 3-min context presentation, respectively. Fear response to the CS tone or context was assessed by measuring freezing behavior. Freezing behavior was recorded using a camera placed in front of each chamber. Digital video was saved on and automatically analyzed by VideoFreeze software (MedAssociates system) installed on a computer system located in an adjoining room. Freezing was only scored if the mouse was immobile for at least 1 s. For each session, the freezing observations were transformed to a percentage of total observations. Freezing response was measured in 20-s period following the CS's presentation or during the whole presentation to context A.

Statistical Analysis

Statistical analyses of c-Fos expression levels and performance in the Audiobox were performed with Statistica 8.0 (StatSoft). When the datasets met the criteria for parametric analyses one-way and two-way ANOVAs followed by Fisher's LSD post-hoc tests were applied. When the criteria were not met the data were subjected to nonparametric testing with Kolmogorov–Smirnov test. For the statistical analysis of the multiunit activity we used a linear mixed-effects model (MATLAB, routine fitlme). As fixed effects we typically (but see description in Results) specified group (LV_sh_c-fos vs LV_sh_luc), training (naive vs. trained) and tone frequency. Sound intensity and mouse identity were specified as non-interacting random effects. The full model was first compared with one in which group was not included to confirm that the presence of a significant effect of group. Further analysis was performed in the full model using ANOVA. The criterion for statistical significance was a probability level of $P < 0.05$.

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