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Multisensory Integration of Natural Odors and Sounds in the Auditory Cortex

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SUMMARY

Motherhood is associated with different forms of physiological alterations including transient hormonal changes and brain plasticity. The underlying impact of these changes on the emergence of maternal behaviors and sensory processing within the mother's brain are largely unknown. By using in vivo cell-attached recordings in the primary auditory cortex of female mice, we discovered that exposure to pups' body odor reshapes neuronal responses to pure tones and natural auditory stimuli. This olfactory-auditory interaction appeared naturally in lactating mothers shortly after parturition and was long lasting. Naive virgins that had experience with the pups also showed an appearance of olfactory-auditory integration in A1, suggesting that multisensory integration may be experience dependent. Neurons from lactating mothers were more sensitive to sounds as compared to those from experienced mice, independent of the odor effects. These uni- and multisensory cortical changes may facilitate the detection and discrimination of pup distress calls and strengthen the bond between mothers and their neonates.

INTRODUCTION

In rodents, the interaction between a mother and her neonates is mediated by a set of characterized sounds emitted by pups that elicit specific maternal behaviors (Ehret, 2005). For example, wriggling calls (WCs) are emitted by mouse pups struggling in the nest. The mother responds by licking the pups, changing her nursing position, and reorganizing the nest (Ehret, 1975; Ehret and Riecke, 2002). A second example are the ultrasonic vocalizations (USVs) produced by young pups that are unable to maintain their body temperature when they are isolated from the nest (Noirot, 1966; Sewell, 1970). These distress calls alert the mother, which prompts her to search for and retrieve the isolated pup back to the nest (Haack et al., 1983; Sewell, 1970). Both WC- and USV-induced maternal behaviors are a hallmark of rodent mothers but not of naive virgins (Leuner et al., 2010; Noirot, 1972).

Maternal behaviors can be regulated by stimuli of different sensory modalities. Olfaction, for example, is a central sense by which rodents communicate with each other. Indeed, pup odors efficiently trigger maternal behaviors and inform the mother of the presence of her pups (Lévy and Keller, 2009; Lévy et al., 2004; Smotherman et al., 1974). Thus, mothers use both auditory and olfactory cues to identify and locate their pups. Because pup calls are always perceived by a lactating mother in an environment enriched with the scent of her pups, it may learn the contingency between these different stimuli. Although there is ample behavioral evidence supporting the value of each sense separately, the merging of these senses in a natural context has not been studied. Even less is known about how neurons respond to multisensory stimuli either before or after maternal associations form. We hypothesized that the odors of pups will modulate the way pup calls are processed by the mothers. Given that the primary auditory cortex (A1) is involved in auditory object recognition and is a known site of neuronal plasticity (Miranda and Liu, 2009; Nelken, 2004; Nelken and Bar-Yosef, 2008; Romanski and Averbeck, 2009; Weinberger, 2004), we tested whether it serves as an early processing station for multisensory integration of pup odors and pup calls. To test this hypothesis, we introduced pup odors to both naive and experienced female mice while monitoring the spiking output of neurons in A1. We found that pup odors triggered robust modulation of auditory processing only in females that interacted with pups. This olfactory-auditory integration had a particularly strong effect on detection and discrimination of pup distress calls, suggesting that it is experience dependent.

RESULTS

Using in vivo cell-attached recordings, we monitored both spontaneous and sound-evoked neuronal activity in A1 of anesthetized female mice. We chose this configuration because it is an unbiased sampling technique that provides stable recordings with excellent single unit isolation for long durations (DeWeese et al., 2003; Hromádka et al., 2008). Single-cell response profiles allowed us to monitor whether and how pup odors modulated sound processing (Figure 1A). In total, we recorded 471 neurons from 60 mice (see Table S1 available online). We first surveyed spontaneous activity and pure tone responses in two experimental groups: primiparous "lactating mothers" (PD4; postpartum day 4) and age-matched naive virgins.

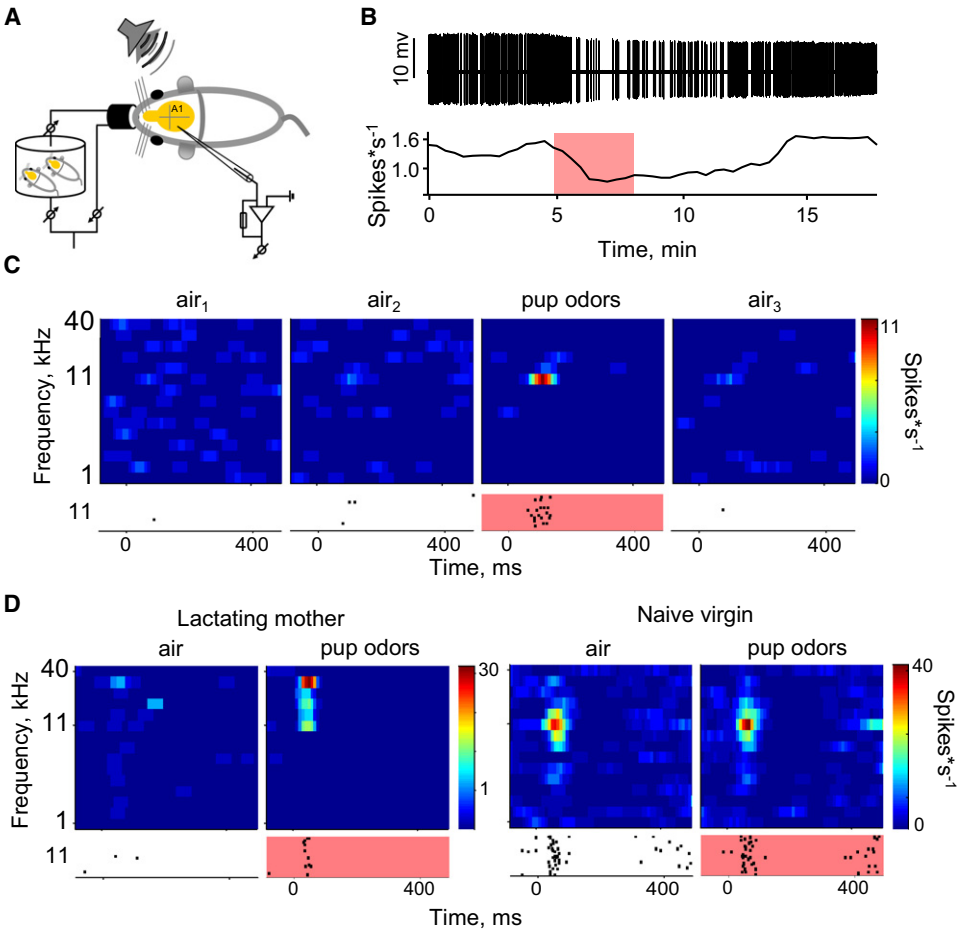

Figure 1. Pup Odors Modulate Neuronal Activity in A1 of Lactating Mothers

(A) Schematic illustration of the experimental setup for in vivo recordings from A1 while playing sounds and presenting pup odors. The presentation of pup odors in (B), (C), and (D) is indicated by a red rectangle.

(B) Representative cell-attached recording (17 min long) from a neuron in A1 of a lactating mother (top) and its PSTH (bottom, running average). Continuous flow of pup odor induced a reversible decrease in spontaneous activity.

(C) Spectro-temporal response fields (STRF) of a neuron from a lactating mother. The first, second, and fourth panels are under clean air flow conditions (air_{1-3}). The third panel (pup odors) is under continuous flow of air containing pup odors. Color code: rate of spikes during 10 ms bins over all trials. The raster plots below show all spikes over all trials of an 11 kHz pure tone for which this neuron responded to under odor condition (time 0 denotes the onset of the stimulus).

(D) Representative STRFs from a lactating mother (left) and a naive virgin (right). Left panels, clean air flow; right panels, pup odors flow. The raster plots on the bottom of each panel show spikes over all trials of an 11 kHz stimulus.

In lactating mothers, a continuous presentation of pup odors induced notable alterations of spontaneous firing rates (increases or decreases) in A1 neurons, which recovered within less than 10 min after odor stimulation offset (Figure 1B). Only long (several dozens of seconds) exposure to the pup odors induced evident changes in spiking activity. To rule out the possibility that these slow changes may be simply a result of a slow buildup of responses in the olfactory epithelium, we carried out EOG recordings from the axonal nerve in between the nasal epithelium and the olfactory bulb while presenting pup odors. Pup odor presentation induced rapid (<10 s) onset and offset axonal responses of olfactory receptor axons (data not shown), suggesting that although odor responses in the olfactory system are fast, A1 cortical changes are slow. In addition, spiking activity in A1 was not synchronized to the breathing cycle (data not shown), further ruling out the possibility of fast and direct synaptic interaction between olfactory inputs and auditory neurons. On a slower time scale, pup odors robustly altered the tone-evoked responses of neurons in lactating mothers. For example, we encountered a few neurons that did not respond significantly to any of the pure tones while the animal was presented with clear air (Figure 1C, air_1 and air_2), but then showed robust responses to a narrow range of tones during pup odor stimulation (Figure 1C, pup odors). Pup odor-mediated auditory responses normally returned to baseline after termination of the odor stimulation (Figure 1C, air_3). We systematically recorded from neurons with different baseline responses and tested how these changed during pup odor stimulation. Pup odors affected different parameters of sound-evoked responses, such as response probability, latency to respond,

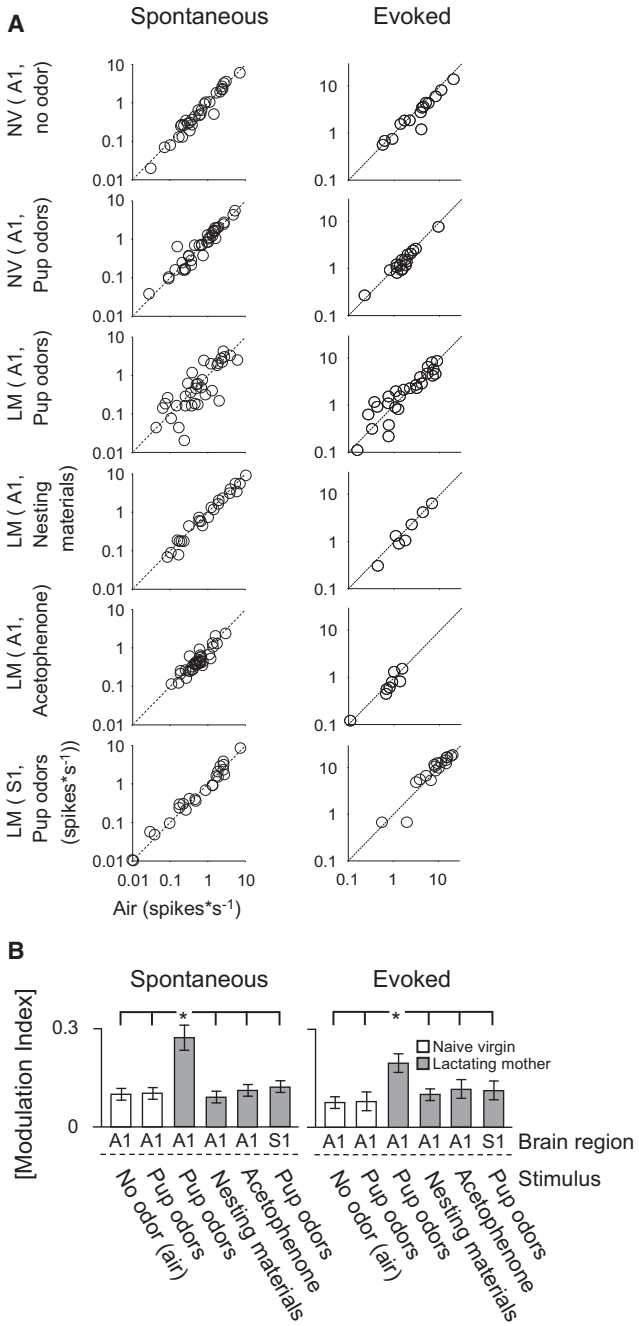

Figure 2. Pup Odors Modulate Spike Rates Specifically in Lactating Mothers

(A) Population scatter plots for six experimental groups comparing the spontaneous (left column) and evoked (right column) spike rates under clean air conditions (*x* axis) and various odor conditions (*y* axis). Each row is a separate experimental group. Each circle in each plot is data from a single neuron under two conditions. In total, data from 181 neurons are plotted. Lactating mothers, LM; naive virgins, NV.

(B) Histogram of the mean modulation index of all experimental groups and control groups for spontaneous and evoked spike rates. Absolute modulation index was calculated as ([spike rate$_{pup\ odor}$ – spike rate$_{air}$]/[spike rate$_{pup\ odor}$ + spike rate$_{air}$]). Pup odors induce modulation of both spontaneous and sound-evoked spike rates in A1 of lactating mothers (left: left to right, n = 30, 36, 34, 35, 24, and 22 neurons, respectively; right: left to right, n = 15, 17, 26, 8, 7, and 20 neurons, respectively). Error bars are mean ± SEM.

Modulation index was significantly higher for neurons in A1 of lactating mothers compared to naive virgins and to all other controls (spontaneous, *p = 0.046, Kruskal-Wallis, df = 5, Mann-Whitney U post hoc contrast with Holm correction; LM (A1, pup odor) versus NV (A1, no odor), p = 0.006; LM (A1, pup odor) versus NV (A1, pup odor), p = 0.015; LM (A1, pup odor) versus LM (A1, nesting materials), p = 0.01; LM (A1, pup odor) versus LM (A1, acetophenone), p = 0.015; LM (A1, pup odor) versus LM (S1, pup odor), p = 0.05; evoked, *p = 0.006, Kruskal-Wallis, df = 5, Mann-Whitney U post hoc contrast with Holm correction; LM (A1, pup odor) versus NV (A1, no odor), p = 0.002; LM (A1, pup odor) versus NV (A1, pup odor), p = 0.002; LM (A1, pup odor) versus LM (A1, nesting materials), p = 0.05; LM (A1, pup odor) versus LM (A1, acetophenone), p = 0.028; LM (A1, pup odor) versus LM (S1, pup odor), p = 0.008).

and response bandwidth (Figures 1C and 1D and see Figure S1 for 10 more examples of neurons from lactating mothers). Here, we describe and quantify odor-induced changes only in terms of spontaneous and sound-evoked spike rates.

Pup odors induced alterations (increases or decreases) of spontaneous and/or tone-evoked spike rates (and combinations thereof) in the majority of neurons from lactating mothers. To describe how pup odors modulated auditory responses, we plotted the average firing rate of each neuron we recorded under both "air" and "odor" conditions. Data from lactating mothers and several control groups are plotted in Figure 2A. Firing rate values for spontaneous and evoked periods are plotted separately (Figure 2A, left and right columns, respectively). Data points that fall on the diagonal correspond to neurons that experienced no change between the air and odor conditions (Figure 2A). Accordingly, neurons above or below the diagonal increased or decreased their firing rates in the presence of pup odors. As shown in Figure 2A, pup odors induce changes largely in neurons from lactating mothers (Figure 2A, third row). To quantify the changes in spontaneous and evoked spike rates, we calculated an index of modulation for each neuron ([modulation index] = [(spike rate$_{pup\ odor}$ – spike rate$_{air}$)/(spike rate$_{pup\ odor}$ + spike rate$_{air}$)]). Pup odors induced significant modulation of spontaneous and sound-evoked spike rates specifically in A1 of lactating mothers (Figure 2B, closed bar, "pup odors A1"). In contrast, neurons from naive virgins were not affected by pup odor stimulation (Figures 2A and 2B, open bars, compare "pup odors A1" and "no odor A1"). To verify that odors of the pups rather than other associated odors are indeed the source of changes, we also tested two control odorants—a strong unfamiliar odorant (0.1% acetophenone) and odors from the nesting material (cotton wool and wood shaving volatile odorants). Unlike pup odors, neither acetophenone nor nesting material affected neuronal spiking activity in lactating mothers (Figures 2A and 2B, closed bars, "acetophenone A1" and "nesting material A1"). These data reveal that neurons in A1 of lactating mothers integrate between olfactory and auditory cues. The robust nature of the pup odor-induced changes suggests that after pregnancy, parturition, and/or maternal experience, olfactory cues may act as a context-switch during which the neocortical network is transiently reorganized.

Because lactating mothers are known to be in an upregulated hormonal state (Brunton and Russell, 2008; Mann and Bridges, 2001), we tested whether our findings were the result of a global

modulation of neuronal activity throughout the neocortex. To this end, we recorded from the somatosensory cortex (S1-barrel field) of lactating mothers before, during, and after pup odor stimulation. In S1, pup odors did not induce changes in either spontaneous activity or air puff-evoked responses (Figures 2A and 2B, closed bar, "pup odors S1"). Although we did not examine other cortical regions, this result indicates that under our experimental conditions, pup odors do not induce global changes in neuronal activity across the neocortex. To further test whether pup odor induced a general physiological arousal, we monitored both heart and breathing rates (n = 5 mice). Neither heart nor breathing rates showed any consistent change during pup odor presentation (data not shown), suggesting that pup odors do not modulate the arousal levels of lactating mothers (at least not in the anesthetized state).

We next asked what triggers the plastic changes in A1 of lactating mothers. Are changes persistent? What impact do they have on the processing of natural sounds that are behaviorally relevant to mothers? To address these questions, we tested two additional experimental groups: "experienced virgins" and "mothers following weaning." "Experienced virgins" are virgins that joined the cage of a primiparous lactating mother and her pups for 4 days starting immediately after parturition (tested at the end of this 4 day period), a priming known to trigger pup retrieval behavior (Ehret et al., 1987; Noirot, 1972). We used this group to test whether olfactory-auditory integration can be instigated in naive virgins by direct interaction with pups, independent of pregnancy and parturition. "Mothers following weaning" are primiparous mothers 1 week after the weaning of and separation from their pups (at PD28). We used this group to test whether the olfactory-auditory integration is a long-lasting phenomenon that is still manifested in experienced mothers when the estrus cycle has been fully restored. Notably, mothers following weaning have recently been shown to process natural calls differently than naive virgins (Galindo-Leon et al., 2009; Liu et al., 2006; Liu and Schreiner, 2007), prompting the question whether olfactory-auditory integration contributes to the known repertoire of changes in these animals.

We first compared the behavioral performance of these two additional experimental groups to those of lactating mothers and naive virgins. In a pup retrieval behavioral assay, all animals that had previous interaction with pups retrieved all the pups back to the nest, while only naive virgins did not retrieve any of the pups (Figure 3A; Movie S1, Movie S2, Movie S3, and Movie S4). Thus, the pup retrieval behavior is independent of pregnancy and parturition because it is evident in experienced virgins. In addition, this behavior is maintained for the long term even after the mice are no longer engaged in it, as evident in mothers following weaning. Notably, however, lactating mothers were still more efficient than the other groups at retrieving their pups back to the nest (Figure 3B; Movie S1, Movie S2, Movie S3, and Movie S4). To challenge the impact of pup odors on retrieval behavior in lactating mothers, we manipulated pup odors by washing the pups. We reasoned that simply washing the pups with warm water may perturb the natural odor emitted from a pup (at least transiently) but will not affect its vocalizations. Interestingly, washing the pups prior to the bioassay hindered pup retrieval performance in lactating mothers (Figure 3; Movie S5). Only 60% of lactating mothers

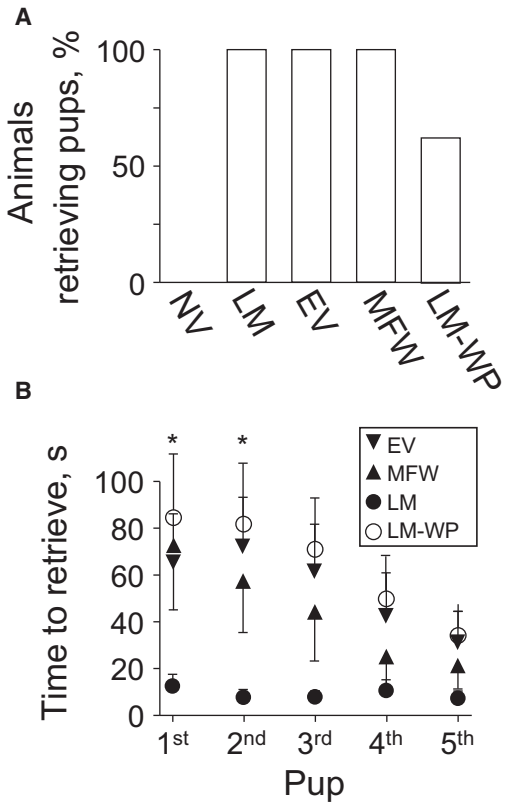

Figure 3. Pup Retrieval Behavior of the Different Experimental Groups

(A) The percentage of mice that retrieved pups from the different experimental groups (see Movie S1, Movie S2, Movie S3, Movie S4, and Movie S5 for examples). Lactating mothers (LM), experienced virgins (EV), and mothers following weaning (MFW) retrieved all pups to the nest whereas naive virgins (NV) did not retrieve any of the pups (n = 7, 7, 8, and 8 mice, respectively). Only ~60% (5/8) of lactating mothers retrieved the pups if these were washed prior to the test (LM-WP) (n = 8 mice).

(B) Chronological pup retrieval latency of EV, MFW, LM, and LM – WP (mean ± SEM). For all groups but LM, the latency to retrieve varied considerably. Relative to LM, it took significantly more time for EV, MFW to retrieve the first and second pups (one-way ANOVA; p = 0.04, Bonferroni post hoc contrast with Holm correction; EV 1st pup*p = 0.0125 and 2nd pup*p = 0.027; MFW 1st pup*p = 0.015 and 2nd pup*p = 0.033).

retrieved washed pups back to the nest (Figure 3A). Furthermore, even when lactating mothers retrieved the washed pups, they did so slower than they retrieved untreated pups. This experiment suggests that pup odor is a powerful cue triggering this behavior. Notably, this result is consistent with a careful behavioral study conducted more than three decades ago (Smotherman et al., 1974).

Next we compared the effects of pup odor stimulation on sound processing in A1 of all four experimental groups (i.e., naive virgins, lactating mothers, mothers following weaning, and experienced virgins). In these experiments, we recorded the spike response profiles to a series of sounds composed of broad band noise (BBN) and natural sounds known to be salient to mothers, such as artificial WCs and recorded USVs, (Ehret, 2005; Ehret and Riecke, 2002) (see Experimental Procedures for the full stimulus array). As expected from the pure tone

experiments, pup odors altered both spontaneous and sound-evoked spike rates of neurons in lactating mothers but not in naive virgins (Figure 4). In lactating mothers, pup odor effects were frequent but heterogeneous. Increases or decreases in evoked spike rates were evident, as well as changes in the sensitivity to stimulus intensity (Figure 4A, left top). Here, too, the heterogeneous effects of pup odor stimulation were largely transient (e.g., see Figure S2 for three complete examples from a lactating mother). Remarkably, pup odor stimulation also induced marked changes in neurons from experienced virgins and mothers following weaning, affecting both spontaneous and sound-evoked spike rates (Figure 4 and see Figures S3A–S3D for 16 additional neurons from the various experimental groups).

Because pup odors had diverse effects on both spontaneous and sound-evoked spike rates, each neuron could have a different combinatorial effect that will determine the exact effect on the neuron's sound detectability (see Figure S3E for examples). Sound detectability is calculated here as the ratio between evoked and spontaneous spike rates (evoked/spontaneous). Detectability >1 implies that the neuron is excited by the auditory stimulus (increase its spike rate). Detectability <1 implies that the neuron is inhibited by the auditory stimulus (decrease its spike rate). The larger the ratio, the stronger the neurons' detectability. To asses how pup odors modulated detectability, we plotted this ratio for each neuron under both conditions (Figure 5A). To quantify these changes, we calculated an index of how detectability was modulated (modulation index = (detectability$_{\text{pup odor}}$ − detectability$_{\text{air}}$)/(detectability$_{\text{pup odor}}$ + detectability$_{\text{air}}$); Figure 5). The average modulation index of all experimental groups having previous experience with pups was slightly higher and more variable as compared to naive virgins, but not significantly (Figures 5A and 5B). Notably, this analysis may underestimate the size of the effect, where averages are considered. For example, neurons with altered receptive fields that were modulated in a nonhomogeneous manner might have a low modulation index score because for some stimuli the response decreased whereas for others it increased (see for example in Figure S1, two bottom neurons; Figure S3B, left neuron; and Figure S3C, second neuron from left, and see below). To study whether neurons in specific laminae were particularly affected, we tested whether the modulation index of detectability was more prominent in a given depth in the cortex. We found no systematic variation between neurons from upper layers as compared to neurons from deeper layers (data not shown; but see Discussion for reservations).

Next, we analyzed whether a particular cell type was more prone to be affected by pup odors. Because fast spiking neurons (FSNs) have been implicated in regulating global network state, response gain, and attention-dependent response modulation (Mitchell et al., 2007; Sun, 2009; Yazaki-Sugiyama et al., 2009), we analyzed odor-induced changes for these neurons separately. By using spike waveforms, we differentiated between regular spiking neurons (RSNs, mostly pyramidal neurons) and FSNs (Figure 5C; Niell and Stryker, 2008). FSNs made up approximately 10% of our data set (28/298 neurons) (Markram et al., 2004). All FSNs recorded in lactating mothers that were responsive to sounds (n = 6 neurons) increased their sound detectability in the presence of pup odors (Figure 5D). Electrophysiological data from all these six FSNs is presented in the different figures (Figure 5D, rasters; Figure 4A, left top; Figure S1, asterisks; and Figure S3E ii, iii). Notably, similar effects were observed for experienced virgins and mothers following weaning but not in naive virgins (Figure 5D, red bars). The comprehensive nature of this effect suggests that FSNs may play a key role in the regulation of the plastic changes occurring in A1 of females exposed to pups.

Although the pup retrieval behavior was evident in all animals after maternal experience, lactating mothers were significantly faster at retrieving the pups (Figure 3B). Consistent with these results, and independent of the olfactory-auditory integration, a significantly higher percentage of neurons from lactating mothers responded to at least one sound (Figure 6A, left; Table S1). This increase was significant when considering both responses to pure tones and to natural sounds separately (Figure 6A, middle and right). Because this amplified response of the population to sounds is not evident in experienced virgins, it could not be explained by mere exposure to pup odors. Because this increase also did not appear in mothers following weaning, we consider it to be a transient effect. This transient effect may well be associated with the transient endocrinal changes that occur during pregnancy and after parturition (Brunton and Russell, 2008; Miranda and Liu, 2009).

Because different sounds bear different behavioral meanings, we analyzed how the different sounds (two different natural sounds and one reference nonnatural sound) were represented in A1 of all four experimental groups. BBN and WCs were overrepresented by ~2-fold in lactating mothers as compared to all other groups (Figure 6B, left and middle). USVs were considerably overrepresented (by ~10-fold) in lactating mothers relative to their representation in naive virgins (Figure 6B, right). Interestingly, USVs were also overrepresented (by ~4-fold, relative to their representation in naive virgins) in experienced virgins and in mothers following weaning, albeit still significantly lower than in lactating mothers (Figure 6B, right). This analysis indicated that maternal-induced plasticity may facilitate responses to specific sounds rather than act as a general gain control to the whole circuit. Therefore, we next analyzed whether the olfactory-auditory integration also induced specific effects on the detection and discrimination of the sounds we presented. To that end, we isolated the effects of pup odors on BBN-, WC-, and USV-evoked responses in lactating mothers (this analysis was limited to this group because of the relatively low sample size of neurons responding to USV stimuli in all other groups; Table S2). Pup odors did not significantly affect the average detectability of neurons to BBN and WC stimuli because some neurons increased and others decreased their detectability (Figure 6C). In contrast, pup odors had a more homogeneous effect on responses to USV stimuli. Specifically, pup odors induced consistent increase for USV detectability in most neurons (Figure 6C; Table S2). For example, 12/15 neurons from lactating mothers increased their responses to USVs (Figure 6D, neurons marked with arrows pointing upwards, and Figure 4A, top left). These analyses indicate that the multisensory cortical changes in lactating mothers may promote high acuity to this specific, context-dependent stimulus as we discuss next.

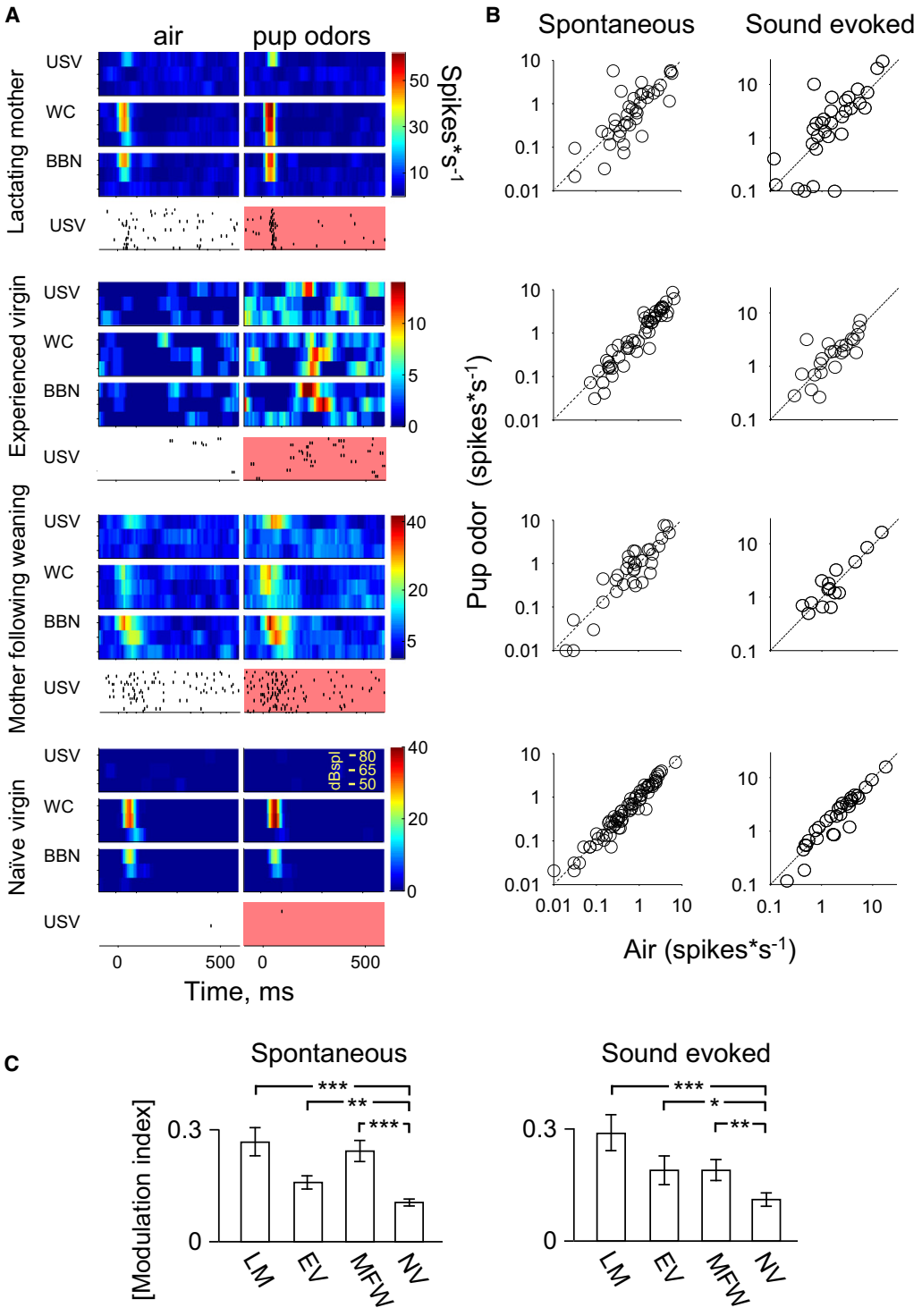

Figure 4. Multisensory Interaction between Pup Odors and Sounds in Animals Having Had Experience with the Pups

(A) Representative STRFs to three stimuli (BBN, WC, and USV, played at three attenuation levels) of one representative neuron from each of the four experimental groups. Each row in the STRF is the sum of 20 trials before (left, air) and during (right, pup odors) exposure to pup odors. The raster plots below show all spikes over all trials of a single stimulus (USV at 80 dB SPL) of the particular neuron.

(B) Population scatter plots of the spontaneous (left) and sound-evoked (right) spike rates under clean air (*x* axis) and pup odor (*y* axis) conditions. Each circle is data from one neuron. Circles above the diagonal increase their spike rate during pup odor presentation. Circles under the diagonal decrease their spike rate during pup odor presentation.

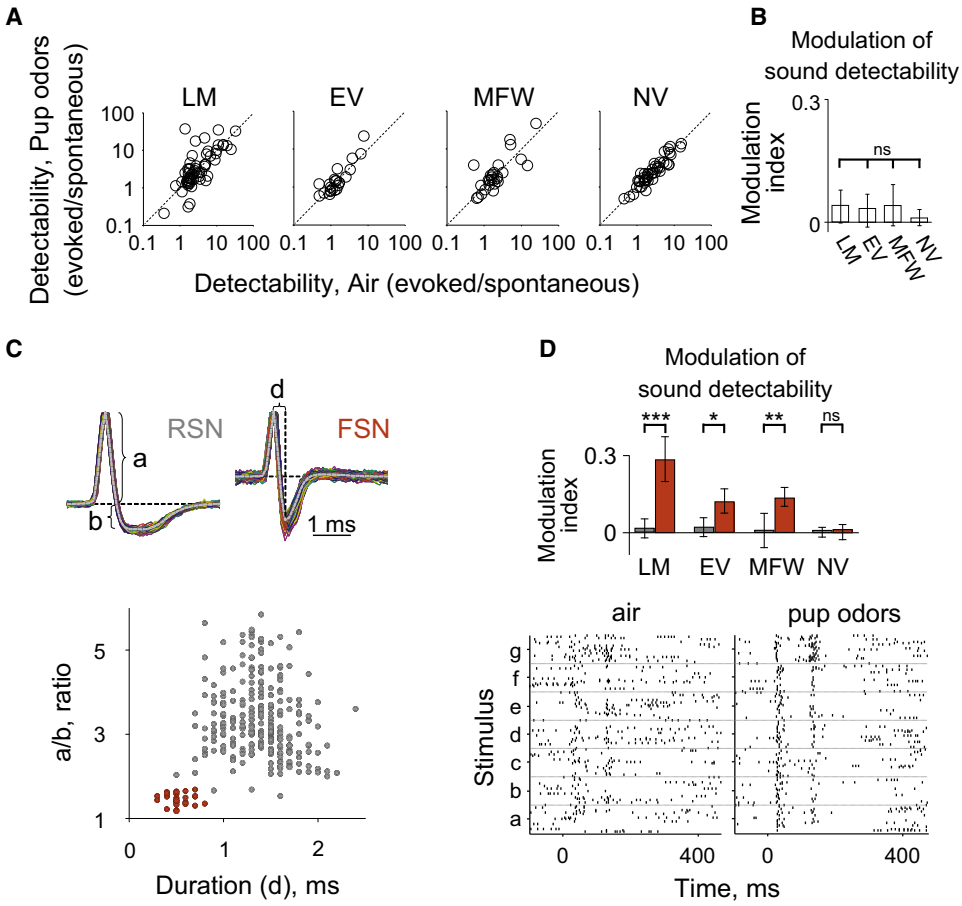

Figure 5. Pup Odors Increase Sound Detectability of Fast Spiking Neurons in A1

(A) Population scatter plots comparing each neuron's detectability (sound-evoked/spontaneous spike rates) under clean air (*x* axis) and pup odor (*y* axis) conditions. Each circle is data from a single neuron; circles falling close to or on the diagonal are neurons that were not affected by the pup odor stimulus.

(B) A summary histogram of detectability modulation index of all four experimental groups. Modulation index for detectability was calculated as $(\text{detectability}_{\text{pup odor}} - \text{detectability}_{\text{air}})/(\text{detectability}_{\text{pup odor}} + \text{detectability}_{\text{air}})$. There is no significant difference between the different experimental groups ($p = 0.46$; Kruskal-Wallis, df = 3). Error bars are mean ± SEM.

(C) Pup odors increase the detectability of FSNs. Top: representative spikes waveform from a RSN and a FSN (colored lines, individual spike waveforms; dashed gray line, mean waveform of the neuron). Bottom: FSNs were identified by spike peak amplitude (a) to volley (b) ratio, relative to the spike peak to volley duration (d). Neurons that had an a/b ratio < 1.8 and a spike duration < 0.7 ms were considered FSNs (n = 28, red). All other neurons (n = 270, gray) were considered RSNs.

(D) Histogram comparing the detectability modulation index of FSNs (red bars n: LM, EV, MFW, and NV = 6, 4, 6, 3 neurons, respectively) and RSN (gray bars n: LM, EV, MFW, and NV = 43, 22, 21, 52 neurons, respectively) in the different experimental groups (LM $p < 0.001$, EV $p = 0.05$, MFW $p = 0.01$; Mann-Whitney U test). Bottom: representative raster plots of a FSN from a lactating mother (stimuli: a–f; 3.8 + 11.4, 7.6, 3.8 + 7.6, 7.6 + 11.4, 11.4, and 7.6 + 11.4 kHz, respectively; g, BBN). Note the increased responsiveness of this neuron and improved discrimination of its on and off responses to 100 ms tones presentation. Error bars are mean ± SEM.

DISCUSSION

In this work we found that motherhood is associated with an appearance of multisensory cortical processing in A1 that was not evident during virginity. We show that neurons in A1 of mothers and other care givers integrate between pup odors and sounds. This multisensory integration was evident in animals that had previous interaction with pups, suggesting that this plasticity is experience dependent. We further demonstrate that this multisensory integration enhances the detection of USVs in A1.

(C) Histogram of the absolute modulation index of the four experimental groups. Pup odors induce modulation of spontaneous and sound-evoked spike rates in A1 of all groups of females that had previous experience with pups (left; n: LM, EV, MFW, and NV = 38, 52, 32, 69 neurons, respectively; right; n: LM, EV, MFW, and NV = 29, 32, 23, 16 neurons, respectively). The modulation index of neurons in NV was significantly lower compared to all other groups. Spontaneous; $p < 0.001$; Kruskal-Wallis, df = 3, Mann-Whitney U post hoc contrast with Holm correction; LM versus NV $p < 0.0001$, EV versus NV $p = 0.013$, MFW versus NV $p < 0.001$. Sound-evoked; $p = 0.003$; Kruskal-Wallis, df = 3, Mann-Whitney U post hoc contrast with Holm correction; LM versus NV $p = 0.0005$, EV versus NV $p = 0.025$, MFW versus NV $p = 0.005$. Error bars are mean ± SEM.

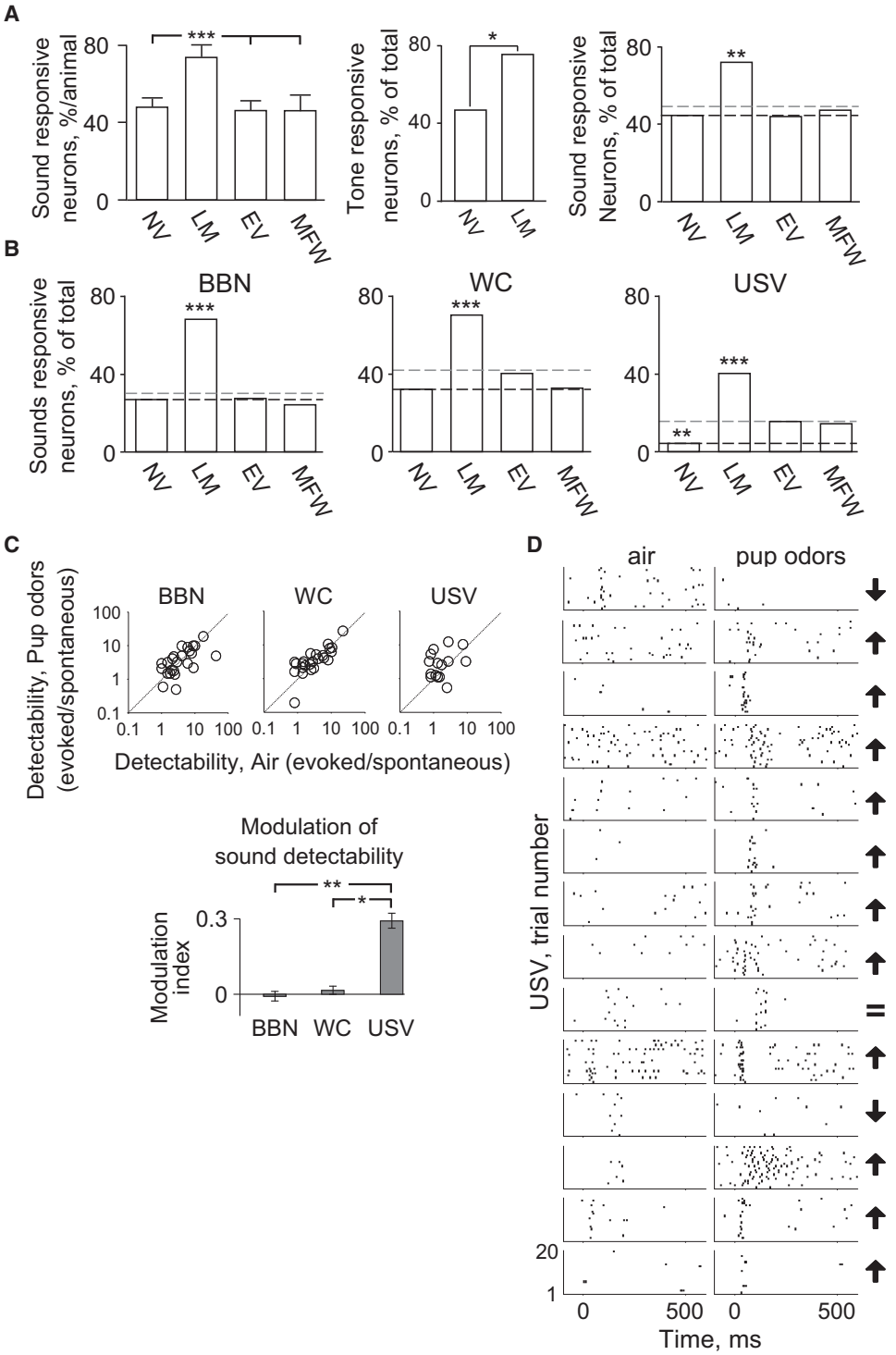

Figure 6. Experience with Pups Facilitates Stimulus-Specific Plasticity for USVs

(A) Left: the response probabilities (per animal tested) of all FSNs and RSNs to respond to any of the sounds in the different experimental groups (mean ± SEM). More neurons respond to sounds in LM as compared to NV, MFW, and EV (n/N, neurons/mice: = 136/16, 136/16, 85/11, 55/8, respectively, ***p = 0.005; Kruskal-Wallis, df = 3, Mann-Whitney post hoc contrast with Holm correction; LM versus NV p = 0.0005, LM versus MFW p = 0.002, LM versus EV p = 0.017). Middle: response probabilities of all neurons to respond to at least one pure tone in the series (*p = 0.006, chi-square test, df = 1). Right: response probabilities of all neurons to respond to at least one stimulus in the natural sound stimulus series (**p = 0.005, chi-square test, df = 3). Dotted gray line, expected count; dotted black line, baseline value of naive virgins.

Multisensory Integration of Smells and Sounds in A1

It is well accepted that the cerebral cortex processes multisensory cues (Ghazanfar and Schroeder, 2006; Stein and Stanford, 2008). In the auditory cortex (including in A1), both imaging and electrophysiological studies revealed that neurons integrate auditory-visual or auditory-somatosensory cues (Bizley et al., 2007; Kayser et al., 2007, 2009; Lakatos et al., 2007; Murray et al., 2005). These forms of multisensory integration have been suggested to improve auditory processing and modulate the way the animal perceives its acoustic environment (Musacchia and Schroeder, 2009; Stein and Stanford, 2008). For example, in humans, for whom vision is a central sense, audiovisual integration has been linked to specific perceptual benefits such as improved speech understanding and better localization accuracy and reaction time (Besle et al., 2008; Schroeder et al., 2008; Schröger and Widmann, 1998; Sekiyama et al., 2003). However, integration of visual or auditory information with olfactory cues remains largely unstudied. Although evidence for multisensory integration between olfaction and audition is scarce, it is not without precedent (Halene et al., 2009). In addition, recent work showed that the opposite interaction also exists. Namely, auditory cues have an influence on olfactory processing and perception (Wesson and Wilson, 2010; Seo and Hummel, 2011). Thus, it seems that olfactory and auditory information can converge in a biologically meaningful way. Our findings support this notion and provide direct neurophysiological evidence for the functional integration of natural odors and sounds in the mammalian cerebral cortex.

The auditory-olfactory integration we detected is different than previous canonical examples of multisensory integration in a significant way. Namely, the auditory-olfactory integration in A1 is slow, taking dozens of seconds to develop and minutes to disappear. Neurons in A1 do not respond to odor stimuli in a classical way (i.e., in a time window of a few hundred milliseconds after stimulus onset). Rather, neuronal firing properties are modulated by the continuous presence of the odor. The slow nature of this interaction implies that there are no direct projections from olfactory centers directly into A1 (Budinger and Scheich, 2009). In contrast, canonical examples of multisensory integration are fast and thought to be mediated by direct connectivity (Stein and Meredith, 1993). In A1, for example, visual stimuli induce direct spiking responses in auditory neurons, which were suggested to be mediated by direct projections from the visual cortex (Bizley et al., 2007). Another possible difference is that the integration we describe here was not evident in naive animals and appeared more robustly in animals exposed to pup odors. Although we cannot rule out different integration pathways between smells and sounds in naive animals, other forms of multisensory integration do seem to be part of the normal repertoire of "naive circuits" (e.g., auditory neurons responding to behaviorally "insignificant" cues like light flashes) (Bizley et al., 2007). However, even simple audiovisual integration changes with experience. For example, the ontogeny of multisensory integration in the superior colliculus was shown to be rudimentary during early postnatal life and developed as connections matured (Wallace et al., 2006; Wallace and Stein, 2007). Moreover, recent evidence suggest that sensory experience can shape the way neurons integrate audiovisual information even after simple exposures (Yu et al., 2010). Our data demonstrate that neurons in A1 integrate behaviorally relevant olfactory and auditory stimuli, possibly in an experience-dependent manner.

Is Maternal Plasticity Experience Dependent?

Maternal behaviors emerge immediately after the birth of the offsprings (Brunton and Russell, 2008). The establishment of maternal behaviors requires interaction with the newborn and repeated exposure to the pups is sufficient to induce them (Figure 3; see also Ehret et al., 1987; Mann and Bridges, 2001; Noirot, 1972). Because direct interaction with the pups is both necessary and sufficient to instigate maternal care, we infer that the plasticity we observed may have been attributed to the experience as well. This argument is supported by several lines of evidence. First, the pup-odor-induced physiological changes were not evident in naive animals (Figures 1D, 2, and 4). Moreover, the physiological changes are correlated with pup retrieval performance of the different experimental groups (Figures 3 and 4). Second, the cortical changes are not triggered just by any odor, but rather by the novel scent of the pups that the animals were exposed to while caring for the pups (Figure 2B). It is difficult to rule out the possibility that some other odor will induce similar effects because odor space is infinitely large, making it experimentally intractable. Third, out of all the sounds that we tested, A1 responses to a particular natural sound (USV) that the caregivers were exposed to was particularly affected by pup odors. Are USVs (like pup odors) novel to the mother?

By the end of the second week of life (postnatal days 12–13) when their eyes and ear canals open, pups are able to maintain their body temperature. At that time, they stop emitting distress USVs (Noirot, 1972; Scattoni et al., 2009). However, the social WCs persist beyond the second week of postnatal life, well after the onset of hearing. Therefore, WCs are not truly novel to the mothers (Ehret and Bernecker, 1986). In contrast, adult mice normally do not hear USVs prior to their experience with the pups as parents. As a result, primiparous females are first exposed to their pup USVs in the context of their body

(B) Response probabilities to any one of the three stimuli that were presented (BBN, WC, USVs) in the different experimental groups, independent of odor effects. BBN and WCs are overrepresented only in LM as compared to all other groups (***$p < 0.0001$, chi-square test, df = 3). USV are overrepresented in LM (~10-fold) as well as in EV and MFW (~4-fold), relative to their representation in NV (n: NV, LM, EV, MFW = 69, 38, 52, 32, respectively, **$p = 0.018$, ***$p = 3 \times 10^{-6}$, chi-square test df = 3, dotted gray line, expected count; dotted black line, baseline value of naive virgins).

(C) Top: population scatter plots comparing detectability of neurons in lactating mothers to BBN, WC, and USVs (sound-evoked/spontaneous spike rates) under clean air (*x* axis) and pup odor (*y* axis) conditions. Each circle is data from a single neuron. Circles falling close to or on the diagonal are neurons that were not affected by pup odor stimulus. Bottom: a summary histogram of detectability modulation index for BBN, WC, and USVs (mean ± SEM). Modulation index for detectability was calculated as in Figure 5. There is a significant increase in the detectability of USVs under pup odor stimulus ($p = 0.039$; Kruskal-Wallis, df = 2, Mann-Whitney U post hoc contrast with Holm correction; BBN versus USV $p = 0.008$, WC versus USV $p = 0.017$). Error bars are mean ± SEM.

(D) Raster plots of 14 neurons in lactating mothers responding to USVs and their modulation by pup odors. An additional neuron from a LM is shown in Figure 4A (top left).

odors. This novel combination may promote high acuity to this specific, context-dependent combination of stimuli contingent with stressed pups.

It is well established that the auditory cortex can discriminate sounds that acquire behavioral meaning (Fritz et al., 2003; Weinberger, 2004). In line with these classical forms of experience-dependent plasticity, the percentage of units responding to USVs was higher (relative to that in naive virgins) in all experimental groups that had previous interaction with the pups (Figure 6B). These changes were seemingly independent of pup odors and may well be a result of the change in acoustic environment related to the presence of pups (i.e., USVs). Both the odor-dependent and the odor-independent changes promote higher detection levels of USVs (Figures 6B–6D) and possibly better discrimination by the mother. Whether these changes follow a mechanism of classical association learning between sounds and smells remains to be seen.

Long-Term Changes in A1 of Mothers and Other Caregivers

As a general observation, we show that pup odor induced modulation of sound detectability. In particular, the representation of USVs in A1 increased. What may be the neural mechanism underlying this long-term change in A1?

Neurons in A1 (as in any neocortical circuit) process information differently across layers (Harris et al., 2011; Sakata and Harris, 2009). Thus, one may expect that the long-term changes in sensory responses would have unique signatures in different layers and interactions therein. Unexpectedly, we did not observe any particular pattern of change based on the depth of our neuronal recordings (not in spontaneous or in evoked firing and not in the odor-evoked changes; analyses not shown). Notably, the lack of layer specificity may still be a limitation of our recording method, which yields relatively low numbers of neurons from each layer in our data set. Dense recording techniques or imaging techniques may be a more informative way to measure odor-induced effects across layers (Happel et al., 2010; Rothschild et al., 2010; Sakata and Harris, 2009).

Pup odors affected the excitatory responses of all cells with no particular reference to their spontaneous or sound-evoked spike rates (Figures 5A and 5B; Figures S1–S3). However, modulation did not affect all neuronal cell types in the same manner. The majority of FSNs showed consistent changes in the form of an increase in their sound detectability (Figure 5B). Moreover, FSNs had a higher probability to respond to sounds compared to RSNs (19/28 versus 132/270). Could FSNs be central to the mechanism of change? Emerging data in the field suggest that they may.

FSNs are the major source of inhibition onto RSNs (i.e., excitatory pyramidal neurons) in the neocortex (Freund and Katona, 2007). Both RSNs and FSNs receive an approximately similar range of synaptic inputs from thalamo-cortical axons, but FSNs convert a broader range of these synaptic inputs into spikes (Cruikshank et al., 2007; Wehr and Zador, 2003; Wu et al., 2008). As a result, FSNs sharpen the frequency tuning of RSNs in a feed-forward manner (Wehr and Zador, 2003; Wu et al., 2008). Feed-forward inhibition ensures high selectivity of RSNs and therefore greater contrast and precision of responses in A1 (Hromádka and Zador, 2009). Interestingly, new sounds that acquire behavioral meaning show increased representation in A1, a change that was suggested to be mediated via feed-forward inhibition (Galindo-Leon et al., 2009; O'Connell et al., 2011). In recent work, Liu and colleagues studied A1 responses in "mothers following weaning" versus "naive virgins" and found that inhibition was earlier, longer, and stronger in RSNs of mothers (Galindo-Leon et al., 2009; Liu and Schreiner, 2007). Those changes were attributed to FSNs and their role in side-band inhibition. Our data support this conclusion and extend this argument to explain the increase in USV representation and detection in experienced animals.

One principal experimental group that was not tested in earlier studies is the group of "lactating mothers." The lactating mother must respond to USVs emitted by her pups promptly and consistently because it is crucial for their survival. Accordingly, we found that the neurophysiological changes in lactating mothers were not only larger as compared to all other experimental groups but also unique in some measures like the general increased sensitivity to all sounds (Figure 6A). Sensory cues emitted by pups may also modulate the hormonal state of the lactating mother. Alternatively or jointly, endocrinal alterations in the lactating mother may have profound effects on sensory processing (Brunton and Russell, 2008). One possible candidate is oxytocin, which is an important modulator of female reproductive functions, including maternal behavior. Little is currently known about the role oxytocin in auditory processing. However, anatomical studies suggested that oxytocin is involved in auditory processing because, in mustached bats, oxytocin-expressing neurons are predominantly localized within the auditory cortex, auditory brain stem nuclei, as well as in the olfactory bulb (Kanwal and Rao, 2002; Prasada Rao and Kanwal, 2004). Oxytocin itself may be regulated by other hormones like estrogen and in turn regulate the rhythmus of other peptide hormones like prolactin (Bertram et al., 2010; Shughrue et al., 2002). Whether oxytocin (or other hormones) serves to bridge between senses remains an open question to explore. Endocrinal or not, when heightened cortical sensitivity is combined with the constant exposure to the pup odors + USVs, transient changes may translate to long-term plastic modifications. The maternal cortex is thus tuned to detect and discriminate USVs exactly when nature calls.

Odor effects were highly heterogeneous and probably be attributed to changes in both inhibition and excitation, not to just one or the other. The balance between excitation and inhibition can be tested directly in the future by measuring synaptic inputs into RSNs and FSNs simultaneously. Although such recordings are still technically challenging, recent improvements in methods like targeted two-photon patch clamp are expected to increase the yield of dual recordings from specific neuronal subtypes even in awake attentive animals (Gentet et al., 2010). Such future experiments may provide insight into the synaptic nature of the cortical changes in spike rates that we report here.

Finally, we show that the olfactory-auditory interaction is evident early in the processing stream, as early as A1. However, maternity-induced changes may still be tracked either earlier or later in the processing stream. For example, changes in responses of thalamic neurons may be a source of an earlier

bottom-up effect. Changes in intracortical connectivity or changes in neuronal gene expression patterns may contribute to local plasticity intrinsic to A1. Multisensory centers may also be a source of change and induce top-down effects (Schroeder and Foxe, 2005). Indeed, A1 is no longer thought to be a sole unisensory center but rather a multisensory hub (Bizley and King, 2008; Budinger and Scheich, 2009; Musacchia and Schroeder, 2009; Schroeder and Foxe, 2005). Because there are no known direct anatomical interactions between early olfactory centers like the olfactory bulb or piriform cortex into A1, functional connectivity is probably relayed indirectly (Musacchia and Schroeder, 2009).

In conclusion, we show that motherhood is associated with a rapid and robust appearance of olfactory-auditory integration in A1 co-occurring with stimulus-specific plasticity to pup distress calls. These uni- and multisensory plastic processes provide substrate for a mechanistic explanation of how changes in neocortical networks facilitate efficient detection of pups by their caring mothers.

EXPERIMENTAL PROCEDURES

Experimental Groups and Surgical Procedures

All experimental procedures used in this study were approved by the Hebrew University Animal Care and Use Committee. Female NMRI mice (total of n = 60 mice, 8–12 weeks old) were anesthetized with ketamine/medetomidine (i.p.; 100 and 83 mg/kg, respectively). Naive virgins are female mice that were never housed with males or pups after they had been weaned at PD21. Lactating mothers are females 4 days after parturition (PD4 ± 12 hr), nursing a litter of at least five pups.

Depth of anesthesia was monitored by the pinch withdrawal reflex and ketamine/medetomidine was added to maintain it. Dextrose-saline was injected subcutaneously to prevent dehydration. Rectal temperature (36°C ± 1°C) was monitored continuously. In five animals, we also monitored the heart rate and/or the breathing rate. In none of these animals did the pup odor stimulus induce a long-lasting change in either heart or breathing rate. Therefore, these physiological measures were not strictly monitored in further experiments. A custom-made metal pin was glued to the skull via dental cement and connected to a custom stage to allow precise positioning of the head relative to the speaker (facing the right ear). The muscle overlying the left auditory cortex was removed and a craniotomy (~2 × 2 mm) was performed over A1 as previously described (Stiebler et al., 1997; Rothschild et al., 2010). We verified that our recordings are from A1 both anatomically and physiologically. Anatomically we injected herpes simplex virus (HSV-GFP) into the site of recording and verified somata labeling in the thalamus (data not shown; see also our tracing experiment with ionophoresis [Rothschild et al., 2010]). We cannot assert the exact position of our recordings within the tonotopic axis of A1. However, most of our neurons had characteristic frequencies between 10 and 20 kHz, consistent with our anatomical targeting to the center of A1. The dura over A1 was gently removed and the cortical surface was kept continuously moist. A similar procedure was applied to the barrel field (at 3 mm lateral, 1–1.5 mm posterior to bregma). After each experiment, animals were sacrificed by an overdose of sodium pentobarbital.

Electrophysiology

Cell-attached recordings were obtained with blind patch-clamp recording. Electrodes (4–7 MΩ) were pulled from filamented, thin-walled, borosilicate glass (outer diameter, 1.5 mm; inner diameter, 1.0 mm; Hilgenberg GmbH, Malsfeld, Germany) on a vertical two-stage puller (PC-12, Narishige, East-Meadow, NY). Internal solution contained (in mM):140 K-gluconate, 10 KCl, 10 HEPES, 10 Na_2-Phosphocreatine, 4 MgATP, 0.4 Na_2GTP, 0.5 EGTA adjusted to pH 7.25 with KOH) and 2%–3% low melting agar (type IIIa, Sigma-Aldrich, St. Louis, MO) was placed over the craniotomy to minimize pulsations. An increase of the pipette resistance to 10–200 MΩ resulted in most cases in the appearance of spikes. The detection of a single spike was the only criterium to start the olfactory/auditory protocol. All recordings were acquired with an intracellular amplifier in current clamp mode (Multiclamp 700B, Molecular Devices), acquired at 10 kHz (Digidata 1440A, Molecular Devices, Sunnyvale, CA) and filtered with a 50 Hz high pass filter.

Auditory, Olfactory, and Somatosensory Stimuli

USVs were recorded with a 1/4 inch microphone, connected to a preamplifier and an amplifier (Bruel & Kjaer, kindly provided by I. Nelken) from P3–P5 pups isolated from their mother and placed in a custom-built sound-shielded box. Vocalizations were sampled at 500 kHz with a Digidata 1322A (Molecular Devices, Sunnyvale, CA). USVs were identified offline, and a library of single calls was created. "Pure tones protocol" is a series of pure tones at 15 frequencies (1–40 kHz logarithmically spaced) at two attenuation levels (55 and 80 dB SPL, 50 ms duration, 550 ms interstimulus interval). Each combination of frequency attenuation was presented 28 times (840 stimuli in total). "Natural sounds protocol" includes: BBN, one synthesized WC, all the possible combinations of the pure tones composing it (3.8, 7.6, and 11.4 kHz), and two played-back USVs (Figure S2). All stimuli were played at three attenuation levels (50, 65, and 80 dB SPL). Each stimulus-attenuation combination was repeated 20 times (600 stimuli in total) with a 600 ms interstimulus interval. All stimuli series were randomly shuffled and had a 5 ms onset and offset linear ramps. The sound series were delivered with custom-written software (Matlab, MathWorks, Natick, MA) through an electrostatic loudspeaker driver and a programmable attenuator (ED1, PA5, Tucker Davis Technologies). The loudspeaker (ES1, TDT) was placed 10 cm from the right ear of the mouse during the electrophysiological recordings.

Pup body odors were delivered through a custom-built 2-channel olfactometer, one channel for clean air and a second (completely separated to avoid contamination) channel for pup odors. For pup odors stimuli, three to five healthy postnatal day 4 pups were placed in a closed glass container on a cotton wool and wood shaving bedding. The void volume of this container was the "pup odor" (Figure 1A). Both air and pup odors were delivered at a constant low flow rate (0.2–0.4 l/min) directly to the nose of a freely breathing mouse. In control experiments, the closed glass container was empty or alternatively contained only the cotton wool and wood shaving bedding ("nesting materials") or 0.1% acetophenone diluted in mineral oil.

Air puffs (100 ms) were delivered at 0.5 Hz (a total of 540 trials) and directed directly at the whisker pad. Stimuli were controlled by an electrical valve triggered by a programmable stimulator (Master-8, A.M.P.I., Israel).

The Olfactory-Auditory Experimental Procedure

Several minutes after achieving cell-attached configuration, we initialized the olfactory-auditory protocol, which lasted for at least 20 min. The olfactory-auditory protocol consisted of playing a series of sounds in the first epoch ("pure tones" or "natural sounds"), followed by 1 min of pup odor delivery before playing the reshuffled sound series again while the odors were continuously presented (second epoch). To assess the reversibility of the odor effect, we presented in a few experiments no odor (clean air) to the animal for 10 min at the end of the second epoch before playing the reshuffled sound series again (Figures 1C and S2). A minimum of 20 min "wash" of pup odors was routinely preformed before continuing to the next neuron in the same animal. Normally, several neurons were recorded from each electrode penetration. We recorded from 7.8 ± 2.8 (mean ± SD) neurons per animal (N = 60). In rare cases, in which the spontaneous firing rate increased suddenly or the electrode "broke in," we analyzed only the stationary epoch of the recording.

Data Analysis

Data analysis was carried out with custom-written code in Matlab, and further statistical analyses were preformed with SPSS software (SPSS, Chicago, IL).

Spikes recorded in cell-attached mode were extracted from raw voltage traces by thresholding. Spike times were then assigned to the local peaks of suprathreshold segments and rounded to the nearest millisecond.

Spontaneous Firing Rate

All trials were assigned to a raster plot according to their chronological order. The neuron's spontaneous firing rate was calculated based on the 200 ms preceding each stimulus presentation (250 ms for the natural sounds series

or 1000 ms for S1 barrel field neurons). The stability of the recording was assessed by continuously monitoring the spontaneous firing rate during the first epoch of the protocol (420–504 s long depending on the sound series, and 360 s for the olfactory-somatosensory protocol). Less than 5% of the neurons in our data set were not stable (significant drift in spontaneous spike rate or the electrode "broke in"). These neurons were not included in the odor-effects statistics but were included in the analysis of probability of neurons to be auditory responsive.

Auditory Responsiveness

A neuron's responsiveness to sound was assessed by calculating the firing rates over all trials of all stimuli (PSTH). The half maximum half width time window of the summed auditory response was defined as the neuron's response window. If the firing rate within the response window was significantly different (>±1.5 SD) from the neuron's spontaneous firing rate, the neuron was considered "auditory responsive." Similar analysis was performed on S1 barrel field neurons' response to air puffs.

To assess the stability of our recordings, we tested the full-length protocol but without pups in the olfactometer chamber ("no odor"). A similar analysis was performed for all other A1 control experimental groups and for neurons in the barrel field presented with pup odors (Figure 2).

Behavioral Experiments

All of the pup retrieval experiments were conducted in the first 6 hr of the light cycle and videotaped. Animals were placed one at a time in a clean plastic chamber (26 × 42 cm) with standard wood chip bedding and a red transparent plastic shelter (mock nest) in one corner and allowed 30 min of free exploration. Five pups at postnatal day 4 were placed in the cage consecutively with 30–40 s intervals. To test lactating mother retrieval of washed pups, each pup was gently washed with warm PBS solution and dried on a clean soft paper towel immediately before it was placed in the cage. The experiment was terminated when all pups were retrieved or after 5 min. The probability to retrieve pups and the latency of each pup retrieval was scored manually from the videotape.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and five movies and can be found with this article online at doi:10.1016/j.neuron.2011.08.019.

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