Title: Plasticity in the Rat Posterior Auditory Field following Nucleus Basalis Stimulation

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ABSTRACT

Classical conditioning paradigms have been shown to cause frequency-specific plasticity in both primary and secondary cortical areas. Previous research demonstrated that repeated pairing of nucleus basalis (NB) stimulation with a tone results in plasticity in primary auditory cortex (A1), mimicking the changes observed after classical conditioning. However, few studies have documented the effects of similar paradigms in secondary cortical areas. The purpose of this study was to quantify plasticity in the posterior auditory field (PAF) of the rat after NB stimulation paired with a high frequency tone. NB-tone pairing increased the frequency selectivity of PAF sites which were activated by the paired tone. This frequency-specific receptive field size narrowing led to a reorganization of PAF such that responses to low- and mid-frequency tones were reduced by 40%. Plasticity in A1 was consistent with previous studies - pairing a high frequency tone with NB stimulation expanded the high frequency region of the frequency map. Receptive field sizes did not change, but CF’s in A1 were shifted after NB-tone pairing. These results demonstrate that experience-dependent plasticity can take different forms in primary and secondary auditory cortex.

KEYWORDS

Non-primary auditory cortex
Basal forebrain
Cholinergic
Map reorganization
Receptive field
Neural plasticity in both primary and secondary sensory cortex has been associated with learning. After long-term operant training in primates and rats, large frequency map expansions can develop in response to trained tone frequencies in primary auditory cortex (Brown et al. 2004; Buonomano and Merzenich 1998; Polley et al. 2006; Recanzone et al. 1993; Rutkowski and Weinberger 2005), but see (Brown et al. 2004). Long-term operant training can also cause frequency-specific plasticity in secondary cortical areas (Polley et al. 2006). Classical conditioning shifts receptive fields in both primary and secondary auditory cortices towards the conditioned stimulus (Diamond and Weinberger 1986, 1984; Weinberger et al. 1984). A greater proportion of neurons in secondary cortical areas are altered by classical conditioning compared to A1 (Diamond and Weinberger 1984; Weinberger et al. 1984). Receptive field plasticity has also been observed in the somatosensory and visual systems, indicating that learning-induced plasticity is a general cortical phenomenon (Buonomano and Merzenich 1998; Ghose 2004; Gilbert 1996; Recanzone et al. 1992a; Recanzone et al. 1992b). In all of these cases, experience-dependent plasticity is specific to the stimuli which were attended to during behavior, and passive exposure does not cause enduring changes in neuronal responses (Recanzone et al. 1993; Weinberger 1998; Weinberger and Bakin 1998). These results indicate that stimulus-specific plasticity occurs during learning and significantly alters sensory information processing in multiple cortical areas.

Many studies have demonstrated that attention or the presence of a neuromodulator such as acetylcholine is necessary to generate lasting cortical plasticity. Nucleus basalis (NB), which has cholinergic and GABAergic projections to all areas of the neocortex, is active when animals are learning new tasks (Butt and Hodge 1997; Richardson and DeLong 1991). Lesions of cholinergic NB neurons or pharmacological blockage of acetylcholine prevent both learning and plasticity in the motor cortex (Conner et al. 2003) and in the primary auditory cortex (Ji et al.
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2001). Direct stimulation of NB paired with sensory inputs is sufficient to create stimulus-specific cortical plasticity in A1 (Bakin and Weinberger 1996; Kilgard and Merzenich 1998a). For example, NB stimulation paired with a single tone results in an increased representation of the paired frequency in primary auditory cortex (Kilgard and Merzenich 1998a). Therefore, similar to classical conditioning and operant conditioning protocols, NB-stimulation pairing leads to stimulus-specific plasticity in A1. The extent of NB-stimulation induced receptive field reorganization outside of A1 has not been well characterized.

In this study, we implanted rats with NB stimulating electrodes, repeatedly paired a high frequency tone with NB stimulation, and documented differences in posterior auditory field (PAF) responses to tones in experimental and control rats. Since PAF responses are much less selective for frequency than A1 (Bao et al. 2001; Doron et al. 2002; Pandya et al. in press, in review; Rutkowski et al. 2003), we expected that plasticity following NB-tone pairing would differ substantially from observations in A1. One possibility, given the broad frequency tuning, was that NB-tone pairing would generate a frequency-specific reduction in the response to frequencies distant from the paired tone. If observed, such plasticity would suggest that the increased response to events associated with neuromodulator release observed in primary sensory and motor cortex may not be characteristic of plasticity in other fields (Buonomano and Merzenich 1998; Conner et al. 2003; Weinberger 2007).

MATERIALS AND METHODS

Six adult female Sprague Dawley rats (250-350g) were implanted with platinum bipolar stimulating electrodes and then exposed to a 19 kHz tone paired with NB stimulation for a period of 20-25 days. Twenty-four hours later, animals were anesthetized and the right auditory cortex was densely mapped by recording tuning curves. Receptive field properties and topographic organization of both primary (A1) and posterior (PAF) auditory fields were quantified. Data from naïve controls (n=14) served as comparisons for all the NB-stimulated animals.
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Additionally, mapping data from an animal that experienced a 9 kHz tone paired with NB-stimulation during a previous study was analyzed to ensure that plasticity in PAF is frequency-specific. The A1 map for this animal is depicted in Figure 1C of (Kilgard and Merzenich 1998a). Analysis of thresholds and frequency range of neural responses indicate every rat had normal hearing (data not shown). The implantation, stimulation and mapping methods described here are identical to those used in previous studies (Kilgard and Merzenich 1998a; Kilgard et al. 2001; Moucha et al. 2005; Pandya et al. 2005). All procedures were approved by the University of Texas at Dallas Animal Care and Use Committee.

**Implantation with NB Stimulating Electrode**

Each experimental animal was implanted with a bipolar stimulating electrode (SNE-200, Rhodes Medical Instruments, Woodland Hills, CA) which was lowered 7 mm below the cortical surface from a location 2.3 mm posterior and 3.3 lateral to bregma in the right hemisphere. This stereotaxic location has been used in previous studies of plasticity after NB-stimulation pairing (Bakin and Weinberger 1996; Dimyan and Weinberger 1999; Kilgard and Merzenich 1998a, 2002, 1998b; Kilgard et al. 2001; Moucha et al. 2005; Pandya et al. 2005), and was not histologically confirmed in the current study. A bone screw was placed approximately 5 mm posterior to the stimulating electrode and another screw placed above the cerebellum to record an EEG. A four-channel connector was used to monitor EEG and deliver current to the stimulating electrode. The entire implant assembly was stabilized with additional bone screws and held in place with dental acrylic. In addition to the pentobarbital anesthetic (50 mg/kg), all animals were given a dose of ceftriaxone antibiotic (20 mg/kg) to prevent infection and atropine (1 mg/kg) and dexamethazone (4 mg/kg) to reduce fluid accumulations in the lungs immediately following anesthetization and after completion of the surgery. All animals were allowed to recover for 1-2 weeks before beginning NB stimulation. Animals were singly housed in wire cages in the
animal care facility after the NB-stimulation implant procedure and between NB stimulation sessions.

**NB Stimulation Procedure**

NB stimulation took place in a 25x25x25cm wire cage located inside of a 50x60x70 cm chamber lined with acoustic insulating foam. Sounds were presented from a speaker hanging above the wire cage. The paired stimulus was a 19 kHz tone (50 dB SPL, 250 ms duration, 5 ms rise-fall time) that was presented approximately every ten seconds 275-350 times per day for a period of 20-25 days. Silent intervals were inserted at random to prevent habituation, and each pairing session lasted approximately three and a half hours. Each tone presentation was accompanied by a short burst of current delivered to the bipolar stimulating electrode (20 biphasic pulses, 0.1 ms duration at 100 Hz) approximately 50 ms after tone onset. The current amplitude ranged from 120-200 µamps for each animal, and was selected to be the level that would reliably elicit brief EEG desynchronization while the animal was in slow wave sleep.

**Dense Electrode Mapping of Auditory Cortex**

Approximately 24 hours after the tone-NB pairing phase ended, animals were anesthetized with pentobarbital (50 mg/kg), and the right auditory cortex was mapped. Similar mapping procedures were used to collect data from 14 experimentally naïve control animals. Because only NB-stimulated animals received connectors and acrylic headcaps used for NB-stimulation, it was impossible to be blind to experimental group during data collection. Briefly, a tracheotomy was performed to prevent breathing problems and head noise, and a cisternal drain was made to minimize cerebral edema. Then the right auditory cortex was exposed and the dura resected. The cortex was maintained under a thin film of silicone oil to prevent desiccation. Fluids with Ringer’s solution and supplemental anesthetic (pentobarbital - 8 mg/ml) were periodically administered throughout the mapping procedure to maintain the animal’s health and a state of areflexia.
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All recordings were performed in a shielded, double-walled sound chamber. Frequency and intensity calibrations were performed with an ACO Pacific microphone (PS9200-7016) and Tucker-Davis SigCal software. The speaker (Motorola model #40-1221) was positioned directly opposite the contralateral ear at a distance of 10 cm. Tucker-Davis Technologies hardware and software (SigGen) were used for stimulus generation. Multiunit responses were recorded using parylene-coated tungsten electrodes (250 µm separation, 2 MOhm at 1 kHz, FHC Inc., Bowdoinham, ME) which were lowered ~550 µm below the cortical surface (layer IV/V). The neural signal was filtered (0.3-8 kHz) and amplified (10,000x). Action potential waveforms were captured using a software program (Brainware, Tucker-Davis Technology, Alachua, FL), and each recording site location was logged on a detailed digitized photo of the exposed auditory cortex using blood vessels as landmarks. At each site, a tuning curve consisting of 81 frequencies spanning from 1 to 32 kHz at 16 intensities spanning from 0 to 75 dB SPL was presented (1,296 tones, 25 ms duration, 5 ms rise and fall time, 1 repetition of each). The tones were randomly interleaved and presented every 500 ms. The total duration of each mapping experiment was similar in naïve and experimental groups (31 ± 1.2 and 26 ± 1.9 hours, p > 0.1).

Data Analysis

In total, 493 experimental sites (6 animals, 295 A1, 198 PAF sites) were compared to 588 sites from naïve controls (14 animals total, 390 A1, 198 PAF sites). For the naïve control groups, the A1 group consisted of 8 animals, while the PAF group contained 9 animals (3 naïve controls belonged to both the A1 and PAF control groups). The control group for each cortical field was selected so that every animal had at least 10 recording sites in the given auditory field. All experimental animals met this criterion for both A1 and PAF.

Tuning Curve Analysis

All sites from control and experimental rats were analyzed together in a blind, randomized batch to prevent experimenter bias. Several receptive field and latency
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characteristics were defined at each site. The lowest intensity that evoked a reliable neural response was defined as the threshold, and the frequency at which this response occurred defined the characteristic frequency (CF). Four bandwidths (BW10-BW40) were calculated as the range of frequencies (measured in octaves) which evoked reliable responses at 10, 20, 30 and 40 dB above threshold (Fig. 1 A & C). A post-stimulus time histogram (PSTH) was constructed from all of the responses to tone-intensity combinations within the receptive field using 1 ms width bins. The spontaneous firing rate at each site was estimated as the spike rate in the first 9 ms recorded after tone onset (before any neural response to sounds). The peak latency for each site was calculated as the time of the maximum number of spikes in the PSTH (Fig. 1 B & D). The onset latency was the first time point in the PSTH when response strength reached 2 standard deviations above the spontaneous firing rate for 2 consecutive milliseconds. Onset latency was only examined in sites with thresholds below 35 dB SPL to ensure that the presented tones were sufficiently loud to evoke the shortest possible response latency. For all of these measures, experimental and control groups were compared using two-tailed unpaired Student’s t-tests to determine if there were statistically significant differences in receptive field or response characteristics after NB-stimulation pairing.

Topography and map expansions

A1 was functionally defined on the basis of latency and tonotopy (Kilgard and Merzenich 1999). In general, sites with peak latencies less than 20 msec were classified as A1. Only 4% of A1 sites in this study exhibited longer latencies. The A1-PAF border was defined using the previously characterized change in topography and latency (Bao et al. 2001; Doron et al. 2002; Pandya et al. in press, in review; Rutkowski et al. 2003). The dorsal and ventral borders of A1 were also defined by changes in topography and latency, while the anterior border of A1 was defined primarily by a reversal in topgraphy. Other boundaries were defined using sites non-responsive to auditory stimuli, or in some cases the limits of data collection.
Changes in cortical tone frequency representation were quantified by comparing the distribution of receptive field tuning (CF’s) in experimental animals to naïve control animals. Since the percent of sites with CF’s in any given frequency range could be biased if the spacing of recording sites was not even across the cortical surface (i.e. due to blood vessels), we also used the percent of cortical area measure used in earlier studies (Kilgard and Merzenich 1998a). Briefly, Voronoi tessellation was used to transform the discretely sampled surface into a continuous map using the assumption that each point on the map has the response characteristics of the nearest recording site. Since regions with above average sampling density have smaller tessellations, they do not bias estimates of the cortical response. The percent of the cortical area responding to each tone was estimated as the sum of the areas of all tessellations from sites (in A1 or PAF) with receptive fields that included the tone divided by the total area of the field.

Changes in PAF and A1 frequency representation were quantified by comparing the percentage of the cortical area of each field which responded to all presented tones in the tuning curve in naïve and experimental animals. Two-tailed unpaired Student’s T-tests were used to determine the statistical significance of all comparisons.

**RESULTS**

Pairing NB-stimulation with a 19 kHz tone shifted the response of A1 and PAF neurons to favor high frequency tones over low frequency tones and increased frequency selectivity in PAF. In A1, the bias for high frequencies results from receptive fields shifts with little change in bandwidth. In PAF, the bias results from a reduction of the PAF response to low frequency tones.

**Classification of Primary and Auditory Cortical Fields**

The primary auditory cortex (A1) of each animal was defined by its location, tonotopic organization, narrow bandwidths and short latencies. The CF of sites increases systematically from posterior to anterior across the surface of A1. Receptive field sizes in A1 were narrower
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than PAF sites (Fig. 1 A and C). In both control and experimental animals, the bandwidths 30 dB above threshold of A1 neurons were approximately 2 octaves (Fig. 2 C and D). The peak latencies for A1 sites were shorter than PAF, usually within 10-20 ms after tone onset (Fig. 1 B). There was no significant change in A1 latencies in experimental animals compared to control animals (onset latency: control, 12.3 ± 0.2; experimental, 12.0 ± 0.2, p = 0.19; peak latency: control, 18.0 ± 0.4; experimental, 19.4 ± 0.7, p = 0.06).

The posterior auditory field (PAF) was defined based on its location posterior to A1, poor tonotopic organization, large receptive field sizes, and long response latencies. The anterior border of PAF was defined by the low frequency edge of A1, while dorsal and ventral borders of PAF were defined by sites which were non-responsive to tones. The far posterior border of PAF was often not as well demarcated because access was limited by the edge of the craniotomy. While the CF’s of A1 sites in controls were fairly evenly distributed (in log space) between 1 and 32 kHz, a relatively large proportion of PAF sites were tuned to frequencies between 5 and 8 kHz (Fig. 2 A). Receptive fields in PAF of control animals often spanned more than 3 octaves at BW30 (see example map in Fig. 2 C), and many sites responded to all tones within the rat hearing range (Fig. 1 C). The clustering of PAF CF’s towards mid-frequencies may result from the large receptive field sizes and lower mid-frequency hearing thresholds of rats (Kelly and Masterton 1977). The peak latencies in PAF for both control and experimental animals were significantly longer than A1, between 30 and 80 ms (Fig. 1 B and D, and Fig. 2 E and F). NB-tone pairing did not significantly alter response latencies in PAF (peak latency, control: 53.1± 2.2 ms, experimental: 54.1± 2.3 ms, p = 0.75, onset latency, control: 35.0 ±1.7, experimental: 31.8 ± 1.2, p = 0.14).

Example of Plasticity in Primary and Posterior Auditory Fields

Data from an example control and an example experimental map are shown in Figure 2 to demonstrate the effects of NB-tone pairing in A1 and PAF (Fig. 2). NB-stimulation paired with
a 19 kHz tone increased the area of A1 that responded to high-frequency tones. While 35% of A1 responded to a 19 kHz 60 dB SPL tone in the example control animal (Fig. 2 A,C), 54% of A1 responded to the same tone in an NB-stimulated animal (Fig. 2 B,D). In our example experimental animal, NB-stimulation paired with a 19 kHz tone decreased the area of the PAF cortex which responded to low frequency tones, but did not increase the total area of PAF that responded to high-frequency tones. While 82% of PAF responded to a 2 kHz 60 dB SPL tone in the example control animal (Fig. 2 A,C), only 56% of PAF responded to the same tone in an NB-stimulated animal (Fig. 2 B,D). In the control and experimental animals, 94% and 82% of PAF responded to a 19 kHz 60 dB SPL tone, indicating that the major effect of NB-tone pairing may have been to decrease responses to unpaired tones rather than increase responses to the paired tone. After NB stimulation, PAF tuning curves in the experimental animal were narrower than the control map, and many more sites had CF’s between 11 and 20 kHz (Fig. 2 B). In the representative control animal, the average bandwidth of PAF sites was 3.9 ± 0.17 octaves, while the average bandwidth of PAF sites in the representative experimental animal was only 2.92 ± 0.16 octaves (Fig. 2 C and D). These two example maps illustrate that both A1 and PAF exhibit shifts in their frequency tuning to favor high frequencies after NB-stimulation paired with a 19 kHz tone. However, only the secondary cortical area (PAF) showed increased frequency selectivity.

**Topography in A1 and PAF**

All A1 maps in the control group had large, significant coefficients of determination between anterior-posterior location and CF (, average r² for A1 in control animals 0.79 ± 0.02). NB-tone pairing did not eliminate the tonotopic organization of A1 (average r² for A1 in experimental rats: 0.79±0.05). PAF showed evidence of weak tonotopic organization in control animals that was eliminated after NB-tone pairing. When all PAF sites from all nine control animals were examined together, we found a weak, but statistically significant, correlation between each site’s
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anterior-posterior position (relative to the A1-PAF border) and CF ($r^2 = 0.09$, $p < 10^{-5}$, Fig. 3 A).

When each animal was considered separately, only 3 out of the 9 control rats showed significant correlations between position and CF (average $r^2$ for PAF in control rats: $0.15 \pm 0.0.05$). In the experimental group, there was no evidence of tonotopic organization when all PAF sites from all animals were grouped together ($r^2 = -0.09$, $p = 0.23$, Fig. 3 B). Only one of the six experimental animals showed any evidence of tonotopic organization in PAF when considered individually (average $r^2$ for PAF in experimental rats: $0.07 \pm 0.05$). We did not observe significance increases in the overall area of either A1 or PAF after NB-stimulation pairing (A1: $1.94 \pm 0.26$ vs. $1.48 \pm 0.17 \text{ mm}^2$, $p=.13$; PAF: $1.28 \pm 0.22$ vs. $0.89 \pm 0.18 \text{ mm}^2$, $p = .15$).

**NB Stimulation Reduced PAF Responses to Low Frequency Tones**

NB-stimulation paired with a 19 kHz tone resulted in an increase in the proportion of sites in PAF which were tuned to high frequencies (Fig. 4). In experimental animals, a larger percentage of sites had CF’s which fell within half an octave of 16 kHz than control animals (mean percentage of total PAF sites in the 16 kHz bin for each animal, controls: $22\% \pm 5$, experimental: $37\% \pm 10$, $p=0.11$). In experimental animals, $28\% \pm 4$ of PAF sites had CF’s within a half an octave of 8 kHz, compared to $41\% \pm 6$ in naïve controls ($p = 0.11$). There was no difference in the percentage of sites falling into the 2 or 4 kHz centered bins (Fig. 4). These results are consistent with a shift in the CF of initially mid-frequency tuned sites (i.e. in the 8 kHz bin) towards the tone frequency paired with NB-stimulation. In the one animal that experienced a 9 kHz tone paired with NB-stimulation, the percentage of PAF sites in the 2 and 4 kHz bins ($4\%$ and $0\%$, respectively) were also decreased compared to naïve controls, providing further indication that the shift in tuning in PAF following NB-tone pairing was frequency-specific.

Pairing NB stimulation with a 19 kHz tone increased tone frequency selectivity in PAF. The average bandwidth $30 \text{ dB above threshold (BW30)}$ decreased by $17\%$, from $3.6 \pm 0.08$ octaves in controls to $3.0 \pm 0.08$ octaves in experimental animals ($p<10^{-7}$). Sites tuned to higher
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Frequencies exhibited the largest reduction in BW. For sites with CF’s within one half octave of 16 kHz, receptive fields were significantly narrower at each analyzed suprathreshold intensity (Fig. 5). For example, BW30 of sites in the 16 kHz bin decreased by 30%, from 3.8 ± 0.14 octaves in control animals to only 2.7 ± 0.18 octaves in experimental animals (p<10^-6). Mid-frequency sites (8 kHz bin) showed a more modest bandwidth decrease and the low frequency bins did not show any change in bandwidths at any intensity above threshold (Fig. 5).

The frequency-specific nature of the bandwidth decrease in PAF suggests the possibility that only sites that were activated by the 19 kHz tone paired with NB stimulation became more frequency selective, while sites that did not respond to the 19 kHz tone were unaffected. All PAF sites in both the control and experimental groups were subdivided into those whose receptive fields included 19 kHz played at 50 dB SPL (Tone In) and those that did not (Tone Out) (Fig. 6). As expected based on the selection criteria, the average bandwidth of the Tone In subset of sites was significantly larger than the average bandwidth of the Tone Out subsets in both control and experimental animals (Fig. 7). The Tone In subset included not only sites which had CF’s near the paired tone, but also virtually all broadly tuned sites (i.e., with bandwidths greater than 4 octaves) regardless of their assigned best frequency (Fig. 6). NB-tone pairing decreased the bandwidth of the Tone In subset while the Tone Out subset of PAF sites remained unchanged (Fig. 7). The average bandwidth 40 dB above threshold (BW40) of Tone In sites dropped by 0.79 octaves following NB-tone pairing, from 4.23 ± 0.12 octaves in control animals to 3.44 ± 0.12 octaves in experimental animals (p < 10^-5). Pairing did not alter the average bandwidth of Tone Out sites (BW40 in octaves of Tone Out sites, controls: 2.62 ± 0.16, experimental: 2.58 ± 0.16, p = 0.85). These results imply that plasticity in PAF may have been restricted to those sites which were able to respond to the paired tone before NB-tone pairing began.
The increase in frequency selectivity in sites that responded to the 19 kHz tone paired with NB stimulation produced a decrease in the percentage of PAF which responded to low frequency tones. While 87% ± 3.2 of PAF responded to a 2 kHz tone played at 60 dB SPL in the control rats, only 53% ± 6.1 of PAF responded after NB-stimulation paired with a 19 kHz tone (p = 0.0001, Fig. 8B). Thus, 39% fewer sites responded to the 2 kHz, 60 dB SPL tone in experimental animals compared to naïve control animals. This decrease in responsiveness to low-frequency tones was observed for all tones with frequencies below 5 kHz played at intensities within the rat’s hearing range. In contrast, there was only a modest increase in the percentage of PAF cortex which responded to high-frequency tones (Fig. 9). For example, the percentage of PAF which responded to a 16 kHz 60 dB SPL tone increased from 65.7% ± 6.9 in control animals to 73.7% ± 6.3 of PAF in experimental animals (p = .41, Fig. 8B). Therefore, after NB-stimulation paired with a 19 kHz tone, the percentage of PAF cortex which responded to high-frequency tones increased only modestly, while the percentage of PAF cortex which responded to low-frequency tones decreased significantly (Fig. 9).

**NB Stimulation increased A1 response to high frequency tones**

Primary auditory cortex (A1) showed an expanded representation of high-frequency sounds following NB stimulation paired with the 19 kHz tone, confirming previous studies of NB-tone pairing (Kilgard and Merzenich 1998a). The percentage of A1 sites which responded to a 16 kHz 60 dB SPL tone increased, from 40% ± 3.62 in control animals to 54.08% ± 5.29 in experimental animals (p = 0.02) (Fig. 8A). There were similar increases in the percentage of A1 sites which responded to tones between 10 and 17 kHz (at intensities between 50 and 70 dB SPL). In addition, the percentage of A1 sites which responded to low-frequency tones decreased slightly (percentage of A1 cortex responding to 2 kHz, controls: 41.7% ± 3.62, experimental: 31.54% ± 6.45, p = 0.13, Fig. 8A). Unlike the plasticity observed in PAF, A1 reorganization
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was not accompanied by significant alterations in receptive field size (BW 30, controls: 1.82 ± 0.05, experimentals: 1.78±0.05, p = 0.61). Therefore, NB-tone pairing in A1 appears to have created a shift of tuning curves towards the paired tone frequency, without changing receptive field sizes.

**Frequency-Specific Changes in PAF Firing Rate**

It is possible that NB-tone pairing increased the number of action potentials elicited by the paired (19 kHz) tone without significantly increasing the area of PAF cortex which responded to that tone. The percentage of cortex measure used in this and previous publications (Kilgard and Merzenich 1998a; Kilgard et al. 2001; Pandya et al. 2005) would not detect such an increase, because the measure only takes into account receptive field boundaries (i.e. tones are ‘in’ or ‘out’) and not variations in the evoked response to different tones within each site’s receptive field. To determine whether NB-tone pairing increased responses to high-frequency tones, we quantified the mean response of all PAF sites in experimental and control animals to every presented frequency-intensity tone combination. At each site, the number of spikes evoked by each tone was averaged with the neighboring frequency and intensity steps (5 dB above and below and 1/16th of an octave above and below each tone).

Pairing NB-stimulation with a 19 kHz tone did not increase the number of spikes elicited by high frequency tones, but did result in a large decrease in the number of spikes elicited by low frequency tones. In control animals, a 16 kHz 60 dB SPL tone elicited 0.69 ± 0.07 spikes per PAF site, while in the experimental group the same tone elicited 0.64 ± 0.06 spikes (p = 0.64). In contrast, a 2 kHz 60 dB SPL tone evoked 0.97 ± 0.09 spikes per site in controls compared to only 0.54 ± 0.06 spikes in experimental animals (p = 0.0002, Fig. 10 A). Changes of similar magnitude and statistical significance were observed in the responses to most tones played below 5 kHz (Fig. 11). These results support our initial observation that NB-tone pairing caused a reduction in the bandwidth of PAF sites, but did not necessarily shift receptive fields towards the
paired tone. If the reduction of bandwidth in PAF sites was due to shifts of individual neurons within the multi-unit clusters toward the paired frequency, we would have expected to see increased evoked responses to the paired tone. We saw no indication of increased responses to any high-frequency tone (Figure 11). Previous studies of A1 that systematically evaluated this issue observed that changes in selectivity of multi-unit responses closely mirrored the changes of well sorted single units (Kilgard and Merzenich 1998a).

When sites were subdivided by whether they responded to the paired stimulus (Tone In) or not (Tone Out), the decrease in response to low-frequency tones was found to originate primarily from the Tone In subset of sites (Fig. 10 B and C). In control animals, Tone In sites responded to a 2 kHz 60 dB SPL tone with 0.99 ± 0.11 spikes per PAF site, but after NB-tone pairing, the same tone elicited only 0.52 ± 0.06 spikes (p = 0.0002). Although there was some evidence for decreased response strength to low-frequency tones in the Tone Out subpopulation (spikes per PAF site, controls: 0.89 ± 0.11, experimentals: 0.68 ± 0.09, p = 0.16, Figure 10 C), this decrease was small and not statistically reliable. The response to high frequency tones was not increased in either the Tone In or Tone Out subgroups (Fig. 10 B and C). This analysis, which required no judgments about tuning curve edges, supports our proposition that the primary effect of NB-stimulation pairing in PAF was to cause sites which responded to the 19 kHz tone to become more selective by decreasing their responses to low and mid-frequency tones (Figure 11).

Spontaneous firing increased in both PAF and A1 after NB-tone pairing. In PAF, the control group had a spontaneous firing rate of 2.75 ± 0.16 Hz, while the experimental group had a spontaneous rate of 4.68 ± 0.25 Hz (p < 10⁻⁴). In A1, the control group’s spontaneous firing rate was 3.74 ± 0.16 Hz, while the experimental group had a spontaneous rate of 4.62 ± 0.21 Hz (p=0.0009). The increase in spontaneous firing showed no evidence of frequency-specificity in either PAF or A1.

**DISCUSSION**
Summary of Results

NB-stimulation paired with a 19 kHz tone increased the cortical response to high frequency tones relative to other tone frequencies in both primary and non-primary auditory cortex. Consistent with earlier observations, the A1 representation of high frequencies increased without a significant decrease in the representation of low or mid frequency tones or change in average receptive field sizes (Kilgard and Merzenich 1998a; Kilgard et al. 2001). PAF showed a pattern of plasticity in response to NB-tone pairing which was distinctly different from A1. Rather than shifting tuning curves toward the paired tone, PAF sites became more selective. The principal effect in PAF after NB-stimulation pairing was not an increase in the representation of the paired tone, but a decrease in the representation of low or mid-frequency tones. While we did not record from the same neurons before and after NB stimulation, it appears that sites which were responsive to the paired tone before NB stimulation increased their selectivity while low-frequency sites were left unaltered.

Changes in Spontaneous Firing and Cortical Plasticity

We observed increased spontaneous activity in both PAF and A1 after NB-stimulation pairing. Increased spontaneous activity has been observed in several studies of NB-stimulation pairing using diverse types of paired auditory stimuli (Metherate and Ashe 1993; Pandya et al. 2005), although not all studies of NB-stimulation pairing have observed increased spontaneous firing rate(Kilgard et al. 2001). Direct application of acetylcholine onto cortical neurons increases spontaneous firing in some cells(Metherate and Weinberger 1990), implying that increased spontaneous activity may be a generalized effect of NB-stimulation. In contrast, changes in receptive field properties such as frequency selectivity and response latency seem to be dependent on specific attributes of the auditory stimuli which were paired with NB-stimulation (Kilgard et al. 2001).
Receptive field narrowing may be a common feature of plasticity in broadly tuned non-primary cortical areas. Neurons in secondary cortical fields were more prone to bandwidth decreases than primary cortical neurons after tone-shock conditioning (Diamond and Weinberger 1986). In the ventroposterior area, which is similar to PAF in terms of response characteristics and location relative to A1, receptive fields were narrowed after stimulation of the ventral tegmental area (VTA) (Bao et al. 2001). These results are analogous to our observation that receptive field sizes in PAF were decreased after NB-tone pairing. However, secondary cortical areas do not always show receptive field narrowing when undergoing plasticity. Extensive training on an auditory frequency discrimination task led to frequency-specific reorganization of secondary field SRAF (suprarhinal auditory field) in trained rats, but did not affect receptive field sizes (Polley et al. 2006). This difference may have been because receptive fields in SRAF are relatively narrow even in naïve animals or because of the sensory statistics associated with the behavioral tasks.

Receptive field size decreases have also been observed in secondary fields of the visual system after behavioral training. Training on an orientation discrimination task leads to smaller receptive fields in V4 (Yang and Maunsell 2004), and discriminating complex stimuli led to smaller receptive fields in the inferotemporal cortex of monkeys (DiCarlo and Maunsell 2003). In both of these cases, plasticity was limited to neurons which were active during performance of the discrimination task, similar to our finding that PAF plasticity after NB-tone pairing may have been limited to sites which were activated by the paired stimulus.

Models of Plasticity

The PAF plasticity observed in this study could result from changes occurring in at least three different locations: A1, auditory thalamus, or PAF. Plasticity in PAF could directly reflect map reorganization in A1. A1 neurons send direct, physiologically active projection to PAF
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(Hishida et al. 2007; Mascagni et al. 1993; Romanski and LeDoux 1993). These connections are necessary to produce driven activity in cat PAF (Kitzes and Hollrigel 1996). Therefore, PAF receptive fields could be constructed from inputs from A1 neurons which are tuned to several different frequencies, from many locations across A1. After NB-tone pairing, receptive fields in A1 are shifted towards the paired frequency, and large areas of A1 cortex become tuned to the same range of frequencies. This would in turn cause PAF neurons to develop smaller receptive fields which are biased towards the paired frequency, because large numbers of A1 neurons are now tuned to the paired tone. This model could explain the pattern of plasticity which we observed in PAF, and could be used to make predictions about the results of future plasticity studies in PAF using more complex sounds based on known A1 results (Kilgard and Merzenich 2002, 1998b; Kilgard et al. 2001; Moucha et al. 2005; Pandya et al. 2005).

It is also possible that reorganization of the non-leminiscal thalamus could explain the observed plasticity in PAF. Secondary cortical areas such as PAF receive direct inputs from the non-lemniscal (medial and dorsal) subdivisions of the thalamus (Arnault and Roger 1990; Kimura et al. 2003; Malmierca 2003; Winer et al. 1999). The medial and dorsal divisions of the thalamus have receptive field properties that are similar to PAF, such as large receptive fields and long response latencies (Aitkin and Webster 1971; Bordi and LeDoux 1994; Calford 1983). Both the medial and dorsal divisions of the thalamus exhibit shifts in receptive field tuning and sometimes reductions in receptive field sizes after classical conditioning (Edeline and Weinberger 1992, 1991). Even though NB does not have direct anatomical connections to the auditory thalamus, (Hallanger et al. 1987) NB-tone pairing could alter receptive field properties in the MGB through cortico-fugal connections from A1 to the auditory thalamus (Hazama et al. 2004; Kimura et al. 2005; Winer and Larue 1987).

Finally, PAF plasticity could be explained by reorganization of the intrinsic circuitry of PAF after NB-tone pairing. PAF receives inputs directly from NB and could undergo plasticity...
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at the circuit level (Arnault and Roger 1990). Based on studies of A1 plasticity after NB-tone pairing, reorganization of PAF receptive fields could result from sharpening of excitatory fields, increased levels of inhibition in the cortical circuit or a combination of the two (Dykes 1997; Froemke et al. 2006; Metherate and Ashe 1993; Metherate and Weinberger 1990). The plasticity which we observed in PAF after NB-tone pairing is likely due to some combination of changes in A1, MGB, and PAF.

Predictions for Future Studies

Our earlier studies have shown that the type of sounds paired with NB stimulation determines the form of plasticity generated in A1 (Kilgard and Merzenich 2002, 1998b; Kilgard et al. 2001; Moucha et al. 2005; Pandya et al. 2005). Pairing temporally modulated tones decreases frequency selectivity in A1, while pairing tones with many different frequencies sharpens frequency tuning. If the A1 feed forward model outlined above is correct, PAF receptive field narrowing would follow any A1 frequency map expansion, but would not necessarily follow plasticity that narrows frequency bandwidth in A1 without changing tonotopy. Sounds that broaden A1 tuning curves would have relatively little effect on PAF neurons which already receive convergent inputs.

Technical Considerations

The methods of our current study did not allow us to record from the same neurons before and after NB-stimulation pairing. The finding that only the Tone In subsets of neurons were different between control and experimental animals implies that PAF plasticity was restricted to neurons which were responsive to the paired tone before NB-stimulation. However, it is also possible that the observed plasticity was caused by more generalized effects in which some PAF neurons shifted away from the paired tone (and thus joined the Tone Out group) while other PAF neurons shifted towards the paired tone (joining the Tone In group). While we favor the first explanation because it is less complicated (i.e., one type of plasticity limited to the
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activated population), the second explanation cannot be excluded without recording from the same neurons before and after NB-tone pairing.

It should be noted that the map expansion recorded in A1 was not centered directly on the paired tone frequency (19 kHz), but was instead centered on approximately 14 kHz and extended from approximately 10 kHz to 17 kHz. It is possible that 20 days of NB-tone pairing was not sufficient to induce a complete shift in tuning to the 19 kHz tone. However, we feel that because of the challenge of characterizing two cortical fields in each individual animal, the full high-frequency border of A1 may not have been fully delineated in each experimental map. Prior studies of NB-stimulation induced plasticity which were focused on mapping A1 exclusively observed A1 map expansions which were centered on the paired tone after a 20 day period of NB-tone pairing.

We did not control for the effect of sound exposure apart from NB-stimulation. Previous plasticity studies have documented that the primary auditory cortex of adult rats is not changed after sound exposure to tones (Bao et al. 2004; Polley et al. 2004; Recanzone et al. 1993; Rutkowski and Weinberger 2005). However, there is still a possibility that the observed plasticity in PAF might have occurred apart from stimulation of NB.

All of the physiological recordings for this study were made under pentobarbital anesthesia. Several studies have indicated that anesthesia can change the response properties of auditory neurons (Gaese and Ostwald 2001; Rennaker et al. 2007; Zurita et al. 1994). In addition, some forms of plasticity are only expressed during the awake state, such as shifts in response properties during a difficult behavioral task (Fritz et al. 2005; Ito and Gilbert 1999; Li et al. 2004). Our observation that the changes in both A1 and PAF cortex caused by NB-tone pairing were present under deep anesthesia implies that the observed changes reflect plasticity in the circuitry of the auditory system, and that feedback activity from higher associational areas may not be necessary for some forms of experience-dependent plasticity to be expressed.
Conclusions

This study demonstrates that stimulus-specific plasticity occurs in a non-primary cortical area following NB-tone pairing. PAF sites showed a tendency to become selective for the paired stimulus at the exclusion of other frequencies. It has been speculated that secondary fields may be specialized for extracting behaviorally relevant features from complex stimuli (Rauschecker 1998), and that learning-induced plasticity in these areas enhances this specialization (Ghose 2004). The finding that receptive field narrowing has been observed in secondary cortical areas after both behavioral training and NB-tone pairing shows that this form of plasticity occurs after sensory experience paired with neuromodulatory inputs. Explicit task knowledge may not be necessary to achieve the increased specialization for trained stimuli which was observed in secondary cortex after behavioral training (DiCarlo and Maunsell 2003; Ghose 2004; Yang and Maunsell 2004). The plasticity which we observed in PAF differs significantly from the concurrent plasticity which we observed in A1, indicating that even simple stimuli can create complex patterns of plasticity across many stations of the auditory system. Recent behavioral and physiological studies have investigated how the primary auditory cortex processes complex, naturalistic stimuli, including speech sounds (Ohl and Scheich 1997; Reed et al. 2003; Sakai and Kudoh 2005; Steinschneider et al. 2003; Toro et al. 2005; Wang and Kadia 2001; Wetzel et al. 1998). To fully understand experience-dependent plasticity in the auditory system, it will be critical to systematically explore plasticity in both primary and non-primary sensory cortex after exposure to complex stimuli. NB-stimulation pairing provides a strong experimental framework from which to understand how stimulus-specific plasticity impacts all stages of sensory processing.

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**Figure 1.** Representative tuning curves and PSTHs demonstrating the larger receptive field sizes and longer latencies of posterior auditory field (PAF) sites compared to primary auditory cortex (A1) sites. (A & C) The following receptive field characteristics which were quantified for each site: characteristic frequency (CF), threshold, bandwidth 10 dB above threshold (BW10), BW20, BW30, and BW40. (B & D) A PSTH for each site was constructed from all of the responses within each site’s receptive field. The peak, onset and end of peak latency for each site was then calculated from the PSTH (see Methods).

**Figure 2.** Maps of A1 and PAF in an example control (left column) and experimental animal (right column) demonstrate the high frequency map expansion and decreased bandwidths after NB stimulation paired with a 19 kHz tone. Each polygon represents a single electrode penetration. The color of each polygon represents the value of the characteristic frequency (CF) (panels A & B), bandwidth 30 dB above threshold (panels C & D), or peak latencies (panels E & F) of the site. The thick black line indicates the border between A1 and PAF. Responses that did not respond to tones are indicated by an open circle (panel F). Sites that responded to tones but did not meet our definition of A1 or PAF are indicated by an X (panel F). The example control map did not have any sites which belonged to other auditory fields or any non-auditory sites. This map was selected for this illustration because of the large number of sites in both A1 and PAF. Most control maps focused on either A1 or PAF and were bounded by recording sites that were either non-responsive, non-A1, or non-PAF. The scale bars in panels A and B indicate a distance of 0.125 mm.
**Figure 3.** Scatter plots of A1 and PAF with best-fit lines demonstrate that PAF is more loosely tonotopically organized than A1. In order to normalize for irregular borders and differing sizes of the A1 and PAF fields between animals, the A1 and PAF border was chosen as the mean position between the most posterior A1 site and most anterior PAF site. Because the border between A1 and PAF is frequently irregular, some A1 sites were located further posterior than the most anterior PAF site. A1 sites are denoted by open circles while PAF sites are denoted by points in the plot for controls (panel A) and for NB-stimulated animals (panel B).

**Figure 4.** Histogram of the proportion of PAF sites (±SEM) for each control or experimental group with characteristic frequency (CF) falling into each of 4 frequency bins, demonstrating the shift in tuning of PAF sites following NB-tone pairing. The one-octave wide bins are centered on 2, 4, 8, and 16 kHz.

**Figure 5.** Average bandwidth of PAF experimental sites decreased compared to controls in high frequency bins across all 4 bandwidth measures (BW 10-40). Each plot shows the average bandwidth (± SEM) in control and experimental sites with characteristics frequencies within each frequency bin for a different bandwidth measure. Asterisks indicate significance values of a t-test comparing control and experimental sites in each frequency bin (* - p<0.05, ** - p<0.01, *** - p<0.001).

**Figure 6.** NB-tone pairing narrows frequency tuning and shifts characteristic frequencies (CF) toward the paired tone frequency (19 kHz). Every PAF recording site for controls (A) and experimental animals (B) is shown sorted by the highest frequency contained
within its receptive field for 50 dB SPL tones. Sites which included 19 kHz within their receptive field are plotted in dark gray, while sites which did not are depicted in light gray. The black circles denote the assigned CF of each site. The vertical black line on each panel is located at 19 kHz.

**Figure 7.** The average bandwidth of PAF sites which contained the paired tone (19 kHz, 50 dB SPL) in their receptive field (Tone In) were decreased in control versus experimental animals. Tone Out sites did not change their receptive field sizes. BW40 was chosen for this measure because the paired tone fell closer to BW40 than other bandwidth measures in the receptive field of most PAF sites. However, the same pattern of results was observed for all bandwidth measures. Asterisks indicate level of significance (* - p<0.05, ** - p<0.01, *** - p<0.001). The average bandwidths of Tone In sites were significantly larger than the average bandwidths of Tone Out sites in both control and experimental animals (see Results).

**Figure 8.** Percentage of A1 and PAF that responds to several tones presented at 60 dB SPL. The average percentage of each field which responded to each tone frequency presented at 60 dB SPL is depicted. While A1 (panel A) showed an increase in the number of neurons which responded to high-frequency tones (such as 16 kHz), PAF (panel B) sites showed a decrease in the number of neurons which responded to low-frequency tones (2 and 4 kHz). Stars indicate level of significance in a comparison between controls and experimentals (* - p<0.05, ** - p<0.01, *** - p<0.001).

**Figure 9.** The percentage of PAF that responded to all low-frequency tones decreased after NB-tone pairing. (A) The average percentage of PAF cortex in controls which
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responds to each tone frequency-intensity combination is shown. Contour lines on the plot indicate the tones which elicit responses from 0, 20, 40, 60 and 80% of PAF neurons.

(B) The average percentage of PAF cortex in experimental animals responding to each tone frequency-intensity combination. The 60 and 80% contour lines were shifted towards high-frequencies in the experimental animals compared to controls. (C) The difference in the percentage of PAF responding to each tone-intensity combination (experimental – controls) is shown. White lines delineate the frequency-intensity combinations with a significant decrease in the percent of cortex responding after NB-stimulation pairing (solid – p<0.05, dashed – p<0.01). No increases in the percentage of cortex responding were statistically significant. The white point in Panels A-C indicates the tone which was paired with NB-stimulation in experimental animals (19 kHz @ 50 dB SPL).

Figure 10. Depiction of the number of spikes elicited by tones played at 60 dB SPL. Panel A shows the average evoked response for all PAF sites. Panels B and C show the responses of those subsets of sites which contained the paired tone within their receptive field (panel B) or did not (panel C). A decrease in the number of spikes elicited by low-frequency tones (panel A) was primarily caused by a decrease in responsiveness of the Tone In subset of sites (panel B). Asterisks indicate level of significance (* - p<0.05, ** - p<0.01, *** - p<0.001).

Figure 11. Average number of spikes responding to each frequency-intensity combination per PAF site. For each site, the spontaneous rate (calculated as the activity to 0 dB SPL tones) was subtracted from the response to all the other frequency-tone combinations. Panel A shows the average number of spikes evoked from all control sites.
while panel B shows the average number of spikes from all experimental sites. The black lines in panels A & B delineate the 0.5, 0.75 and 1 spike per tone boundaries on each plot. Panel C shows the subtraction of the average of the control sites (panel A) from the average of the experimental sites (panel B). A zero (orange) indicates that there was no difference between the two groups in the number of spikes evoked by the frequency-intensity combination. Panel D shows the result of t-tests between the number of spikes evoked by each frequency-intensity combination in control and experimental sites. The color bar indicates degrees of significance (a value of 3 denotes p<0.001, while a value of 4 denotes p<0.0001).
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