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Cholinergic modulation incorporated with a tone presentation induces frequency-specific threshold decreases in the auditory cortex of the mouse

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Abstract

Learning-induced or experience-dependent auditory cortical plasticity has often been characterized by frequency-specificity. Studies have revealed the critical role of the cholinergic basal forebrain and acoustic guidance. Cholinergic facilitation of specific thalamocortical inputs potentially determines such frequency-specificity but this issue requires further clarification. To examine the cholinergic effects on thalamocortical circuitry of specific frequency channels, we recorded the responses of cortical neurons while pairing basal forebrain activation or acetylcholine (ACh) microiontophoresis with tone presentations at 10 dB below the neuronal response threshold. We found that both basal forebrain activation and acetylcholine microiontophoresis paired with a tone induced a significant decrease in response threshold of the recorded cortical neurons to the frequency of the paired tone, and that this threshold decrease could be eliminated by atropine microiontophoresis. Our data suggest that cortical acetylcholine specifically facilitates thalamocortical circuitry tuned to the frequency of a presented tone; it is the first, fundamental step towards frequency-specific cortical plasticity evoked by auditory learning and experience.

Introduction

Auditory learning induces remarkable changes in the functional organization of the auditory cortex that are highly specific to the frequency content of the learned sound (Suga & Ma, 2003; Yan, 2003; Weinberger, 2004; Ohl & Scheich, 2005). Interest in the role of the cortical cholinergic system has grown as indicated by elegant studies of the human auditory cortex. One such study demonstrates that aversive conditioning enhances the cortical representation of the conditioned sound; in contrast, such enhancement is not seen in subjects that are administered scopolamine, a muscarinic acetylcholine receptor antagonist, before the conditioning (Thiel *et al*., 2002). These results are confirmed by animal studies showing that learning-induced cortical plasticity is augmented by cortical application of acetylcholine (ACh) and conversely, prevented by cortical application of the muscarinic antagonist, atropine, during the conditioning (Ji *et al*., 2001; Ji & Suga, 2003).

The major source of cortical cholinergic inputs is the nucleus basalis (NB) of the basal forebrain. The NB is demonstrably active during learning tasks (Mesulam *et al*., 1983; Richardson & DeLong, 1991). Electrical stimulation of the NB increases the cortical acetylcholine level; it increases the auditory response of cortical neurons in a non-specific manner (Rasmusson *et al*., 1992; Hars *et al*., 1993; Jimenez-Capdeville *et al*., 1997; Yan & Zhang, 2005). It has been repeatedly demonstrated that electrical stimulation of the NB (ES_{NB}) can induce frequency-specific plastic changes in cortical receptive fields only when a tone is simultaneously presented (Bakin & Weinberger, 1996; Bjordahl *et al*., 1998; Kilgard & Merzenich, 1998; Ma & Suga, 2003). This change can be eliminated by blocking cortical muscarinic receptors (Miasnikov *et al*., 2001; Yan & Zhang, 2005). Furthermore, the ES_{NB} paired with a tone (tone-ES_{NB}) or auditory learning also induces frequency-specific plasticity in the subcortical nuclei of the auditory midbrain in a normally functioning auditory cortex (Ma & Suga, 2003; Zhang *et al*., 2005). Finally, other studies have established that NB activation enhances thalamocortical synaptic transmission and that focal thalamic activation induces frequency-specific cortical plasticity (Metherate & Ashe, 1993; Jafari *et al*., 2007). The interaction of cholinergic and thalamocortical inputs within the auditory cortex appears significant to the development of frequency-specific auditory plasticity.

Our previous studies demonstrated that electrical stimulation of the NB paired with a tone of 20 dB above the minimum threshold of a given cortical neuron induces a clear response threshold decrease that is highly specific to the frequency of the paired tone. The frequency-specific threshold decrease was observed in only 36% of the studied neurons (Yan & Zhang, 2005). Our proposal, that cholinergic facilitation of thalamocortical inputs of specific frequency channels is key to the development of frequency-specific cortical plasticity, required more consistent results. Therefore, we extended our previous work by examining the plasticity of cortical receptive field induced by the electrical stimulation of the NB or acetylcholine microiontophoresis paired with a tone presentation. To minimize the number of the activated thalamocortical inputs, the experimental protocol sets the amplitude of the paired tone at 10 dB below the response threshold of selected cortical neurons. Clear decreases in response threshold were

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observed following either the NB activation or acetylcholine microiontophoresis paired with tone presentation. The threshold decrease was highly specific to the frequency of the paired tone and was not observed when atropine was simultaneously injected to the recorded neurons.

Materials and methods

The materials, animal surgery, acoustic stimulation, electrical stimulation of the basal forebrain, recording of neuronal activities and data processing are essentially the same as outlined in our other papers (Yan & Zhang, 2005; Zhang *et al*., 2005; Zhang *et al*., 2006). The essential portion and any notable differences are described below.

General

C57BL/6 female mice aged 4–7 weeks and weighing from 17 to 21 g were used in our experiments. All protocols and procedures followed institutional guidelines and were approved by the Animal Care Committee of the University of Calgary (Protocol Number M02034). Animals were anaesthetized with a mixture of ketamine (100 mg/kg, i.p., Bimeda-MTC Animal Health Inc, Canada) and xylazine (10 mg/kg, i.p., Bimeda-MTC Animal health Inc., Canada). The anaesthetic status was examined approximately every 40 min. If required, additional doses of ketamine (20 mg/kg) and xylazine (2 mg/kg) were injected to maintain the anaesthetic level throughout the surgery and physiological experiments. The head of the animal was fixed in a custom-made head holder by rigidly clamping between the palate and nasal/frontal bones. The hair and the scalp overlaying the dorsal skull were removed. Two holes measuring 2 mm in diameter were made on the skull to expose the left primary auditory cortex and the brain surface above the basal forebrain. The dura mater above the left auditory cortex was carefully removed. Two additional holes measuring 1 mm in diameter were made close to the bregma and lambda on the right skull for conducting the electroencephalogram (EEG) recordings. Throughout the surgery and the experimental protocol, the animal's body temperature was kept at a constant 37 °C by a feedback controlled heating pad.

Acoustic stimulation

Tone bursts (60-ms duration with 5 ms rise and fall times) were digitally synthesized and converted to analogue sinusoid waveforms by a RP2 real-time processor (Tucker-Davis Tech Inc, Alachua, Florida, USA). The output amplitude of the sinusoidal wave was set at 20 V peak-to-peak. The signals were then fed to a digital attenuator (PA5) and presented by a tweeter via a power amplifier. The tweeter was placed 45 degrees deviated to and 130 cm away from the mouse's right ear. The tone amplitude, expressed as dB SPL (ref. 20 μPa), was calibrated with a condenser microphone (Model 2520, Larson-Davis Inc, USA) and a microphone preamplifier (Model 2200C, Larson-Davis Inc, USA). We used the previously determined position of the animal's right ear. Frequencies and intensities of tone bursts were varied either manually or automatically with BrainWare software. A frequency-amplitude scan (FA-scan) was used to sample the excitatory response area of the cortical neurons. In the FA-scan, tone frequency varied from 3 to 40 kHz in increments of 1 kHz and tone amplitude varied from −3 to 80 dB SPL in increments of 5 dB. The interstimulus interval was at least 200 ms. The tone frequency and amplitude in the FA-scan were randomly presented by the BrainWare software. The stimulus was repeated five times per frequency/amplitude set.

EEG recording

The EEG was recorded with two silver electrodes placed on the dura close to the bregma and lambda, filtered with a bandpass of 1–100 Hz, amplified 10 000 times, monitored on an oscilloscope, and archived by SciWork software (DataWave, Inc, Berthoud, Colorado, USA).

The electrical stimulation of the NB

A concentric bipolar electrode with a tip of 125 μm in the outer diameter was placed dorsoventrally into the basal forebrain (1.2 mm posterior to the bregma, 1.5 mm left to the midline and approximately 4.0 mm ventral to the brain surface; Franklin & Paxinos, 1996). A train of electric pulses (0.2-ms long, monophasic constant current square wave, 120 Hz, 200-ms train duration) was used as the electrical stimulation and was generated by a Grass S88 stimulator and a Grass CCU1 constant current unit (Astro-Medical, Inc., West Warwick, Rhode Island, USA). The current was delivered to the NB through the central pole of the concentric electrode. The threshold current for desynchronizing the EEG was tested by using six trains of electrical pulses with 500-ms intervals per attempt. The electrode's position was adjusted until the maximal desynchronization of the EEG was observed. The threshold current was then determined. The level of the stimulation current, which ranged from 92 to 167 μA (126.6 ± 25.1), was set at 10 μA above the threshold current level required to desynchronize the EEG for the NB stimulation.

Multibarrel electrodes for recording of action potentials and for microiontophoretic injection of drugs

A five-barreled carbon-fibre microelectrode was employed in these experiments. The multibarreled electrode was inserted perpendicularly into the left primary auditory cortex approximately 300–400 μm below the brain surface. Its central tubing contained an 8-μm-diameter carbon fibre that was used to record action potentials. Bioelectrical signals were amplified 10 000 times and filtered with a bandpass of 0.3–10 kHz (RA16PA and RA16, Tucker-Davis Tech, USA). Multiunit responses to tone stimuli were displayed on an oscilloscope and recorded by a BrainWare data acquisition system (Tucker-Davis Tech Inc, Alachua, Florida, USA). Single-unit action potentials were isolated through spike sorting (Yan & Ehret, 2002; Yan *et al*., 2002, 2003).

The electrode's central tubing was surrounded by five glass tubes (barrels). The overall diameter of the multibarreled electrode was 15–20 μm at the tip of the barrels, which was 20–40 μm proximal to the tip of the carbon fibre. Three of the five barrels were filled with drugs; saline, atropine or ACh. The remaining two barrels were filled with isotonic saline solution (0.9% NaCl, pH 7.0) for either grounding or balancing. This study used atropine sulphate salt (0.4 M in 0.9% NaCl, pH 4.0, Sigma–Aldrich Co, St. Louis, Missouri, USA) and acetylcholine chloride (1.0 M in 0.9% NaCl, pH 3.5–4.0, Sigma–Aldrich Co, St. Louis, Missouri, USA). Saline (0.9% NaCl, pH 7.0) was used as a control injection.

For microiontophoretic injection of a drug, an electric current with a constant current source was applied to the drug-filled barrels (BH-2, Harvard Apparatus, Inc., USA). The injection current was maintained at a level of +50 nA for each drug, i.e. saline, atropine or ACh. During non-injection periods, a retention current of −20 nA was applied to the drug-filled barrels. The drug-injection times were established as described below. Automatic current balancing was performed through the saline-filled barrel.

Experimental protocol

When the action potential recordings were stable, the responses of cortical neurons to the FA-scan were recorded as the control. The animals were then divided into two groups. Animals in Group 1 were treated by the ES_{NB} paired with a tone (Tone–ES_{NB}). The NB was first stimulated by a train of pulses at a rate of 1/s for 6 min. Both immediately and 1 h after the stimulation, the responses of the given cortical neurons to the FA-scan were recorded. After the frequency/threshold-tuning curve was measured, the frequency and amplitude of a tone to be paired with ES_{NB} were determined according to the best frequency (BF) and threshold of a given isolated unit. The animal was next stimulated by a series of ES_{NB} paired with a tone. This procedure concluded with continuous microiontophoresis of either saline or atropine to the recorded neurons for a period of 6 min. The ES_{NB} and tone were synchronized at the onset and delivered at a rate of 1/s.

Animals in Group 2 were treated by the microiontophoresis of acetylcholine paired with a tone. Acetylcholine was continuously injected to the recorded cortical neurons for 10 min. Both immediately and 1 h after the injection, neuronal responses to the FA-scan were recorded. The frequency and amplitude of a tone to be paired with the microiontophoresis of acetylcholine were determined according to the BF and threshold of a given isolated unit. Then, acetylcholine or a combination of acetylcholine/atropine were continuously injected together with a tone that was presented at a rate of 1/s for 10 min. For both groups, the responses of the cortical neurons to the FA-scan were recorded immediately and every 30 min until 6 h after the treatment or when at least 30% recovery was achieved from the maximal changes in either the BF at which the neuron showed the lowest response threshold or response threshold. The frequency of the paired tone was set between 10 kHz below and 10 kHz above the BF of a given neuron. The amplitude of the paired tone was set at 10 dB below the response threshold of a given neuron to the frequency of the paired tone.

Histology for the identification of stimulated NB sites

Once the physiological experiments were completed, a 30-s DC current of 1 mA was applied to the stimulated site in the NB. While under deep anaesthesia, the animal was then killed with a cardiac perfusion of 10% formalin. The brain was sectioned and the electrolytic lesion point was examined under microscope. Data were excluded from our final analysis if the lesion site was not in the NB.

Data processing

The frequency and amplitude of a continuous 8-s EEG waveform were analysed immediately before, immediately after, and 28 s after the ES_{NB}.

The excitatory response area of single-unit to the FA-scan were eventually displayed by dot-rasters, peristimulus time histograms or cumulative peristimulus time histograms all using a bin width of 1 ms. The excitatory frequency-tuning curve was derived from the response threshold at various frequencies of the FA-scan data (Yan & Ehret, 2002; Yan & Zhang, 2005; Zhang *et al*., 2005). The BF was the frequency showing the minimum threshold (MT). The response latency was the time that the spike number reached 10% of the maximum (peak of peristimulus time histograms or cumulative peristimulus time histograms). Spike numbers were counted for five identical stimuli at the BF and 10 dB above the MT. The auditory response magnitude was expressed by the average spike number (ASN) that was the quotient of the sum of spike numbers at the frequency/amplitude sets in the area of receptive field (RF) divided by the size of RF areas below 50 dB SPL.

The plastic changes of cortical neurons were expressed by the differences in response properties, i.e. BF, MT and threshold to other frequencies, before and after treatments. The minimum detectable changes in the BF and threshold were 1 kHz and 5 dB, respectively. These were the smallest units measurable on the FA-scan. The criterion of BF shift was that the BF changed by 1 or more kHz. The detectable changes in MTs could be as small as 1 dB because of the shifted BF after the treatments as well as the uneven frequency response curve produced by the loudspeaker. The maximal changes in the response properties of cortical neurons after the treatments were eventually compared with those of the control data.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). A *t*-test and chi-square test were used to compare the differences between individual values and groups of data. A *P*-value of less than 0.05 is considered to be statistically significant.

Results

In total, 149 cortical neurons from 102 mice were studied. These neurons commonly displayed frequency/threshold tuning curves of various shapes but had clear BFs and MTs. The BFs of sampled cortical neurons ranged from 7 to 30 kHz (17.45 ± 5.71 kHz) and the MTs ranged from −4.15 to 44.38 dB SPL (19.53 ± 10.61 dB SPL). These results fell within the central hearing range of C57 mice and were consistent with previous findings (Yan & Zhang, 2005; Zhang *et al*., 2005; Jafari *et al*., 2007). After the excitatory responses of given cortical neurons to the FA-scan were recorded as control, one of the following procedures was used; (i) the microiontophoresis of saline to recorded neurons during tone–ES_{NB} (saline/tone–ES_{NB}); (ii) the microiontophoresis of atropine to recorded neurons during tone–ES_{NB} (atropine/tone–ES_{NB}); (iii) the microiontophoresis of acetylcholine (ACh) to recorded neurons paired with a tone (tone–ACh) and (iv) the microiontophoresis of acetylcholine/atropine to recorded neurons paired with a tone (tone–ACh/atropine).

It has been well established that ES_{NB} increases the level of cortical acetylcholine and desynchronizes global EEG (Bakin & Weinberger, 1996; Jimenez-Capdeville *et al*., 1997; McLin *et al*., 2002; Yan & Zhang, 2005). For the experiments that employed tone–ES_{NB}, the effectiveness of ES_{NB} was first assessed by examining the changes in EEG waveforms. Under anaesthesia, EEG waveforms consisted of large and slow waves. ES_{NB} changed the EEG waveforms to small and fast waves. The EEG waveform mostly recovered at 12 s after ES_{NB}. These results were consistent with our previous data (Yan & Zhang, 2005; Fig. 1).

Two examples of cortical neurons before and after tone–ES_{NB} accompanied with microiontophoresis of saline or atropine

The ES_{NB} paired with a tone of 10 dB below the response threshold of given cortical neurons induced significant changes in the RFs of cortical neurons. A typical example is shown in Fig. 1A. This neuron tuned to 17 kHz with an MT of 22.5 dB SPL (Fig. 1A, a). Microiontophoretic injection of saline alone to this neuron caused little changes in its BF and the response thresholds across frequencies (Fig. 1A, b). Obvious changes were observed after ES_{NB} paired with a

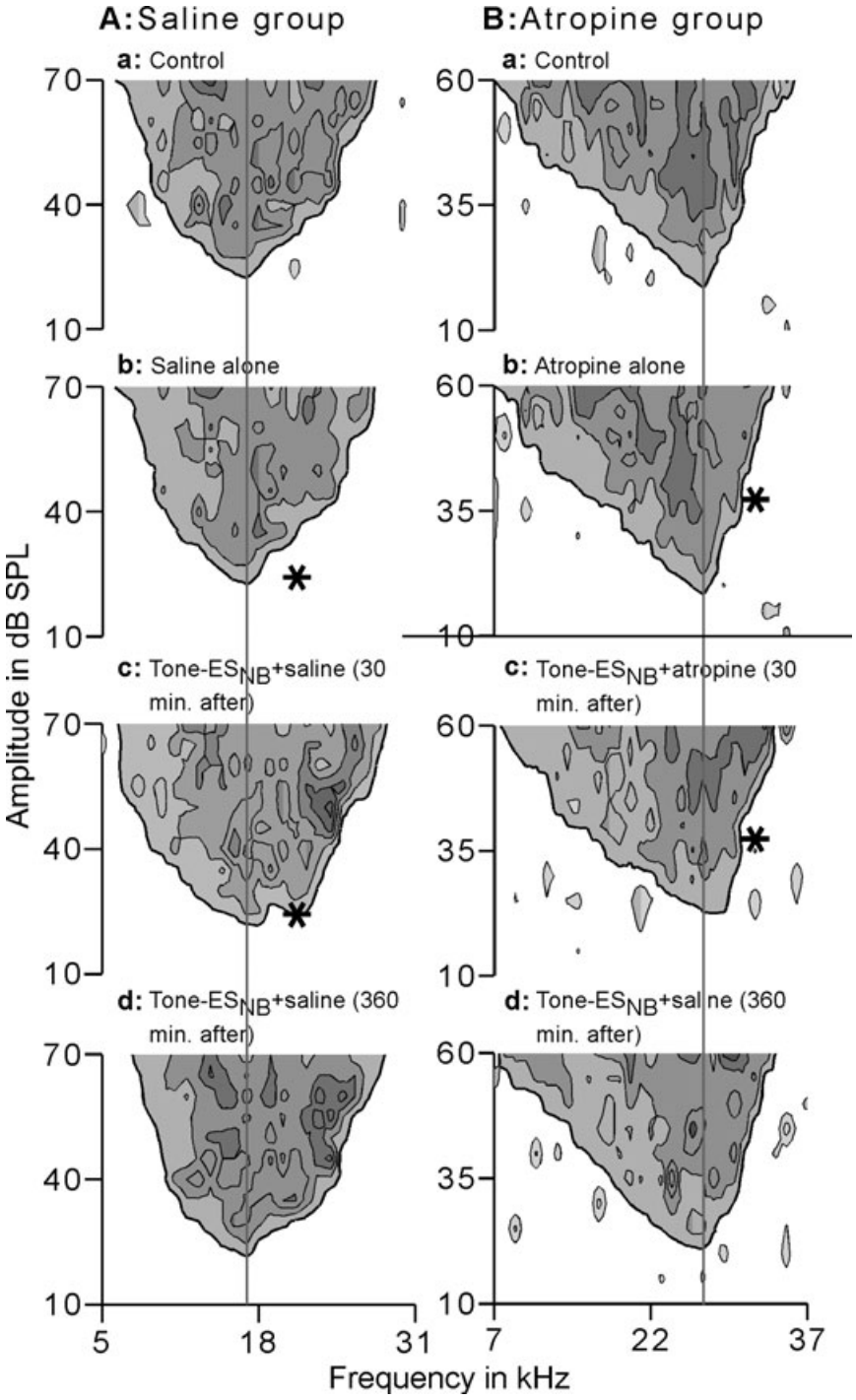

FIG. 1. Examples of tone–ES$_{NB}$ accompanied with saline (A) and atropine (B) microiontophoresis on the RFs of a single cortical neuron. Tone–ES$_{NB}$ induced the BF shift towards the PT frequency and a sharp threshold decrease to the PT frequency (Ac). The effects of atropine microiontophoresis on a second recorded neuron however, did not produce a threshold change (Bc). Contour lines are drawn at 20, 40, 60 and 80% of the maximum spike number. The 20% contour line delineates the major excitatory area. The solid lines indicate the control BF and the asterisks indicate the PT frequency and amplitude.

tone of 21 kHz and 25 dB SPL accompanied with the microiontophoretic injection of saline (Fig. 1A, c). The changes included increases in response magnitude, BF shift and the threshold decrease at the frequency of paired tone (PT). Importantly, tone–ES$_{NB}$ with atropine microiontophoresis induced a similar BF shift but did not cause a clear threshold decrease in response to the PT frequency (Fig. 1B, b and c). The changes in BFs and response thresholds had mostly returned to control levels at 6 h post-treatment (Fig. 1, Ad and Bd).

Frequency-specific shifts in cortical BFs following tone–ES$_{NB}$ with and without atropine

Tone–ES$_{NB}$ accompanied with either saline or atropine microiontophoresis caused significant changes in frequency/threshold tunings of cortical neurons. One of the most pronounced changes was the shift in the BFs of the examined cortical neurons. The BF of the cortical neuron consistently changed as long as the PT frequency was different from the BF of a given neuron after tone–ES$_{NB}$ with either saline or

atropine microiontophoresis. When the PT frequency was higher than cortical BFs, tone–ES_{NB} shifted cortical BFs higher. When the PT frequency was lower than cortical BFs, tone-ES_{NB} shifted cortical BFs lower. However, when the PT frequency was similar to cortical BFs, tone–ES_{NB} did not have an impact. The shifts in cortical BFs were linearly correlated to the difference between PT frequencies and cortical BFs (Fig. 2A and B). The range of the BF shift was obviously different between the saline and atropine treatments; the shifts in cortical BFs evoked by tone–ES_{NB} accompanied with saline microiontophoresis (slope, 0.40) were significantly larger than those evoked by tone–ES_{NB} accompanied by atropine microiontophoresis (slope, 0.25). Statistically, the percentage of BF shift evoked by tone–ES_{NB} in the cortical saline treatment was significantly larger than that in the cortical atropine treatment ($P < 0.001$, Fig. 2C).

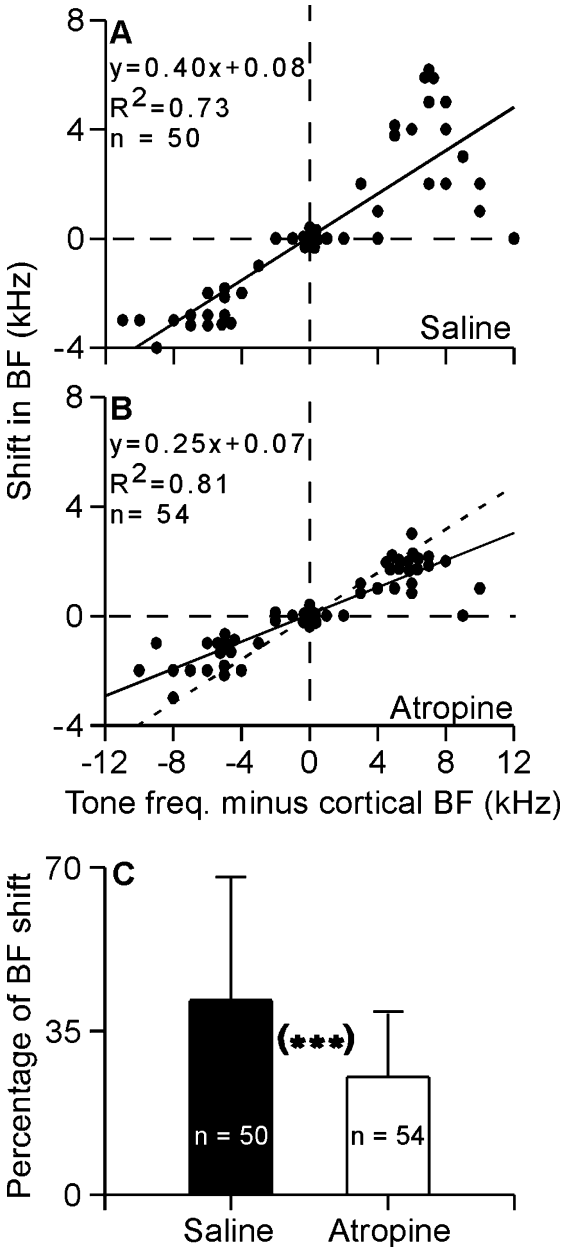

FIG. 2. Effects of atropine microiontophoresis on the BF shift evoked by tone–ES_{NB}. Tone–ES_{NB} accompanied with saline microiontophoresis systematically shifted cortical BFs towards the frequency of the paired tone (A). Cortical microiontophoretic injection of atropine largely reduced the range of the BF shift (B). The percentage of BF shift evoked by tone–ES_{NB} with atropine microiontophoresis was significantly lower than that evoked by tone–ES_{NB} with saline microiontophoresis (C, *t*-test, $P < 0.001$). The dashed line in B represents the regression line in A. ***$P < 0.001$, comparing saline and atropine groups.

Frequency-specific decreases in the response threshold of cortical neurons following tone–ES_{NB} with and without the microiontophoresis of atropine

To evaluate the changes in the response thresholds across frequencies, we focused on the changes in response thresholds to the control BF (BF_{CON}), the new BF after treatment (BF_{NEW}) and the PT frequency. We will use the terms PT neurons to designate cortical neurons that tune to the PT frequency and non-PT neurons for those that tune to other frequencies.

In the case of the PT neurons, the BF_{CON}, BF_{NEW} and PT frequency were the same. Therefore, only the changes in MT were analysed. The tone–ES_{NB} accompanied with the saline treatment typically decreased the MT of the neurons. Out of 12 PT neurons, 11 (91.67%) showed decreases in MT and one (8.33%) showed no change. In contrast, tone–ES_{NB} accompanied with the atropine treatment rarely changed cortical MTs. Out of ten PT neurons, eight (80%) showed no change in MT and two (20%) showed an increase in MT. On average, tone–ES_{NB} with cortical saline treatment decreased cortical MTs by 7.08 ± 4.31 dB whereas tone–ES_{NB} with cortical atropine treatment increased cortical MTs by 0.5 ± 2.7 dB. The changes in MTs were significantly different between the treatments ($P < 0.001$, Fig. 3A).

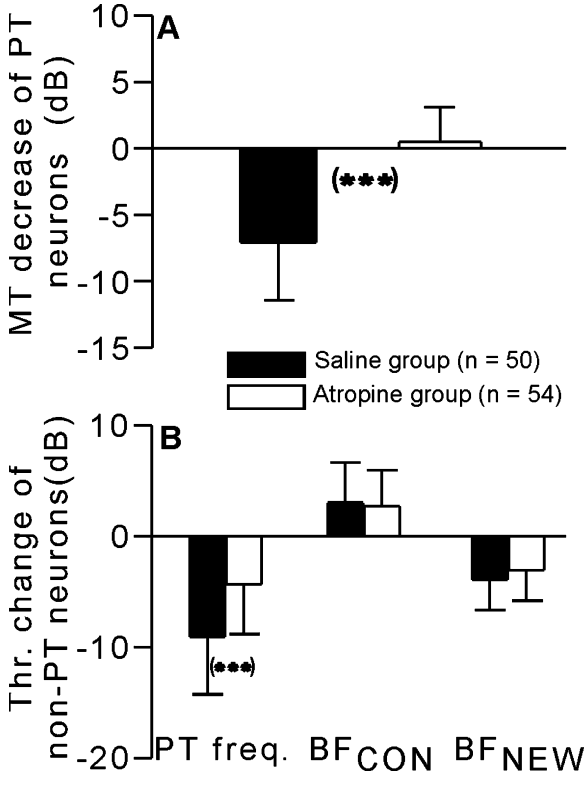

FIG. 3. Changes in response thresholds to different frequencies after tone–ES_{NB}. The MT of PT neurons was significantly decreased by tone–ES_{NB} with saline microiontophoresis (*t*-test, $P < 0.001$) but rarely changed by tone–ES_{NB} with atropine microiontophoresis (A). For non-PT neurons, tone–ES_{NB} largely decreased the response threshold to PT frequency. The threshold decrease to PT frequency was significantly smaller with atropine microiontophoresis to the recorded neurons (*t*-test, $P < 0.001$). Tone–ES_{NB} also increased the response threshold to BF_{CON} and decreased the response threshold to BF_{NEW} (B). These changes were insignificant for both groups ($P > 0.05$). ***$P < 0.001$, comparing saline and atropine groups.

In the case of the non-PT neurons, the changes in response thresholds to BF_{CON} and BF_{NEW} were similar following tone–ES_{NB} accompanied with cortical saline and atropine treatments. Following tone–ES_{NB} accompanied with saline microiontophoresis, the response thresholds to BF_{CON} were slightly increased by 3.0 ± 3.5 dB and those to BF_{NEW} were decreased by 4.0 ± 2.6 dB. Following tone–ES_{NB} accompanied with atropine microiontophoresis, the response thresholds to BF_{CON} were slightly increased by 2.7 ± 3.1 dB and those to BF_{NEW} were decreased by 3.1 ± 2.7 dB. There were no statistical differences in the threshold changes between saline and atropine treatments ($P > 0.05$ for both BF_{CON} and BF_{NEW}, Fig. 3B). Noticeably, tone–ES_{NB} induced more obvious threshold decreases to the PT frequencies. The threshold decrease evoked by tone–ES_{NB} accompanied with saline microiontophoresis was significantly larger than that accompanied with atropine microiontophoresis (9.1 ± 5.1 dB vs. 4.3 ± 4.4 dB, $P < 0.001$, Fig. 3B). The threshold decreases to the PT frequency were significantly larger than the threshold decreases to the BF_{NEW} following tone–ES_{NB} accompanied with saline microiontophoresis ($P < 0.001$).

We further analysed the relationship of the frequency showing the largest threshold decrease (F_{LTD}) to the PT frequency or BF_{NEW}. No matter what the control BFs were, the F_{LTD} evoked by tone–ES_{NB} accompanied with saline microiontophoresis was the same as the PT frequency in 26 out of 38 non-PT neurons (Fig. 4A, a) and was unrelated to the BF_{NEW} (Fig. 4A, b). The deviation of F_{LTD} from the PT frequency was 0.55 ± 0.99 kHz while that from the BF_{NEW} was 2.97 ± 2.05 kHz, which were significantly different ($P < 0.001$). On the other hand, the F_{LTD} evoked by tone–ES_{NB} accompanied with atropine microiontophoresis was the same as the BF_{NEW} in 18 out of 44 non-PT neurons (Fig. 4B, b) and was unrelated to the PT frequency (Fig. 4B, a). The deviation of F_{LTD} from the PT frequency was 3.21 ± 1.71 kHz while that from the BF_{NEW} was 0.82 ± 1.02 kHz, which were significantly different ($P < 0.001$).

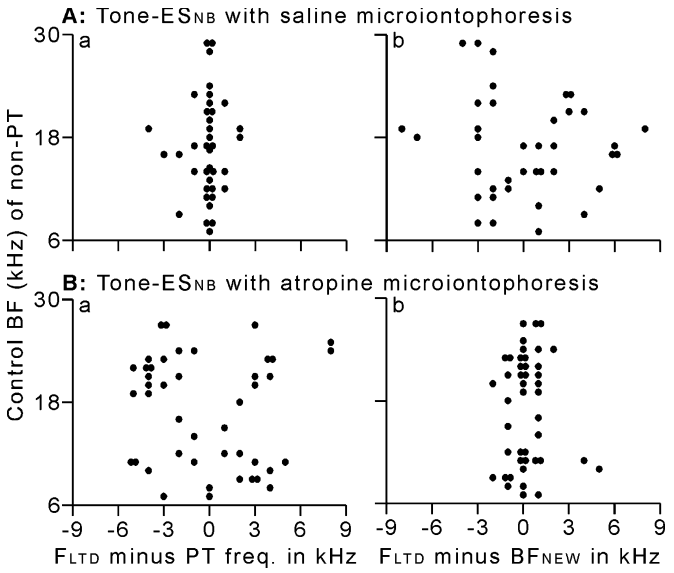

FIG. 4. Relationship of the F_{LTD} to the control BF, PT frequency and BF_{NEW} of non-PT neurons after tone–ES_{NB} accompanied with saline and atropine microiontophoresis. In the *x*-axes of Aa and Ba, zero indicates that the F_{LTD} is the same as the PT frequency. In the *x*-axes of Ab and Bb, zero indicates that the F_{LTD} is the same as the BF_{NEW}. The F_{LTD} evoked by tone–ES_{NB} was independent from the control BF. When the animal was treated by tone–ES_{NB} accompanied with saline microiontophoresis, the F_{LTD} is highly specific to the PT frequency (Aa). When the animal was treated by tone–ES_{NB} accompanied with atropine microiontophoresis, the F_{LTD} is mostly associated with the BF_{NEW} (Bb).

Frequency-specific decreases in response threshold of cortical neurons following microiontophoresis of acetylcholine paired with a tone

The above results demonstrate that tone–ES_{NB} induced significantly larger threshold decreases to PT frequency. The atropine microiontophoresis reduced but did not completely eliminate the threshold decreases (Fig. 3B). This may be due to the global shifts in frequency/threshold tuning through cholinergic facilitation of the cortical PT neurons and not the recorded non-PT neurons. Therefore, we examined the changes in cortical frequency/threshold tuning curves following acetylcholine microiontophoresis to the recorded neurons paired with a tone. We attempted to clarify the impact of cholinergic facilitation of PT thalamocortical inputs on cortical non-PT neurons; this should have induced the response threshold decrease specific to PT frequency but not induced a global tuning shift.

The example in Fig. 5A illustrates the effects of acetylcholine microiontophoresis paired with a tone on the excitatory response area of a cortical neuron to tone stimuli. This neuron tuned to 18 kHz with an MT of 27.5 dB SPL (Fig. 5A, a). Acetylcholine microiontophoresis alone did not alter the BF but broadened the frequency tuning and increased the overall response to tone stimulation (Fig. 5A, b). When acetylcholine was microiontophoretically injected to the neuron paired with a tone of 14 kHz and 20.5 dB SPL, the response threshold to the PT frequency (14 kHz) was reduced by 15 dB and the response magnitude increased. The response threshold to 14 kHz became the lowest threshold across frequencies so that the BF of this neuron shifted 4 kHz downward to 14 kHz (Fig. 5A, c). The whole frequency/threshold tuning curve showed partial recovery 5 h after treatment (Fig. 5A, d).

In most cases, the threshold decrease to PT frequency was not large enough to become the lowest response threshold across frequencies. Therefore, the BFs of most cortical neurons were unaltered throughout the observation period. Out of 24 studied neurons, only seven (29.17%) neurons showed a BF shift to the PT frequency. The BF shift apparently resulted from the threshold decrease to the PT frequency that was close to the original BF (Fig. 6A).

To illustrate better the PT-specific threshold decrease following the acetylcholine microiontophoresis paired with a tone, we first calculated the difference in frequency/threshold tuning curves before and after the acetylcholine microiontophoresis paired with a tone. We next calculated the average of the differences in response thresholds across all frequencies of all samples that were aligned at the PT frequencies. The result showed that the acetylcholine microiontophoresis paired with a tone led to a significant decrease in response threshold to the PT frequency (Fig. 6B). This phenomenon was not seen following microiontophoresis of acetylcholine/atropine paired with a tone (Figs 5B and 6C). The much smaller standard deviation in Fig. 6C indicates that the differences in response thresholds before and after microiontophoresis of acetylcholine/atropine paired with a tone were mostly zero in all of the samples.

Changes in response magnitude and latency following tone–ES_{NB} and tone–ACh

To assess the responses above the threshold, we measured the ASNs below 50 dB SPL. On average, the ASN of cortical neurons was 11.90 ± 6.64 before any treatment. ES_{NB} alone significantly increased the ASN to 18.01 ± 13.05 ($P < 0.01$). Tone-ES_{NB} further increased the ASN to 20.01 ± 13.06 ($P < 0.001$). When tone–ES_{NB} was delivered with atropine microiontophoresis, the ASN did not significantly change

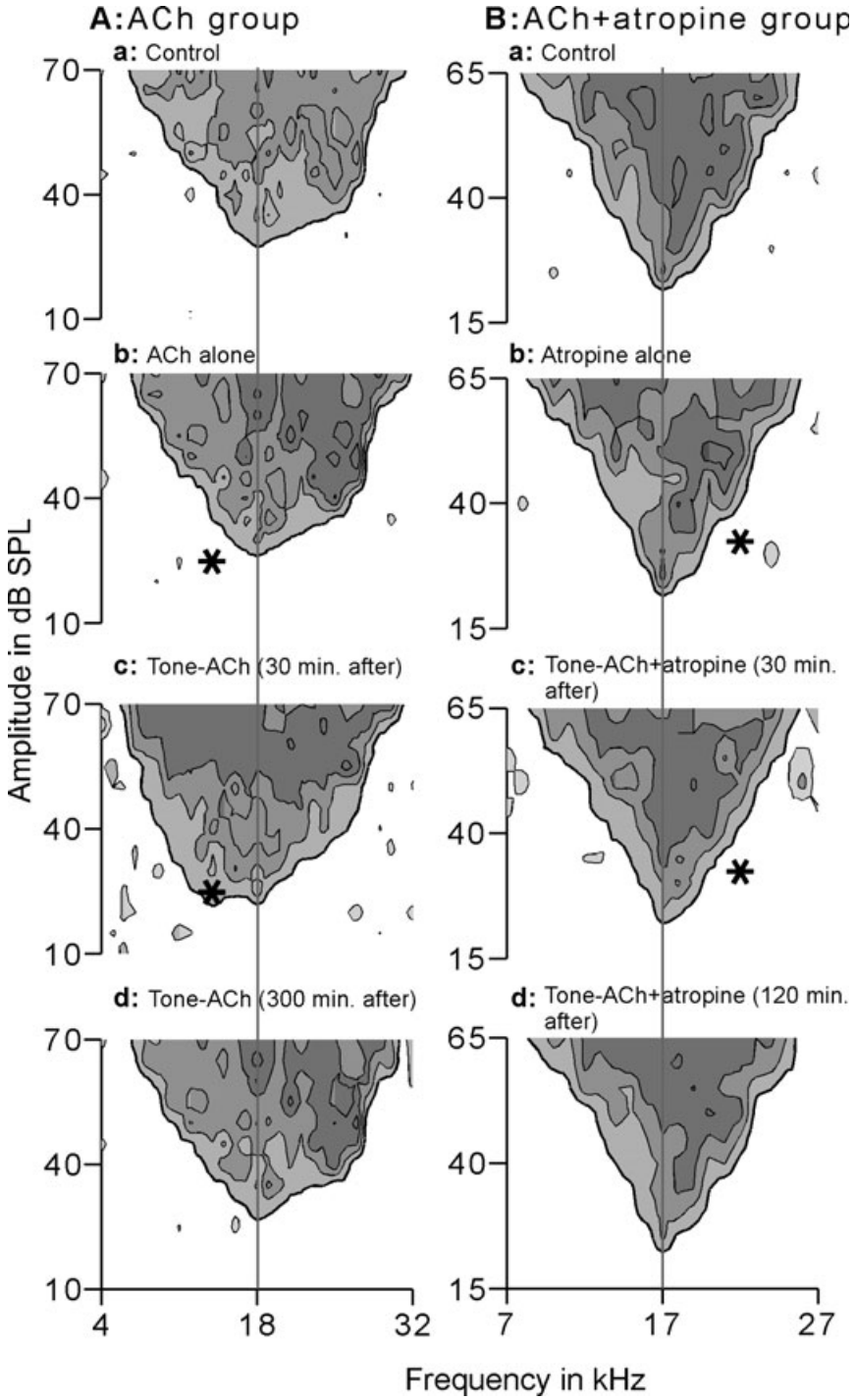

FIG. 5. Effects of acetylcholine (A) and acetylcholine/atropine (B) microiontophoresis paired with a tone on the excitatory frequency response areas of single cortical neurons. The acetylcholine and acetylcholine/atropine microiontophoresis paired with a tone largely decreased the response threshold in response to the PT frequency (Ac). No threshold decrease was observed when acetylcholine was microiontophoretically injected with atropine (Bc). Contour lines are drawn at 20, 40, 60 and 80% of the maximum spike number. The 20% contour line delineates the major excitatory area. The solid lines indicate the control BF and the asterisks indicate the PT frequency and amplitude.

(10.00 ± 5.31, $P > 0.05$). Similarly, microiontophoresis of ACh to cortical neurons significantly increased ASN from 11.90 ± 6.64–20.91 ± 8.81 ($P < 0.001$). Tone–ACh further increased the ASN to 22.97 ± 9.59 ($P < 0.001$). However, tone paired with microiontophoresis of ACh/atropine did not significantly change the cortical ASN (13.35 ± 5.35, $P > 0.05$). Apparently, either external or internal ACh increased the excitability of cortical neurons.

We specifically measured the response latency of given neurons to the frequency of the paired tone at the control threshold level. The average response latencies were 33.27 ± 9.09 ms before and 22.23 ± 9.56 ms after the tone–ES_{NB}, which were statistically significant ($P < 0.001$). Similar results were also obtained for tone–ACh; the average latency was significantly shortened from 27.08 ± 7.21 ms to 22.36 ± 9.48 ms ($P < 0.001$). The changes in response latency to

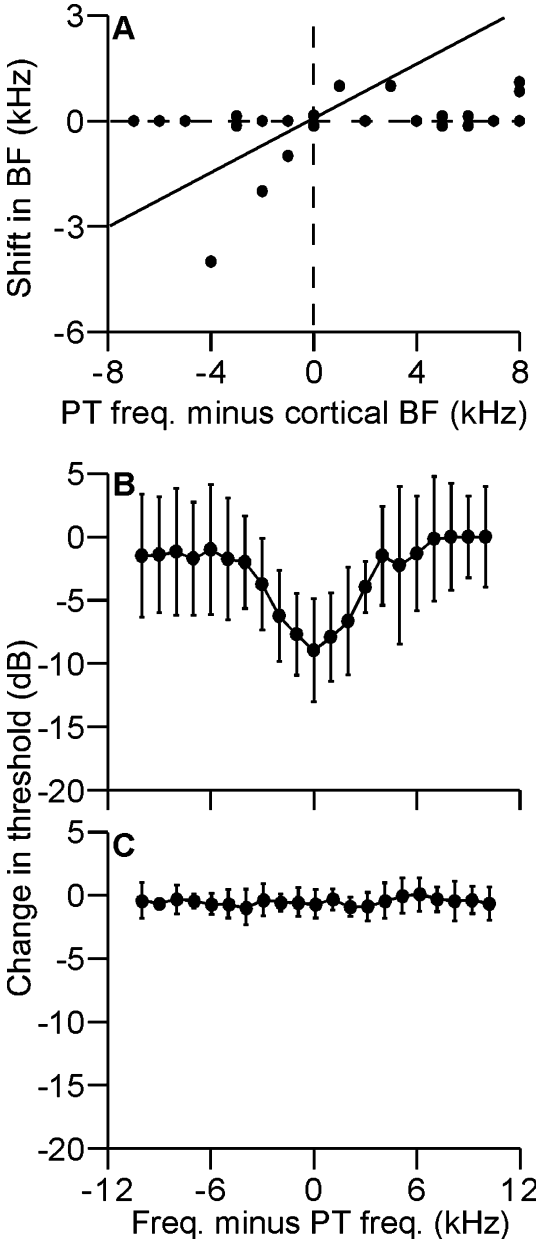

FIG. 6. The effect of acetylcholine microiontophoresis paired with a tone on the shift of cortical BFs and the threshold changes across frequencies. Most cortical neurons showed no BF shift following treatment (A). In B and C, the difference in frequency/threshold tuning curves of all samples before and after treatment was averaged and aligned at the PT frequency ($n = 24$ for B and $n = 21$ for C). This clearly shows that acetylcholine microiontophoresis paired with a tone induced large threshold decreases centred on the PT frequency from approximately 4 kHz below to 4 kHz above the PT frequency (B). This effect was not observed in the presence of atropine (C). The solid line in A represents the regression line as illustrated in previous Fig. 2A.

the frequency of paired tones were not significantly altered when atropine was administrated with tone–ES_{NB} (33.27 ± 9.09 ms vs. 34.02 ± 6.71 ms, $P > 0.05$) or with tone–ACh (31.90 ± 7.68 ms vs. 32.51 ± 7.91 ms, $P > 0.05$).

Discussion

Learning-induced or experience-dependent auditory plasticity can cause frequency-specific changes, i.e. the frequency tunings or receptive fields of auditory neurons shift to the frequency of the learned sound (Bakin & Weinberger, 1990; Recanzone *et al*., 1993; Gao & Suga, 2000). Efforts have been to identify the neural circuitry responsible for frequency-specific changes in the auditory cortex. Studies over the past 20 years have identified the NB as an essential neural substrate for the promotion of the cortical plasticity induced by auditory learning or experience (Kilgard, 2003; Suga & Ma, 2003; Weinberger, 2003; Yan, 2003). Recent studies involving lesions of the cochlea however, suggest that the NB is not essential (Kamke *et al*., 2005).

Immunochemical studies reveal that approximately one-third of NB neurons that project to the cerebral cortex are cholinergic neurons and approximately two-thirds are non-cholinergic neurons. Most non-cholinergic neurons are GABAergic and some are glutamatergic (Manns *et al*., 2001; Sarter & Bruno, 2002). The contribution of non-cholinergic NB projections to cortical plasticity remains largely unknown. Interestingly, the axons of NB GABAergic neurons predominantly synapse with GABAergic interneurons in the cerebral cortex (Freund & Meskenaite, 1992; Sarter & Bruno, 2002). This suggests that the NB non-cholinergic projections mostly mediate the disinhibition of cortical principal neurons. Such disinhibition may be involved in plasticity of cortical principal neurons by increasing their excitability (Dykes, 1997). On the other hand, cortical cholinergic inputs from the NB have repeatedly been shown to be essential for cortical plasticity. The NB itself does not carry any auditory information. Thalamocortical circuitry however, possesses an intrinsic mechanism underlying the frequency-specificity of the auditory plasticity has been recently proposed (Metherate *et al*., 2005; Jafari *et al*., 2007). Therefore, we speculate that the frequency-specific auditory plasticity induced by auditory learning or experience may depend on the incorporation of NB cholinergic projections with thalamocortical circuitry that consists of thalamocortical, cortical lateral and corticofugal projections (Yan & Zhang, 2005).

Cholinergic facilitation of specific thalamocortical circuitry

As the thalamus gates all sensory information to the cortex, thalamocortical inputs may be of considerable importance to the development of frequency-specific plasticity in the auditory cortex. Significantly, an *in vivo* study using extracellular and intracellular recordings demonstrates that NB activation enhances auditory thalamocortical synaptic transmission evoked by the electrical stimulation of the auditory thalamus (Metherate & Ashe, 1993). In this study, cortical acetylcholine was increased by either microiontophoretic injection or electrical stimulation of the NB. The increased cortical acetylcholine, whether from these external or internal sources, induced remarkable decreases in cortical response thresholds only when paired with a tone. The threshold decrease was highly specific to the PT frequency no matter whether the recorded cortical neurons tuned to the PT frequency or the non-PT frequencies (Figs 1A, 4A, 5A and 6B). Why was the threshold decrease highly specific to the PT frequency for both PT and non-PT neurons? A logical explanation is that cortical acetylcholine not only facilitates the responses or increases the excitability of postsynaptic cortical neurons but it may also facilitate specific presynaptic strength that is activated by the paired tone. This is supported by our findings that the F_{LTD} evoked by either tone–ES_{NB} or tone–ACh was highly correlated to the frequency of the paired tone (Fig. 4A and 6B) and that the latency of a given neuron was significantly shortened in response to the PT frequency at the threshold level.

It is known that thalamocortical projections are tonotopically organized (Velenovsky *et al*., 2003) and single neurons in the auditory cortex can receive inputs from a large number of thalamic neurons (Andersen *et al*., 1980; de Venecia & McMullen, 1994; Huang & Winer, 2000). Consequently, it is possible that the activation of a single thalamic neuron potentially influences thalamocortical synaptic inputs to a number of cortical neurons including both PT and non-PT neurons. Unlike previous studies on cortical plasticity evoked by tone–ES_{NB} (Bakin & Weinberger, 1996; Kilgard & Merzenich, 1998; Ma & Suga, 2003; Yan & Zhang, 2005), this study used a tone with considerably lower amplitude; the PT amplitude of 10 dB below the response threshold meant that the activated thalamic neurons tuned to a very narrow frequency range. Therefore, the acetylcholine-facilitated thalamocortical synapses should be those tuned to a narrow frequency range, approximately 8 kHz according to the PT-frequency-aligned average of the threshold decrease across frequencies (Fig. 6B).

Importance of cortical muscarinic ACh receptors

Acetylcholine acts on two families of receptors, muscarinic and nicotinic acetylcholine receptors. It has been shown in the hippocampus that nicotinic acetylcholine receptors enhance glutamatergic synaptic transmission and facilitate long-term changes in synaptic transmission (Radcliffe & Dani, 1998; Vogt & Regehr, 2001; Ge & Dani, 2005). These findings suggest that nicotinic receptors are potentially involved in receptive field plasticity in the auditory cortex. However, a study involving visual cortical neurons shows that neonatal ocular dominance plasticity can be prevented by the muscarinic receptor antagonist but not by the nicotinic receptor antagonist (Gu & Singer, 1993). The role of nicotinic receptors in cortical receptive field plasticity clearly requires further clarification. In contrast, many studies show that the frequency-specific cortical plasticity evoked by auditory learning or tone–ES_{NB} can almost be abolished by muscarinic receptor antagonists such as atropine and scopolamine in animal and human subjects (Bakin & Weinberger, 1996; Miasnikov *et al*., 2001; Thiel *et al*., 2002; Ma & Suga, 2003; Yan & Zhang, 2005). Similar effects are also seen in other sensory cortices (Boroojerdi *et al*., 2001; Verdier & Dykes, 2001).

This study demonstrated that atropine microiontophoresis to the recorded neurons eliminated the frequency-specific threshold decrease of cortical RFs evoked by either acetylcholine microiontophoresis or NB activation paired with a tone (Figs 1B, 5B and 6C). Among the five well-identified muscarinic subtypes (M_{1-5} receptors), the M_1 receptor is the most abundant in the cerebral cortex (Levey, 1993). It has been shown that acetylcholine-facilitated cortical responses to tone stimulation can be eliminated by the M_1 receptor antagonist, pirenzepine, but not by the M_2 receptor antagonist, galamine (Metherate *et al*., 1990). Our recent study in M_1 receptor knockout mice concludes that the M_1 receptor is the most important subtype of muscarinic receptor for the mediation of cortical plasticity evoked by tone–ES_{NB} (Zhang *et al*., 2006). Therefore, cholinergic effects on learning-induced or experience-dependent cortical plasticity are mostly mediated by muscarinic acetylcholine receptors, particularly M_1 receptors.

Studies with brain slices also support this conclusion. The postsynaptic potentiation mediated by the glutamate *N*-methyl-D-aspartate (NMDA) receptor is long lasting and also important for synaptic plasticity. What is interesting here is that acetylcholine significantly enhances NMDA-mediated synaptic transmission through its action on muscarinic receptors, particularly the M_1 receptor (Metherate *et al*., 1990; Aramakis *et al*., 1997, 1999). This indicates that cholinergic facilitation of synaptic strength requires glutamate release, i.e. tone-evoked thalamocortical activities. Consequently, cortical acetylcholine may merely enhance synaptic strength such as these thalamocortical synapses that are activated by the presented tone during auditory learning and experience. Our finding of the shortened response latency and PT-specific response threshold decrease of cortical neurons, in conjunction with the role of M_1 receptors in NMDA-mediated synaptic transmission, confirm that activity-dependent cholinergic facilitation of thalamocortical inputs is fundamental to frequency-specific RF plasticity.

Indication of the involvement of cortical lateral and corticofugal projections in cortical plasticity

It is well known that global application of atropine to the auditory cortex prevents the global RF shift of cortical neurons evoked by auditory learning or tone–ES_{NB} (Bakin & Weinberger, 1996; Ji & Suga, 2003; Yan & Zhang, 2005). A very important observation in this study is that atropine microiontophoresis to the recorded neurons does not prevent the global RF shift towards PT frequency following tone–ES_{NB} even though the shifting range is smaller (Fig. 2). Unlike global application of atropine to the auditory cortex, atropine microiontophoresis blocks the muscarinic receptors of only a few neurons around the recording electrode. It does not impact the muscarinic receptors of other cortical neurons, including those of PT neurons. In other words, atropine microiontophoresis merely prevents the synaptic strengthening of corresponding thalamocortical and/or intracortical inputs to the recorded neurons during tone–ES_{NB}. Considering the frequency-specific cortical plasticity evoked by focal cortical stimulation and thalamic stimulation (Maldonado & Gerstein, 1996; Jafari *et al*., 2007), our data indirectly suggest that the global RF shifts of the recorded cortical neurons evoked by tone–ES_{NB} may result from the facilitation of PT thalamocortical inputs to cortical PT neurons.

What is the neural circuitry that is responsible for the global RF shift of cortical neurons? In addition to thalamocortical projections, anatomical studies reveal that there are innumerable lateral connections between isofrequency bands within the auditory cortex (Wallace *et al*., 1991; Ojima & Takayanagi, 2004). Cortical neurons also send large numbers of tonotopically organized projections to subcortical neurons (Huffman & Henson, 1990; Saldana *et al*., 1996; Winer *et al*., 1998). Physiological studies reveal that focal stimulation of cortical neurons leads to the shift of cortical and subcortical RFs towards the best frequency of the stimulated cortical neurons (Maldonado & Gerstein, 1996; Yan & Suga, 1998; Chowdhury & Suga, 2000; Sakai & Suga, 2002; Yan & Ehret, 2002; Yan *et al*., 2005). A study examining brain slices taken from the auditory cortex shows that activation of muscarinic receptors enhances thalamocortical postsynaptic potentials but suppresses intracortical postsynaptic potentials (Hsieh *et al*., 2000). In other cortical areas, ACh is known to amplify thalamocortical inputs while suppressing associational inputs (Hasselmo & Bower, 1992; Kimura *et al*., 1999). Together with the fact that basal forebrain GABAergic neurons innervate cortical GABAergic interneurons (Freund & Meskenaite, 1992; Sarter & Bruno, 2002), intracortical lateral innervation (e.g. disinhibition) should be involved in the frequency-specific changes in both PT and non-PT neurons induced by tone–ES_{NB} and tone–ACh. It also appears to be involved in strengthening the thalamocortical synapses via glutamatergic mechanisms.

All of these findings allow us to propose that the tonotopic loops of thalamocortical projections and corticofugal projections, incorporated

with cortical lateral projections, form a core circuitry for the frequency-specific RF shift of cortical neurons (Yan, 2003; Yan & Zhang, 2005). Briefly, tone–ES_{NB} facilitates the synaptic transmission of PT thalamocortical inputs to cortical PT neurons. The facilitation of cortical PT neurons subsequently shifts the global RFs of cortical non-PT neurons through cortical lateral projections and corticofugal projections. Therefore, the learning-induced or experience-dependent shifts in cortical RFs are a culmination of cholinergic facilitation of PT thalamocortical inputs to given non-PT and PT neurons. The latter further shifts the given cortical non-PT RFs through cortical lateral projections and cortico-subcortical tonotopic loops. The activities of cortical lateral projections are further balanced by cholinergic suppression of intracortical synaptic transmission and basal forebrain GABAergic disinhibition.

In addition to the effects of atropine microiontophoresis on the plasticity of cortical non-PT neurons evoked by tone–ES_{NB} (Figs 1A, 2A, 2B and 4B), we also show that acetylcholine microiontophoresis to non-PT neurons paired with a tone only induced a frequency-specific threshold decrease but not a global RF shift (Fig. 5A and 6A). Our data suggest that the frequency-specific threshold decrease of non-PT cortical neurons is determined by the facilitation of PT thalamocortical inputs to non-PT cortical neurons. However, global RF shifts of cortical non-PT neurons may result from the cholinergic facilitation of the PT thalamocortical inputs to cortical PT neurons, i.e. subsequent cortical lateral modulation and corticofugal modulation.

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Abbreviations

ACh, acetylcholine; ASN, average spike number of neuronal receptive field; BF, best frequency; BF_{CON}, control BF; BF_{NEW}, new BF; EEG, electroencephalogram; ES_{NB}, electrical stimulation of the NB; F_{LTD}, largest threshold decrease; MT, minimum threshold; NB, nucleus basalis of the basal forebrain; PT, paired tone; RF, neuronal receptive field; tone–ACh, microiontophoresis of acetylcholine to recorded neurons paired with a tone; tone–ACh/atropine, microiontophoresis of acetylcholine and atropine to recorded neurons paired with a tone; tone–ES_{NB}, ES_{NB} paired with a tone.

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