Development of Reorganization of the Auditory Cortex Caused by Fear Conditioning: Effect of Atropine

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Ji, Weiqing and Nobuo Suga. Development of reorganization of the auditory cortex caused by fear conditioning: effect of atropine. J Neurophysiol 90: 1904–1909, 2003; 10.1152/jn.00363.2003. Reorganization of the frequency map in the central auditory system is based on shifts in the best frequencies (BFs; hereafter, BF shifts), together with the frequency-response curves, of auditory neurons. In the big brown bat, conditioning with acoustic stimulation followed by electric leg-stimulation causes BF shifts of collicular and cortical neurons. The collicular BF shift develops quickly and is short term, whereas the cortical BF shift develops slowly and is long term. The acetylcholine level in the auditory cortex must be high during conditioning to develop these BF shifts. We studied the effect of atropine (an antagonist of muscarinic acetylcholine receptors) applied to the auditory cortex on the development of the long-term cortical BF shift in the awake bat caused by a 30-min conditioning session. We found 1) the cortical BF shift starts to develop ~15 min after the onset of the conditioning, gradually increases over 60 min, and reaches a plateau, 2) the cortical BF shift changes from short to long term ~45 min after the onset of the conditioning, 3) the cortical BF shift can plateau at different frequencies between the BF of a given neuron in the control condition and the frequency of the conditioning tone, 4) the maximum BF shift is determined ~70 min after the onset of the conditioning, and 5) acetylcholine plays an important role in the development of the cortical BF shift. Its role ends ~180 min after the onset of the conditioning.

INTRODUCTION

Repetitive acoustic stimulation delivered to the big brown bat (Eptesicus fuscus), without any electrical stimulation, evokes small short-term collicular (Gao and Suga 1998; Yan and Suga 1998) and cortical changes (Chowdhury and Suga 2000) in auditory response, frequency-response curve, and best frequency (BF). In the big brown bat, conditioning with repetitive acoustic stimulation followed by electric leg-stimulation causes large short-term collicular changes that recover within 180 min after the conditioning and large long-term cortical changes that last >26 h. The long-term cortical change evoked by auditory fear conditioning has also been demonstrated in cats (Diamond and Weinberger 1986, 1989) and guinea pigs (Weinberger et al. 1993). (According to our definition, the BF shift that recovers within 3.5 h after the end of a conditioning session is called short term, whereas the BF shift showing <10% recovery at 3.0 h is called long term. The BF shift showing >10% recovery at 3.0 h is called intermediate term.) In the big brown bat, inactivation the somatosensory cortex stops the development of collicular and cortical changes to be caused by the conditioning (Gao and Suga 1998, 2000), whereas electric stimulation of the somatosensory cortex immediately after tone-burst stimulation augments the collicular and cortical changes (Ma and Suga 2001). Inactivation of the auditory cortex during the conditioning stops the development of the collicular changes to be caused by the conditioning (Gao and Suga 1998). Electric stimulation of the auditory cortex evokes the collicular changes that are identical to those evoked by the conditioning (Ma and Suga 2001, 2003; Yan and Suga 1998). Therefore for the changes (plasticity) caused by the conditioning, the auditory cortex, auditory corticofugal system, and somatosensory cortex are necessary (Gao and Suga 1998, 2000). In addition, the amygdala (for review LeDoux 2000; Weinberger 1998) and the cholinergic basal forebrain play an important role in cortical plasticity (Bakin and Weinberger 1996; Bjordahl et al. 1998; Kilgard and Merzenich 1998; for review, Rasmussen 2000; Weinberger 1998).

In the auditory cortex of the squirrel monkey (Foote et al. 1975), guinea pig (Metherate et al. 1990) and big brown bat (Ji et al. 2001), acetylcholine (ACH) applied to cortical neurons augments their responses to acoustic stimuli. In the auditory cortex of the cat, an iontophoretic application of ACH to cortical neurons paired with tone bursts reduces their responses at the frequency of the tone bursts but increases their responses at other frequencies. Such effects of ACH are antagonized by atropine iontophoretically applied to these cortical neurons (McKenna et al. 1989; Metherate and Weinberger 1989, 1990). In the big brown bat, ACH applied to the auditory cortex 5 min before conditioning augments the undetectably small cortical BF shift caused by a 15-min conditioning session and evokes the long-term cortical BF shift. It also augments the undetectably small cortical BF shift and evokes the short-term collicular BF shift (Ji et al. 2001). A 30-min conditioning session evokes the long-term cortical BF shift and the short-term collicular BF shift (Gao and Suga 2000). Atropine applied to the auditory cortex 5 min prior to a 30-min conditioning session blocks the development of the long-term cortical BF shift caused by the conditioning and reduces the short-term collicular BF shift. Atropine applied to the auditory cortex 3 h after the conditioning has no effect on the collicular and cortical BF shifts caused by it (Ji et al. 2001). The aim of our current paper is to report that the effect of atropine on the cortical BF shift is not all-or-none but graded as a function of...
time interval between the conditioning and an atropine application. The time course of the development of the cortical BF shift (i.e., reorganization of a frequency map) caused by conditioning has not been well studied in any species except the big brown bat. Our current data give us an important insight into the development of long-term cortical reorganization caused by conditioning.

METHODS

Experiments were performed with 17 adult big brown bats. Procedures for animal preparation, acoustic stimulation, electric leg-stimulation, recording of action potentials, and drug applications were the same as those previously described (Ji et al. 2001). Therefore our methods are briefly described. The protocol for this research was approved by the animal studies committee of Washington University.

Preparation

Under neuroleptanalgesia (Innovar), a metal post was glued to the dorsal surface of the bat’s skull. Experiments for recording auditory responses from single cortical neurons, conditioning animals and drug applications began 3–4 days after the surgery. The awake bat was placed in a polyethylene-foam body-mold suspended at the center of a soundproof room maintained at a temperature of 31°C. The bat’s head was immobilized by fixing the metal post glued on the skull onto a metal rod with set screws, and it was adjusted to face directly at a loudspeaker located 74 cm away. The bat was neither anesthetized nor tranquilized during experiments.

Acoustic and electric stimuli

Acoustic stimuli (20-ms pure tone bursts with a 0.5-ms rise-decay time) were delivered to the bat at a rate of 5/s. Their frequency and amplitude were manually varied or computer-controlled to measure the BF and minimum response threshold of a given neuron. The computer-controlled frequency scan consisted of 34 150-ms time blocks. In the frequency scan, the frequency of the tone burst was shifted every 150 ms in 0.2-, 0.5-, or 1.0-kHz step. The amplitude of the tone bursts in the scan was set at 10 dB above the minimum threshold (i.e., threshold at the BF) of the neuron, so as to easily detect a BF shift.

To evoke the BF shift of a cortical neuron, the bat was conditioned with a 1.0-s train of acoustic stimuli (conditioning stimulus), followed by a 1.0-s gap and then by an electric leg stimulus (unconditioned stimulus). In the acoustic stimulus, 50 dB SPL and 10-ms tone bursts were delivered at a rate of 33/s. Their frequencies were set 5.0 kHz lower than the BF of a given cortical neuron. In the big brown bat, auditory fear conditioning evokes the largest BF shift of cortical and collicular neurons when the acoustic stimuli is ~5.0 kHz lower than their BFs (Gao and Suga 1998, 2000). Electric leg-stimulus was a 50-ms monophasic electric pulse. That was 0.15–0.57 m/s, just above the threshold for eliciting a leg-flexion. A pair of conditioning and unconditioned stimuli was delivered every 30 s for 30 min (60 times in total). To minimize a cumulative effect of conditioning, only one neuron was studied in a 1-day experiment, and the same animal was used with a 1- to 3-day interval. In each 1-day experiment, tone bursts alone were delivered at a rate of 5/s over 2–3 h to record single-unit activity and to obtain data in the control condition. This period presumably caused extinction of BF shifts, if any, remaining after a previous conditioning experiment.

Drug application

To investigate the role of ACh in the development of BF shifts caused by the 30-min conditioning session, atropine was applied to the auditory cortex before or after the conditioning. Multiple holes of ~50 μm in diameter were made in the skull, and the auditory cortex was first electrophysiology mapped to locate its approximate center. A hole at the center was enlarged to ~1.0 mm in diameter prior to single unit recording and an atropine application. Then, 0.5 μl of 0.4 M atropine sulfate solution (pH 5; dissolved in 0.9% saline, Sigma Chemical) was applied to the exposed surface of the auditory cortex with a 1.0 μl Hamilton syringe. Ji et al. (2001) had shown that such an atropine effect develops within 5 min after the application, reaches a peak ~15 min after, and then disappears ~45 min after and that, at the peak, cortical auditory responses are reduced by ~10%. They also had shown that 0.9% saline solution applied to the auditory cortex instead of an atropine solution has no effect on cortical responses to tone bursts and the cortical BF shift evoked by the conditioning. Therefore we did not repeat such a sham experiment in our current studies.

Data acquisition and processing

Through the hole, a sharpened, vinyl-coated tungsten-wire recording microelectrode with a tip diameter of ~7 μm was inserted orthogonally into the auditory cortex. An indifferent tungsten-wire electrode was placed on the dura matter near the recording electrode. The responses of single cortical neurons to tone bursts were recorded at 200–700 μm depths (layers III–V) in the auditory cortex. A BAK time-amplitude window discriminator was used to select action potentials from a single neuron. Action potentials discharged by the neuron were continuously compared with the template on the screen of the digital storage oscilloscope during data acquisition: before, during and after the conditioning and/or drug application. An array of peristimulus time (PST) histograms was acquired every 15 min at least over 240 min after the onset of the conditioning, as long as action potentials visually matched the template. The array of PST histograms were plotted to display the responses of a single cortical neuron to 50 identical frequency scans. The frequency-response curve based on these responses was also plotted. The BF of the neuron was defined as the frequency at which the frequency-response curve was peaked. To study the development of a BF shift, BFs determined from the frequency-response curves obtained every 15 min were plotted as a function of time. The time courses of BF shifts obtained from several neurons were averaged. Therefore each data point in an averaged time-course curve represents the mean and standard error based on 50 responses multiplied by a number of neurons (N) used for averaging.

RESULTS

A 30-min conditioning session evoked the BF shifts of 95 cortical neurons out of 121 recorded. These 95 cortical neurons were studied to examine the effect of atropine applied to the auditory cortex on the cortical BF shifts. Their BFs in the control condition ranged between 19 and 55 kHz (mean ± SE: 28.5 ± 3.5 kHz). Because the conditioning tone was set at the frequency 5.0 kHz lower than the BF of a given cortical neuron, the BF always decreased toward the frequency of the conditioning tone. In the remaining 26 neurons, auditory responses to tone bursts were not stable and data acquisition was incomplete because of animals’ frequent movements.

Figure 1A shows the arrays of PST histograms displaying the responses of a single cortical neuron to tone bursts at different frequencies ranging from 24.0 to 34.0 kHz. The neuron was tuned to 30.0 kHz. That is, its BF for excitation was 30.0 kHz (Fig. 1A1, ○). The conditioning tone was 25.0 kHz. The BF shifted from 30.0 kHz to 29.0 kHz 35 min after the onset of the conditioning (Fig. 1A2). It further shifted to 28.0 kHz 60 min after the onset (Fig. 1A3, ○). When atropine was applied to the
auditory cortex 70 min after the onset, the responses of the neuron to all tone burst stimuli became smaller and its BF shifted back to 30.0 kHz (Fig. 1A4). However, 105 min after the onset, the responses became stronger and the BF shift redeveloped (Fig. 1A5). The BF eventually became 28.0 kHz 165 min after the onset (Fig. 1A6). No further changes in the response and BF occurred even 240 min after the onset. The changes in the responses to 30.0 kHz (the BF in the control condition) and 28.0 kHz (the BF in the maximally shifted condition) are further shown by the PST histograms plotted on the expanded time axis (Fig. 1B).

The effect of atropine on a BF shift was the most drastic when it was applied to the auditory cortex immediately prior to the conditioning. It completely abolished the development of shifts in the frequency-tuning curve and BF of cortical neurons, as previously reported by Ji et al. (2001). In Fig. 2, a cortical neuron tuned to 27.0 kHz. The conditioning tone was 22.0 kHz. It was expected that its BF at 27.0 kHz shifted to ∼25.0 kHz 70 min after the onset of the conditioning and that the BF shift was maintained at least over 3 h. However, atropine applied 5 min prior to the conditioning caused no shifts in frequency-response curve and BF. It reduced the auditory responses of the neuron over ∼60 min after the atropine application (Fig. 2A, 2 and 3). The reduction of the response at the BF was 34% in A2 and 5% in A3. The effect of atropine in terms of the reduction of auditory responses completely disappeared at 85 min after the application. The frequency response curve of the neuron was acquired ≥210 min after the conditioning. There was no sign of the development of a BF shift (Fig. 2A, 4–6).

Without atropine, the cortical BF shift started to develop 15 min

FIG. 1. Effect of atropine on the development of the shifts of the frequency-response curve and best frequency (BF) of a single cortical neuron caused by a 30-min conditioning session. An atropine application to the auditory cortex 70 min after the onset of the conditioning reduced the auditory responses and the shifts of the curve and BF. In A, each array of peri-stimulus time (PST) histograms displays the responses of the neuron to 50 identical frequency scans, in each of which frequency was shifted from 24.0 to 34.0 kHz by 1.0-kHz steps. The arrays of PST histograms in 1–6 were obtained at −5, 35, 60, 75, 105, and 165 min after the onset of the conditioning. Each rectangle along the frequency axis indicates a 20-ms tone burst delivered at the beginning of a 150-ms time block. The BF of the neuron in the control conditioning (●) was 30.0 kHz. It shifted from 30.0 to 28.0 kHz 60 min after the onset (3) but was temporarily shifted back to 30.0 kHz by atropine (4). It redeveloped back to 28.0 kHz 165 min after the onset (6). ○, the BF in the maximal shifted condition. In B, the responses at 28.0 and 30.0 kHz are shown on the expanded time axis. The conditioning tone was 25.0 kHz (↓ in A2).

FIG. 2. Effect of atropine applied to the auditory cortex 5 min prior to conditioning. Atropine blocked the development of the changes in the frequency-response curve and BF of a single neuron to be caused by a 30-min conditioning session. It reduced the auditory responses of the neuron. In A, each array of PST histograms displays the responses of the neuron to 50 identical frequency scans, in each of which frequency was shifted from 21.0 to 31.0 kHz by 1.0-kHz steps. The arrays of PST histograms 1–6 were obtained at −5, 35, 55, 70, 85, and 115 min after the onset of the conditioning. The BF of the neuron was 27.0 kHz (●). Each rectangle along the frequency axis indicates a 20-ms tone burst delivered at the beginning of a 150-ms time block. The conditioning tone was 22.0 kHz (↓ in A2), which, without atropine, could effectively evoke changes in the frequency-response curve and BF of the neuron. In B, the responses at 27.0 kHz are shown on the expanded time axis.
min after the onset of the conditioning, developed to 53% of the maximum at the end of the conditioning, and slowly reached a plateau ~70 min after the onset. That is, it slowly developed even after the end of the conditioning with the stored information of the frequency of the conditioning tone. The plateau lasted ~3.0 h without showing any sign of recovery (Fig. 3A, curve 1). The BF shift was long term. The effect of atropine on the cortical BF shift was different according to the relation in time between an atropine application and the conditioning. Atropine applied to the auditory cortex 5 min prior to the conditioning abolished the development of the cortical BF shift caused by the conditioning (Fig. 3A, curve 2). Atropine applied to the auditory cortex 35 min after the onset of the conditioning reduced the BF shift, developed to 56% of the maximum BF shift, and made it short term (Fig. 3A, curve 3). Atropine applied to the auditory cortex 55 min after the onset also reduced the BF shift, developed to 66% of the maximum, but the BF shift became long term. The plateaued BF shift was 39% smaller than the maximum BF shift (Fig. 3A, curve 4). That is, the cortical BF shift changed from short to long term ~45 min after the onset of the conditioning. For atropine applied to the auditory cortex 70, 85, or 115 min after the onset of the conditioning, the BF shift, which had developed to the maximum, was reduced by 68–79%, but it redeveloped back to the maximum and plateaued. These observations indicate that the maximum BF shift is determined ~70 min after the onset of the conditioning, so that even the BF shift reduced by atropine redeveloped back to the maximum. The time from an atropine application to the redevelopment back to the plateau was 80 min (Fig. 3A, curves 5–7). Atropine applied to the auditory cortex 160 min after the onset had a very small short lasting effect on the BF shift (Fig. 3A, curve 8). Atropine applied to the auditory cortex 205 min after the onset had no effect at all on the BF shift (Fig. 3A, curve 9). ACh apparently plays an important role in the development of the cortical BF shift. Its role ends ~180 min after the onset of the conditioning.

The time course of the redevelopment of the BF shift to the maximum BF shift after an atropine application was very similar to curves 5–7 (Fig. 3B). Furthermore, the time course of the recovery of the BF shift to the control BF after an atropine application (Fig. 3B, curve 3i) was also similar to that of the above three curves (P = 0.45, ANOVA). In other words, the BF shift for recovery was opposite in direction to that for redevelopment, but it was similar to the latter in time course.

**DISCUSSION**

In the following, we will discuss how our present findings might be related to the collicular BF shift and to the cellular mechanisms for learning and memory.

**Contributions of the collicular BF shift to the cortical BF shift**

The cortical BF shift at the plateau could be reduced by atropine applied to the auditory cortex between 70 and 160 min after the onset of a 30-min conditioning session, but it redeveloped to the plateau. The collicular BF shift evoked by a 30-min conditioning reaches a peak 30 min after the onset of the conditioning (i.e., at the end of the conditioning) and then recovers 180 min after the conditioning (Fig. 4, curve b) (Gao and Suga 1998, 2000; Ji et al. 2001). Atropine applied to the auditory cortex 5 min prior to the 30-min conditioning session completely abolishes the cortical BF shift, and reduces the collicular BF shift by ~26%. Excitatory synaptic transmitter for auditory signal processing is glutamate and/or aspartate (Dori et al. 1992; Fonnum et al. 1981; Karlsen and Fonnum 1978; Nieoullon and Dusticier 1983; Tsumoto 1990), so that the corticofugal feedback is not disrupted by atropine applied to the auditory cortex. Atropine applied to the auditory cortex 70 min after the onset of the conditioning (i.e., during the recovery phase of the collicular BF shift) has no effect on the collicular BF shift (Ji et al. 2001). Because the collicular BF shift can be evoked without the cortical BF shift, the redevelopment of the cortical BF shift after the reduction caused by atropine may depend on the short-term collicular BF shift.
However, as discussed in the following text, an atropine application to the inferior colliculus indicates that this possibility is low.

Atropine applied to the inferior colliculus 70 min after the onset of the conditioning reduces the collicular BF shift and shortens its recovery time so that the collicular BF shift becomes zero 100 min after the onset of the conditioning instead of 180 min after that (Fig. 4, curve d). However, it does not affect the cortical BF shift at all (Fig. 4, curve c; Ji et al. 2001). If the collicular BF shift in its recovery period boosted the cortical BF shift, such changes in the collicular BF shift evoked by atropine should change the cortical BF shift. However, this doesn’t occur. Atropine applied to the inferior colliculus 5 min prior to the conditioning completely abolishes the collicular BF shift to be developed by the conditioning but not the cortical BF shift. It reduces the cortical BF shift by 31% and makes it short term (Ji et al. 2001). Therefore the collicular BF shift is necessary for the development of the large long-term cortical BF shift caused by the conditioning, but it is unnecessary once the cortical BF shift augmented by ACh develops to the plateau. It is not yet known whether this small short-term cortical BF shift is due to cortical intrinsic plasticity and/or auditory thalamic plasticity. In other words, the redevelopment of the cortical BF shift is not due to the collicular BF shift in its recovery period, but due to the intrinsic cortical and/or thalamic plasticity.

**Long-term potentiation and cortical BF shift**

In the hippocampus, long-term potentiation (LTP) of postsynaptic potentials evoked by high-frequency electric stimulation of presynaptic neurons consists of two components: early (E-LTP) and late phases (L-LTP). E-LTP depends on phosphorylation of existing proteins and enhanced release of synaptic transmitters. It lasts 2–3 h, whereas L-LTP depends on protein synthesis for structural changes of synapses and lasts many hours (for review, Kandel 2001; Schafe et al. 2001). The new dendritic spines of hippocampal pyramidal neurons accompanied by LTP appear ~30 min after LTP initiation and grow to the normal size within 60 min (Engert and Bonhoeffer 1999). E-LTP and L-LTP are physiological changes that may be related to the mechanisms for short- and long-term memory, respectively (Schafe et al. 2001 for review).

To evoke LTP, N-methyl-D-aspartate (NMDA) receptors (1 of glutamate receptors) play an essential role. ACh receptors are synergic to NMDA receptors, so that ACh enhances NMDA-induced membrane depolarization (Aramakis et al. 1997, 1999; Aigner 1995 for review). In the hippocampus, an antagonist of the muscarinic ACh receptor blocks the induction of LTP (Hirotso et al. 1989). ACh reduces potassium conductance (McCormick and Prince 1985; Rouse et al. 2000), increases calcium release from intracellular stores via inositol 1,4,5-trisphosphate, and stimulates protein kinase C via diacylglycerol (Markram and Segal 1992) so that NMDA-dependent depolarization and NMDA receptor-dependent events are augmented. Protein kinases phosphorylate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (1 of glutamate receptors) and increase AMPA receptor density at the postsynaptic membrane. As a result, synaptic connectivity becomes stronger after induction of LTP and silent synapses become active. This is a mechanism of synaptic plasticity (for review, Gu 2002; Sheng and Kim 2002). It has been demonstrated that ACh plays an important role in evoking the long-term cortical (Bakin and Weinberger 1996; Ji et al. 2001; Kilgard and Merzenich 1998) and short-term collicular BF shifts (Ji et al. 2001). The preceding cellular and subcellular phenomena may be involved in the development of the cortical BF shift.

For the BF shift, the dendrites of a cortical neuron must receive an array of frequency-labeled inputs. The input determining the BF of the neuron must be gradually shifted along the array of the inputs by an array of frequency-labeled modulatory neurons, which may evoke LTP or LTD at the nerve endings terminating at the dendrites. LTP and LTD, respectively, strengthen and weaken synaptic connectivity, via the depolarization-dependent change in the density of AMPA receptors at the membrane postsynaptic to the modulatory neurons: an increase in the density for LTP and a decrease in the density for LTD. Strengthening and weakening may also be explained by the Hebbian rule and inhibitory neurons, respectively. It is also possible that translocation of the catalytic subunits of cAMP-dependent protein kinase A and mitogen-activated protein kinase to the cell nucleus and the protein synthesis may occur in the auditory cortex within ~50 min after the onset of the conditioning, and that a BF shift is stabilized not only by increasing connectivity of preexisting synapses, but also by forming new synapses.

Cellular and subcellular mechanisms for the plasticity of synaptic connectivity have been extensively studied. However, the mechanisms for a gradual shift of locations for synaptic strengthening and weakening occurring along the array of inputs of cortical neurons during and after the conditioning have not yet been explored. For the understanding of reorganization of the auditory cortex, we have to know the dynamics of neural circuits, in addition to the cellular and subcellular mechanisms.

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