MEMORY FORMATION AND RETRIEVAL IN AUDITORY CORTEX

CHEN XI

Ph. D

The Hong Kong

Polytechnic University

2011

The Hong Kong Polytechnic University

Department of Rehabilitation Sciences

Memory Formation and Retrieval in Auditory Cortex

Chen Xi

A thesis submitted in partial fulfillment of the requirements for the

Degree of Doctor of Philosophy

July 2011

CERTIFICATION OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____ (Signature of the Candidate)

Chen Xi (Name of the Candidate)

_____ (Date)

Abstract

One important aspect of memory is association of different modality components/inputs. Brain imaging and electrophysiological studies have shown that cortex of one modality can show responses to stimulus of another modality after intensive training. In the present study, we focused on the neocortex and explored how cross-modal associative memory was established, whether the established memory could be correlated with behavioral tasks and how the entorhinal cortex participated in the establishment and retrieval of this memory. We produced a conditioned association in rats between electrical stimulation of the auditory cortex and a visual stimulus that was followed by foot shock. The merits of using electrical stimulation include the fact that the site activated by electrical stimulation is more focused than that activated by a natural sound stimulus. Also the activated area in the auditory cortex, which reflects association with the visual stimulus, can be easily monitored by recording electrodes that attached to the stimulating electrode. In contrast to this, the cortical area that activated by the sound stimulus would likely have spread to a greater portion of the auditory cortex and beyond making monitoring difficult. The results showed the most direct evidence that auditory cortical neuron responded to the visual stimulus after the conditioning, and the responses to visual stimulus gradually reinforced when training trials increased. Control sites where no electrical stimulation was given showed no significant change, so the association was built specifically between visual stimulus and the sites in auditory cortex where electrical stimulation was given. To confirm the visuoauditory association behaviorally, we first paired the combined sound and light

stimulus with foot shock. After conditioning, the sound stimulus was then associated with water reward, and the establishment and recall of the association was proven when the rat successfully secured the reward after the sound stimulus was replaced by the visual stimulus for the first time. The control group who did not have such association could not acquire water reward. To examine activities in the auditory cortex during above behavioral paradigm, electrical stimulation of the auditory cortex was used instead of the sound stimulus in a separated group of subjects. Similarly, the association between visual stimulus and electrical stimulation of auditory cortex was first built. Then electrical stimulation was used as cue to train the subjects to gain water reward, and when electrical stimulation was replaced by visual stimulus, the subjects could attain water reward in the first trial. Recording near the stimulation site in auditory cortex showed that when visual stimulus was given, neural activities around stimulation site increased, and such activities extinguished if the training processed. After fully extinction, responses to visual stimulus regained while visual stimulus and electrical stimulation of auditory cortex were associated again followed by foot shock. To explore the role of entorhinal cortex, two electrode arrays were implanted into both sides of auditory cortex respectively and a cannula into one side of entorhinal cortex. Formation of new associative memory in the auditory cortex with classical conditioning was bilaterally abolished when the unilateral entorhinal cortex was temporally inactivated, but returned if the entorhinal cortex was not inactivated. Retrieval of the established associative memory in the ipsilateral neocortex was affected by the inactivation of the unilateral entorhinal cortex, while the contralateral cortex was not

affected, suggesting a less dependence of the hippocampal system in the retrieval than in the formation of associative memory.

This study presents most direct physiological evidences of the establishment of memory traces in the auditory cortex of behavioral rats, showing the association between the auditory and visual modalities. The results filled the gap of the process that neurons of one modality respond to other modality stimulus in highly trained monkeys. The straightforward physiological results from behavioral subjects confirmed these findings and provided four further steps concerning the cross-modal associative memory. First, associative memory can be established at single neuron level after 20 trials of conditioning. Second, neuronal responses reflect the recalling of the established memory. Third, the associative memory could be confirmed behaviorally, and only 10 trials of conditioning were needed. Finally, the extinction process lasts for hundreds trials of testing in several days and reestablishment of the extincted memory happens after a brief reconditioning. The establishment of the associative memory in the auditory cortex needed the involvement of the entorhinal cortex. It was interesting to note that a unilateral inactivation of the entorhinal cortex affected the establishment of the association bilaterally in the cortex. That was also confirmed behaviorally as the animal showed no context learning. This result may reflect the bilateral projection of the entorhinal cortical neurons to the hippocampus. However, the retrieval of the memory was only affected in the ipsilateral cortex to the inactivated entorhinal cortex, suggesting a less dependence of the hippocampal system in the retrieval than in the formation of associative memory. The present results imply that the hippocampal system is necessary for the

formation of associative memory in the auditory cortex from the beginning. Memory retrieval still needs the involvement of the hippocampal system at least at early stage within 10 days, though its dependence on the hippocampal system would be less than that of the formation of associative memory.

Acknowledgements

I would like to show my deepest gratitude to my supervisor, Prof. Jufang He, whose guidance, stimulating suggestions and encouragement from the first day of my study enabled me to accomplish this project.

It is also a pleasure to thank those who made this thesis possible, they are Dr. Yiping Guo, Dr. Xinjian Li, Ms. Zhengli Liao, Dr. Haitao Wang, Mr. Xiao Li, Ms. Chunhua Liu and other colleagues in the lab.

Especially, I would like to thank my parents and my wife whose love enabled me to achieve this work.

Table of Contents

Chapter 1 Introduction

We live in an environment full of all kinds of inputs. Therefore, the brain always receives information from more than one modality, and it is amazing that the brain can integrate information from different modalities to facilitate the outcome – behaviour reaction. For example, in a cocktail party there are a lot of people talking with each other and you are trying to understand what one of your friends is saying by watching the movement of his mouth. This cocktail phenomena well demonstrates that with the help of information from one sensory modality, the brain can perceive information from a different modality more accurately. One early study done by Sumby and Polack in 1954 showed that hearing threshold decreased if there was a visual input assisting[1]. Another example – the McGurk illusion showed how strongly the visual input could affect the auditory perception, as the movement of lips could dramatically change the voices that the subjects perceive[2]. Inspired by above and other cases, a lot of experiments have been done to study them and to see how powerful the multi-modal interaction could be. However, the key questions are where the cross-modal interaction happens in the brain and how such interaction forms.

Role of higher association cortices in cross-modal integration

Traditional views proposed that sensory cortices mainly focus on processing uni-modal information and these sensory cortices send axons to higher association cortices (prefrontal cortex, cortices along superior temporal sulcus, etc.), and association cortices integrate inputs from different modalities to facilitate

behavioural reaction. Numerous studies have been conducted in these areas, and both anatomical[3-5] and physiological data[6-10] support the role of higher association cortices in cross modal integration. In one of these studies, rats were trained to learn using light as the cue to gain a water reward at different locations and each location was associated with a specific odor[7]. The results revealed that neurons in the orbitofrontal cortex fired differentially to different locations or odors and activities of some neurons even reflected the association between the location and the odor, so orbitofrontal cortex could combine information from different modalities and even build association between them[7]. Another study used a sound-colour matching task to train monkey, where first a tone was presented and after a period of delay the subject needed to press a button of a specific colour based on the frequency of the previous tone, and it was found that neurons in the prefrontal cortex could respond to both particular tones and colours and during the delay period the activities were sustained, which may reflect the working memory storing the association between the specific pairing of a tone and a colour[8].

Evidence of sensory cortices participating in cross-modal association

As mentioned above, many experiments proved the important role of higher cortices in cross-modal integration. However, these areas only occupy part of the cerebral cortex and it may not be efficient enough to face this complex world if multimodal association only happens in higher association cortices. Maybe due to the limitation of the recording technique and experiment design, few studies showed uni-sensory cortices have the ability to integrate information from several modalities several

decades ago. Things have changed a lot nowadays. More and more evidence has demonstrated that cross-modal effect happens in lower sensory cortices.

In human subjects, a PET study showed that when using auditory substitution equipment, visual cortex of early blind subjects were activated besides sensory cortex[11], and when early blind subjects did tactile discrimination tasks, their visual cortical areas were also activated[12]. For early deaf people, by using fMRI it was found that visual stimuli could excite auditory cortex[13]. These studies suggest that when one sensory input is deprived, the brain has the ability to redistribute the "blank" areas to process other information. Although such reorganization needs a long period, it reveals that cortices that were considered as uni-sensory previously have the potential to integrate cross-modal information. What is the situation in normal subjects? fMRI studies found that auditory cortical areas of normal subjects were activated during a silent lipreading task but not by the meaningless pseudospeech[14, 15]. When attending to speech gestures, activities in auditory areas to speech sounds were enhanced which showed visual inputs could modulate the perception of auditory information[16]. In addition to evidence related to auditory and visual cross-modal interaction, there is also evidence showing the existence of auditory and somatosensory integration in auditory cortex[17]. Most of above imaging studies used language as their stimuli which could easily evoke cross-modal effect since auditory and visual parts of language always associate with each other. However, after simply exposing normal subjects to bimodal audiovisual stimuli consisting of noise bursts and light flashes, the primary visual cortex was activated by auditory stimuli alone[18]. A further study used partial least-squares analysis to

search for possible sources of visual areas activation, and the results showed that prefrontal cortex and auditory cortex influenced activities in occipital areas and interactions among these structures could account for the learning induced changes in visual cortex[19]. Many imaging studies have proved that sensory cortex even primary sensory cortex could process information from other modalities, but there are several limitations. First, the spatial resolution is rather low for imaging studies and the anatomical structures are not identical for each individual so it is hard to confirm the site where cross-modal integration happens[20]; second, changes of activities revealed by imaging technique only represents the varying of metabolic level but not the neural activities itself, thus it is hard to know whether the cross-modal effect happens at the single neuron level and what kind of neurons are recruited[21].

Animal studies could better answer these questions by directly recording neural activities in desired locations. Kayser and his colleagues demonstrated that in monkey auditory cortex (primary and secondary cortex), neural responses to auditory stimuli were strongly affected by visual stimuli and when visual stimuli were 20-80 ms earlier than auditory stimuli, the modulating effect was strongest[22]. This indicates that neurons in the auditory cortex not only receive auditory information but are also modulated by visual input. A study on ferrets also indicated that visual stimuli changed the firing properties (both firing rate and spike timing) of neurons in auditory cortex to sound and the percentage of neurons which were influenced by visual input were higher in secondary cortices than in primary cortices[23]. Zhou and Fuster used a visuo-haptic task to train monkeys, subjects

needed to differentiate two patterns of visual stimuli and then used the given pattern to choose the paired bar, recordings in the somatosensory cortex showed that many neurons were activated when visual stimuli were presented and activities of these neurons reflected the association between the visual cue and the corresponding haptic stimuli[24]. By using a similar experiment design in which a click sound was added to signal the starting of a trial, Zhou and Fuster also demonstrated that cells in somatosensory cortex could be activated by the click sound[25]. The similar phenomenon also occurred in auditory cortex. In this study, monkeys were extensively trained by an auditory discrimination task during which a cue light was used to hint the beginning of the trial and animals needed to manipulate a bar to complete the task, it was found that a certain percentage of neurons in auditory areas responded to the cue light and to the motion of the hand, and if the task changed from the auditory discrimination task to a visual discrimination task which meant that auditory system was no longer needed in the posterior task, neurons in auditory cortex did not respond to light anymore[26]. These physiological data supported imaging studies in humans and proved that cross-modal integration happened at the single neuron level even in primary sensory cortex. Moreover, through learning neurons in one sensory cortex could be triggered by stimuli of another modality which were used as cues during the task, these activities could lead to the behavioral reaction, and the activation of these neurons depended on the nature of the task.

Therefore neurons in sensory cortex do not merely process information of one modality and then pass the information to higher cortex, they may store associations of stimuli which are needed to complete the task, and once the cue is presented to

the subject, the cue can activate these neurons and let the cortex retrieve stored association (see a different view[27]).

Question 1 - Can cross-modal association be formed by fear conditioning?

Most of these studies done to date have used complex tasks to train the subjects and the subjects needed to go through extensive training then neurons in sensory cortex were recorded. Hence, the course of how neurons build the association between cross-modal stimuli could not be observed. Here touches the first question that the present work focused on. In this study, a simple behavioural paradigm - fear conditioning was used to check whether cross-modal association could be formed by this paradigm, and the time course of the association building was examined.

Question 2 - Can the subjects use association learned through fear conditioning to facilitate the performance in other tasks?

Fear conditioning is one kind of Pavlovian conditioning, and through conditioning the subjects learn the association between a conditional stimulus (CS) and an unconditional stimulus (UCS) [28]. In the present study, cross-modal stimuli were used as the CS, so the subjects needed to learn not only the association between the CS and the UCS, but also the association between the two stimuli that were used as cross-modal CS. After the establishment of an association, another question this study addressed is whether the subjects can use this association to facilitate the performance in other tasks. Following fear conditioning, the subjects were trained to

use the stimulus of one modality as cue to gain a reward, then the cue was replaced by the stimulus of another modality, and the performance of subjects with and without an association were compared to answer above question.

Question 3 & 4 - how does the hippocampal system engages in the establishment of new associative memory in the neocortex in the animal model, and does the retrieval of an established memory depend on the hippocampal system?

Paralleling the increase of imaging or physiological studies showed the cross-modal integration happens in early sensory cortices, more and more experiments were conducted to find the anatomical basis or to investigate this issue from other angles. One study revealed that the microcircuits of rat brain slices could store the temporal patterns it experienced[29]. In this study two sites on the slice were activated in phase or with a certain delay, after the shaping when one site was activated, activities of another site could reflect the delay used during the training[29], and another study provided similar results[30]. Accordingly, the local network of cortex could memorize associations artificially created between two sites at least 2mm away[29], and this may partially afford a mechanism of the cross-modal integration. *In vivo*, the situation is much more complicated. Higher association cortices send projections back to primary and secondary sensory cortices[31-33], and these projections could support the cross-modal integration in the sensory cortices. Some studies even demonstrated there were connections between these sensory cortices[23, 32, 34, 35], but these connections were weak and sparse, so it is highly possible that information sent by

these connections is used as modulating signals and no the cross-modal association messages are passed. Besides above mentioned connections, the projections between the limbic system and sensory cortices are also notable[36-39]. Since the present study tried to build an associative memory between two cross-modal stimuli, the limbic system should be more relevant than the other two sources of projections. Patients with bilateral medial temporal lobe lesions lost almost all ability to form new recent memories[40, 41], but their long-term memory could still be intact [42]. Detail animal studies showed a similar role of the hippocampus system during the formation of memory[43, 44]. Especially, the entorhinal cortex sits between the hippocampus and the neocortex, and its function could be the bridge of information between the hippocampal system and neocortical areas[45, 46]. In one study, the monkeys were trained to remember paired pictures, and then their rhinal cortices were lesioned[47]. After the lesion, the animals' ability to learn new paired pictures was profoundly retarded[47], and recordings in inferotemporal cortex demonstrated that neurons in this area could not represent associations between stimuli anymore[48]. Since damage of the neocortex impairs remote memory[49, 50], it is proposed in the hippocampus-neocortex-transfer model that memory is first stored in the hippocampus, gradually transferred to the neocortex, and eventually independent from the hippocampus[51, 52]. Therefore, the third question arises here how does the hippocampal system engages in the establishment of new associative memory in the neocortex in the animal model, and the fourth question; does the retrieval of an established memory depend on the hippocampal system?

Summary

The present study focused on the neocortex and explored how cross-modal associative memory was established by conditioning and whether the established memory could be correlated with behavioral tasks. With electrophysiological recording from the behavioral animals, the processes of the establishment, extinction and reestablishment of an associative memory in the neocortex were monitored. Then we monitored how the process of establishment and retrieval of the new associative memory was affected by the temporal inactivation of the entorhinal cortex.

Chapter 2 Method

Animal

We used male and female Sprague-Dawley rats (280 – 360 g) with clean external ears and no observable deficit (n=15 for purely behavioral experiments and n=11 for physiological experiments).

Animal preparation for physiological study

Anesthesia was induced intraperitoneally with 50mg/kg pentobarbital sodium (54.7mg/ml solution, Ceva Sante Animale Co., France) and maintained throughout surgery and recording with 25 mg/kg/h pentobarbital sodium. Subcutaneous atropine sulphate (0.05 mg/kg) was administered 15 min before induction of anesthesia to inhibit tracheal secretions. A local anesthetic (xylocaine, 2%) was liberally applied to the wound. Animals were surgically prepared as described previously[53]. Briefly, rats were mounted in a stereotaxic device, and a midline incision was made in the scalp. A craniotomy was performed to access the auditory cortex, and the dura mater was then opened. Animal's body temperature was maintained at 37 – 38°C with a heating blanket. All animal procedures were approved by the Animal Subjects Ethics Sub-Committees of The Hong Kong Polytechnic University.

Tungsten microelectrodes with impedances of 2 – 7 MΩ (Frederick Haer & Co., Bowdoinham, ME) were employed to map frequency tuning properties of the auditory cortex. For recording, electrodes were positioned with a stepping-motor

microdriver controlled outside the soundproof room. Neuronal signals recorded by the microelectrode, together with the acoustic and light signals, were amplified and stored using Tucker-Davis Technologies software (OpenEX, TDT, Alachua, FL) and Axoscope software (Axon Instruments, Sunnyvale, CA). The time of spike occurrence relative to stimulus delivery was calculated with Matlab software (Mathworks, Inc, Natick, MA).

A home-made electrode array normally consisting of 4 (2x2) bundles of electrodes was implanted into the auditory cortex in each hemisphere[54]. In each electrode bundle, one recording electrode was glued closely to a bipolar stimulation electrode. The stimulation electrode was made of insulated stainless steel wire (A-M Systems, USA) with an impedance of < 100 kΩ and the recording electrodes were made of insulated tungsten wires (A-M Systems, INC., USA) with impedances of 0.5-1 MΩ. The array was held by a micromanipulator and lowered to the surface of the cortex. Once the electrode array arrived at the right position, the opening of the skull was covered by a layer of silicone (World Precision Instruments, USA). All electrodes were connected to a 20-pin socket cemented to the skull. The animal was returned for recovery.

Acoustic and visual stimuli

Acoustic stimuli were digitally generated using a computer-controlled TDT Auditory Workstation and delivered to the ear via a coupled electrostatic speaker (EC1, TDT) mounted in a probe. The sound pressure level was calibrated with a condenser microphone (Center Technology, Taipei). Pure tones were used to map

the auditory cortex, while noise-bursts were used for association. White light generated by light-emission diodes was used as the visual stimulus for association experiments.

Association of auditory and visual stimuli

Animals for pure behavioral study were presented with the combined sound and light stimulus as conditional stimulus (CS), followed by 600 ms foot shock of 0.5-0.9 mA for 10 trials. The interval between trials was either 30 or 60 s. White light of 2000 ms was used for the visual stimulus and a white noise of 1800 ms for auditory stimulus. The onset delay between light and sound was 200 ms with light first. Foot shock was presented at the end of CS. In the control group, animals were conditioned only to the visual stimulus with the foot shock for 10 trials.

Animals for physiological study were presented with light (100 ms) first, with 200 ms onset delay electrical stimulation of the auditory cortex was presented, and then followed by 600 ms foot shock of 0.5-0.9 mA for 15 or 20 trials as one session. Foot shock was presented 500 ms (onset) after light. Two pulses of 30- 100 μA were used to activate the auditory cortex. Up to 10 sessions were given to train one subject.

Association of auditory stimulus with water reward

After conditioning, water intake was scheduled before they participated in a reward paradigm[55, 56] designed to train the animal to initiate the paradigm and approach for a water reward after hearing the sound stimulus or sensing cortical stimulation. An

infrared sensor was placed in the central hole of three parallel holes to detect the poking of the animal, which initiated the trial. The animal was given 2 s to move to the right hole for the reward, starting from the onset of the sound or electrical stimulation. No water was delivered if no signal was detected within 2 s. Trial counting was triggered when the animal poked into the central hole. The training consisted of 5 stages. In stage 1, the animal was taught that water was delivered in the hole on the right side. In stage 2, the animal was required to poke into the central hole, before reward reception (see text for stages 3-5). In stage 3, the animals had to hold their head in the central hole before reward reception. The holding time was changed randomly from 200 to 800 ms, and in stage 4, from 400 to 1500 ms, and in the final stage, to random intervals between 100 and 1200 ms. Once the animal had successfully moved to the right hole for the reward for 4 successive trials, the training was promoted to the next stage, and 9 successive trials were given for the final stage. One animal that could not finish stage 3 within one hour was excluded from the study. Since in this session, the animal initiated the trials and moved to the right hole for the reward after hearing the sound or sensing electrical stimulation, we termed this "subject-initiated sound-reward protocol", or "subject-initiated cortical stimulation-reward protocol". Both experimental and control animals in the behavioral experiments participated in this subject-initiated sound-reward protocol training. In the experiment to examine the physiological implication, we adopted the subject-initiated cortical stimulation-reward protocol.

Testing protocol with light stimulus

After mastering the subject-initiated sound-reward protocol or subject-initiated cortical stimulation-reward protocol, the animal was examined with a testing protocol in which the previous sound stimulus/cortical stimulation was replaced by the visual stimulus. This protocol was used to examine the association between the visual stimulus and the sound stimulus or the cortical stimulation. A successful association would lead the animal to move to the right hole for the water reward after the replacement of the auditory stimulus or cortical stimulation with the visual stimulus. This was termed "subject-initiated light-reward protocol".

Those trials in which the animal successfully triggered the visual stimulus and moved to the right hole for the reward were categorized as successful trials, those trials in which the animal triggered the visual stimulus but failed to approach for the reward were categorized as unsuccessful trials, and those trials in which the animal poked into the hole but failed to trigger the visual stimulus were categorized as premature trials. When we calculated the success rate in Figure 13 in reward reception after the visual stimulus was triggered, we counted those successful trials over both successful and unsuccessful trials.

For physiological recordings, the animals were also examined to determine whether they could move to the right hole for the water reward when they were passively exposed to the visual stimulus. As the protocol was initiated with the visual stimulus by the experimenter, it was termed experimenter-initiated light-reward protocol.

Physiological examination of association and extinction

Neuronal responses to auditory and visual stimuli in the stimulation and control sites were recorded by the implanted electrode array after the surgical recovery of the animal (6-7 days). In the first group (n=6) of simple visual-auditory association, the neuronal responses to the visual stimulus were recorded after conditioning. In the second physiological experiment, neuronal responses to the visual stimulus were recorded while the animal was performing the subject-initiated light-reward protocol or experimenter-initiated light-reward protocol for the following days. See text for time course and extinctive process details.

To study the time course of the establishment of the visuoauditory association, testing trials with the visual stimulus only were inserted into the light-cortical stimulation-foot shock protocol. We have inserted 10 testing trials each in the different times of the protocols.

In another experiment with 3 animals, we examined the extinction process of an established association by repeating the testing trials with the visual stimulus alone at different times in the following days. The testing trials included the subject-initiated light-reward protocol and the experimenter-initiated light-reward protocol.

Inactivation of the entorhinal cortex

In order to examine the influence of the hippocampal system on the establishment of new associative memory in the auditory cortex, a guide cannula was implanted into the gateway region of the hippicampal system, the entorhinal cortex

(Rostrocaudal, Bregma -6.6 mm; mediolateral, 4.7 mm; depth, -6.8 mm from skull surface). The region for drug injection was identified in a pilot study in anesthetized rats on which activation of the region showed modulatory effects on the neuronal responses to acoustic stimulus in the auditory cortex. The guide cannula was implanted only to one hemisphere of the entorhinal cortex and was cemented together with the stimulation and recording electrode matrixes on the skull. The guide cannula was blocked with a dummy cannula during resting, extending 0.2 um below the guide, and the injection needle connected with a Hamilton syringe, extending 0.7 um below the guide. Lidocaine (2%,2 ul, 0.5ul/min) was injected to 1 rat, and a glutamate AMPA receptor antogonist (DNQX, 15mM, 1.2 ul, 0.3ul/min) was injected to the other 3 rats to inactivate the entorhinal cortex at 10 min before the conditioning aiming to establish the association between the light stimulus and electrical stimulation at the auditory cortex or at 10 min before the retrieval test during which just light stimulus was presented. Drug microinjection was performed by using QSI-Quintessential stereotaxic injector (Stoelting Co, USA), and the injection needle was held at site for another 5 min before being withdrawn. Retrieval testing trials during which only the light stimulus was given to measure the establishment of the association were inserted before, at 60 min and overnight after each session of classical conditioning.

Data analysis

Neuronal responses in the auditory cortex were recorded on a computer together with the animal's behavioral activities. For those cross-session recordings over days,

there was no guarantee of recording from the same unit. Therefore, we adopted an analysis of Z-scores (mean±SE) to examine neuronal responses and compare the responses across sessions[55].

Chapter 3 Results

Association of cross-modal stimuli

In the first experiment, we produced an association between the auditory cortex and a visual stimulus by conditioning a combined electrical stimulation of the auditory cortex and a visual stimulus of white light with a foot shock. The aforementioned association would be reflected if neurons in the auditory cortex started responding to the visual stimulus after the conditioning. The stimulating site, where electrical stimulation of a doublet pulse was delivered to the auditory cortex through a bipolar stimulating electrode, was monitored with a closely attached recording electrode, while another recording electrode 3 mm away served as the control site. The merits of using electrical stimulation rather than natural sound stimuli include the fact that the cortical area activated by electrical stimulation is more focused than that activated by a natural sound stimulus. Also the activated area in the auditory cortex, which reflects association with the visual stimulus, can be easily monitored by recording electrodesattached to the stimulating electrode. In contrast to this, the cortical area activated by the sound stimulus would likely have spread to a greater portion of the auditory cortex and beyond making monitoring difficult. As the establishment of the associative memory in the auditory cortex between the sound and visual stimuli could store anywhere within the limited regions of the activated area, it is impractical to detect the regions and to monitor the process.

All recordings in the present study were carried out in free moving subjects unless specifically mentioned. Two neurons recorded and sorted from the stimulation site

and the control site respectively are shown in Figure 1. Figure 2 shows the neural responses to electrical stimulation. The left raw signal was recorded by a recording electrode attached to the stimulation electrode and the right signal was recorded by the electrode at the control site. Sampled raw data are shown in the upper row for each neuron, and their raster displays for 10 trials are shown in the lower rows. Arrowheads indicate the trials that the sampled raw data were taken from. Arrows indicate the artifacts of the stimulation pulses. The amplitude of the stimulation artifacts was moderate and the artifacts were easily separated from the neuronal signals. Only neurons in the stimulation site were activated but not the control site. This demonstrated that electrical stimulation only activated a limited area. Before the conditioning, neurons in both the stimulation and control sites responded to the auditory stimulus of a noise burst with short latency (Figure 3). Visual stimulus was also presented to animals. Both stimulation site and control site showed no response to the visual stimulus (Figure 4, raster plot and PSTH in blue). After the conditioning of 120 trials, the neurons in the stimulation site started to respond to the visual stimulus, while the control site neurons did not (Figure 4, raster plot and PSTH in red; with raw data showed in Figure 6 and Figure 7). In the first 2-s window of the repeated visual stimulus of 10 trials, neuronal responses increased significantly compared to the baseline immediately preceding the stimulus only in the stimulation site and only after the conditioning ($P<0.001$, t-test). The neuron at the stimulation site showed a significant increase in its responses to the visual stimulus after the conditioning (Z-scores, 9.60±0.99 vs 0.13±0.56, $P < 0.001$, t-test) (57), while the neuron in the control site showed no significant change (0.67±0.65

vs 0.25±0.48, P = 0.66, t-test). We confirmed these results in six animals. The mean Z-scores of neuronal responses to the visual stimulus showed significant increases in the first 2-s period after conditioning at the stimulation site, but not at the control site (Figure 5, n=6; *, P<0.05, ANOVA; each data point was taken for 200ms).

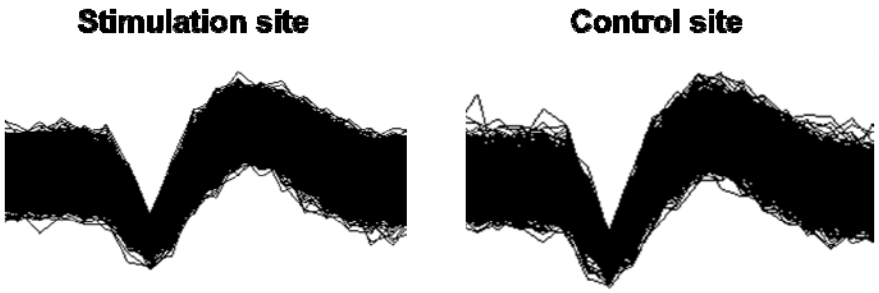

Figure 1 Examples of Neural activities from stimulation site and control site respectively.

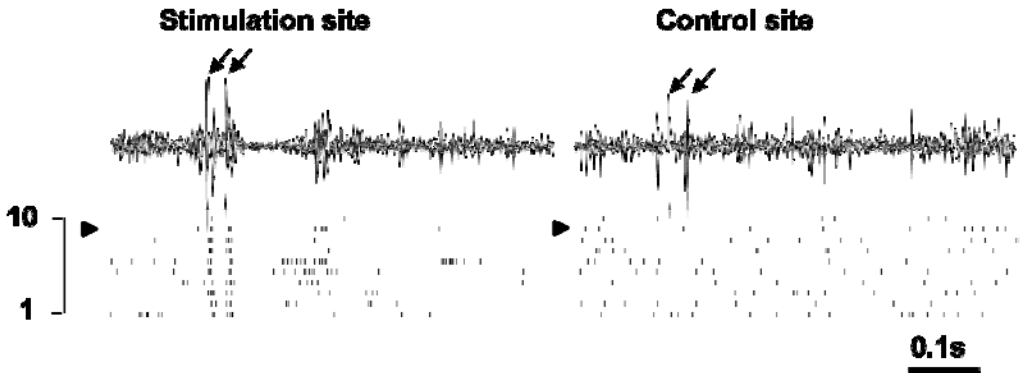

Figure 2 Neural responses to electrical stimulation. The left signal was recorded by a recording electrode attached to the stimulation electrode and the right signal was recorded by the electrode at the control site. Sampled raw data are shown in the upper row for each neuron, and their raster displays for 10 trials are shown in the lower rows. Arrowheads indicate the trials that the sampled raw data were taken from. Arrows indicate the artifacts of the stimulation pulses. The amplitude of the stimulation artifacts was moderate and the artifacts were easily separated from the neuronal signals. Note that the artifacts were not mixed in the raster displays.

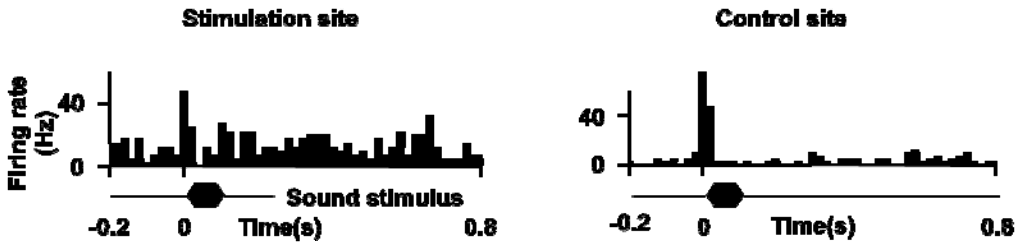

Figure 3 Peristimulus histograms show neuronal responses to the noise-burst stimulus in the auditory cortex. The stimulation and recording electrode combination was implanted in the auditory cortex. A stimulation electrode was implanted together with two recording electrodes at the stimulation site, and one recording electrode was implanted at the control site. One neuron from each site is shown here.

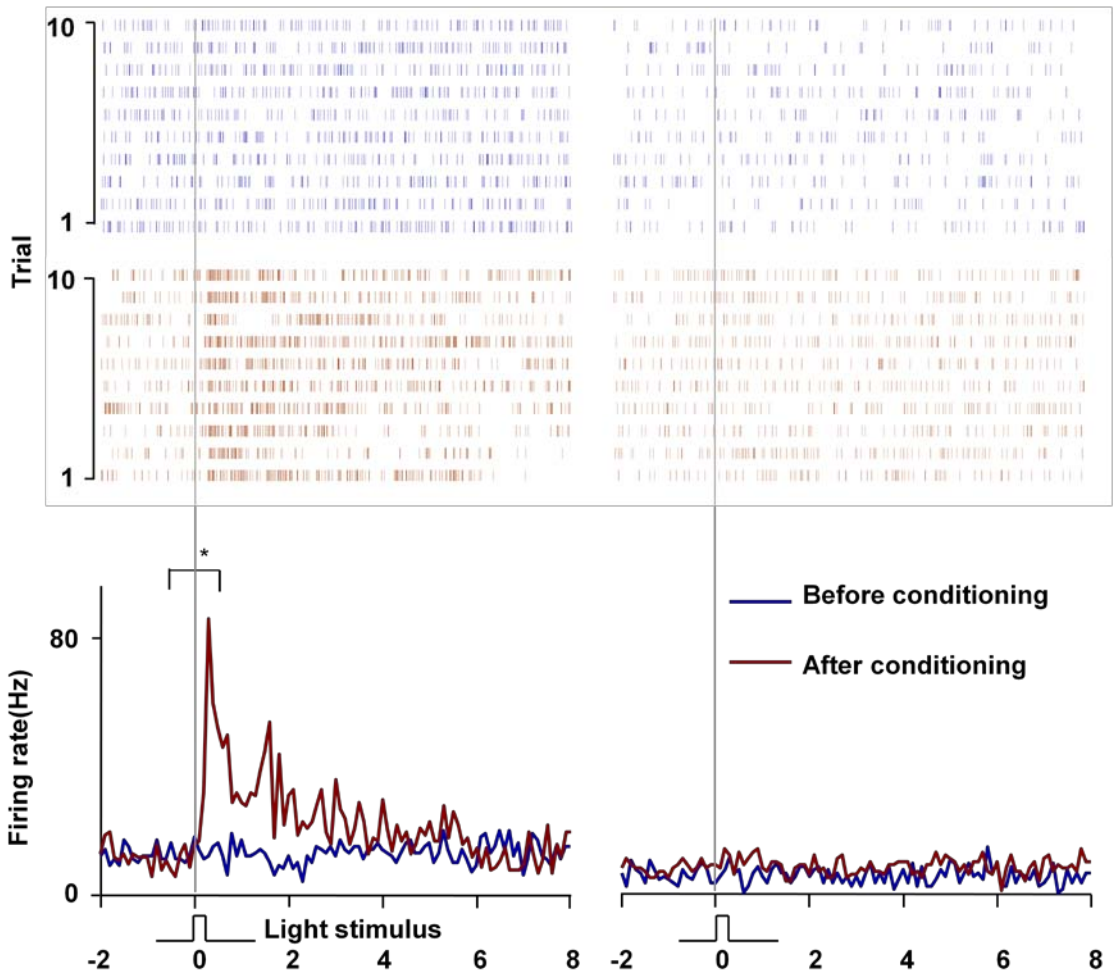

Figure 4 Neuronal responses to repeated light stimulus before (upper raster displays in blue) and after (lower raster displays in red) conditioning in the stimulation and control sites. Statistical comparisons were made between neuronal activities during the 2 s immediately before and after the onset of the visual stimulus (*, $P < 0.001$, t-test).

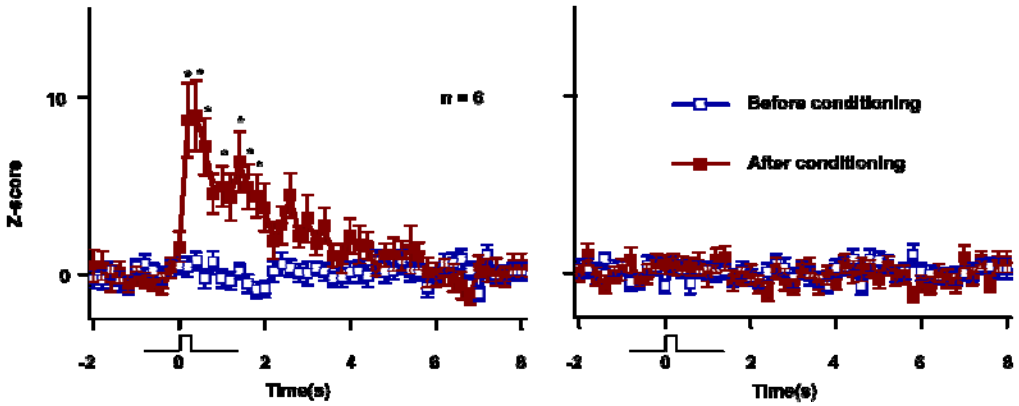

Figure 5 Z-scores (mean±SE) of neuronal responses to the visual stimulus before and after conditioning (n=6) Each data point was taken for 200 ms. Comparisons were made between responses before and after conditioning for both stimulation and control sites (*, $P < 0.05$, repeated-measure ANOVA).

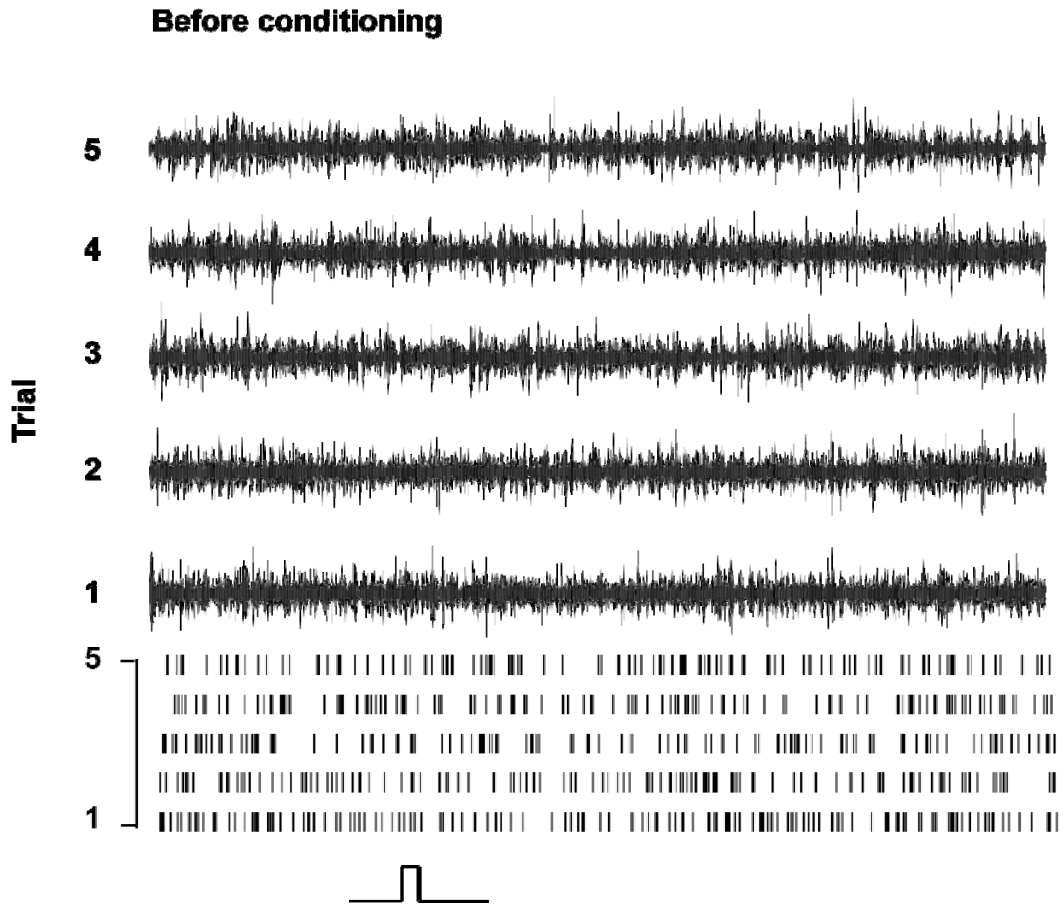

Figure 6 Raw data and detected spikes of neuronal responses to the first five trials of the visual stimulus at the stimulation site before conditioning.

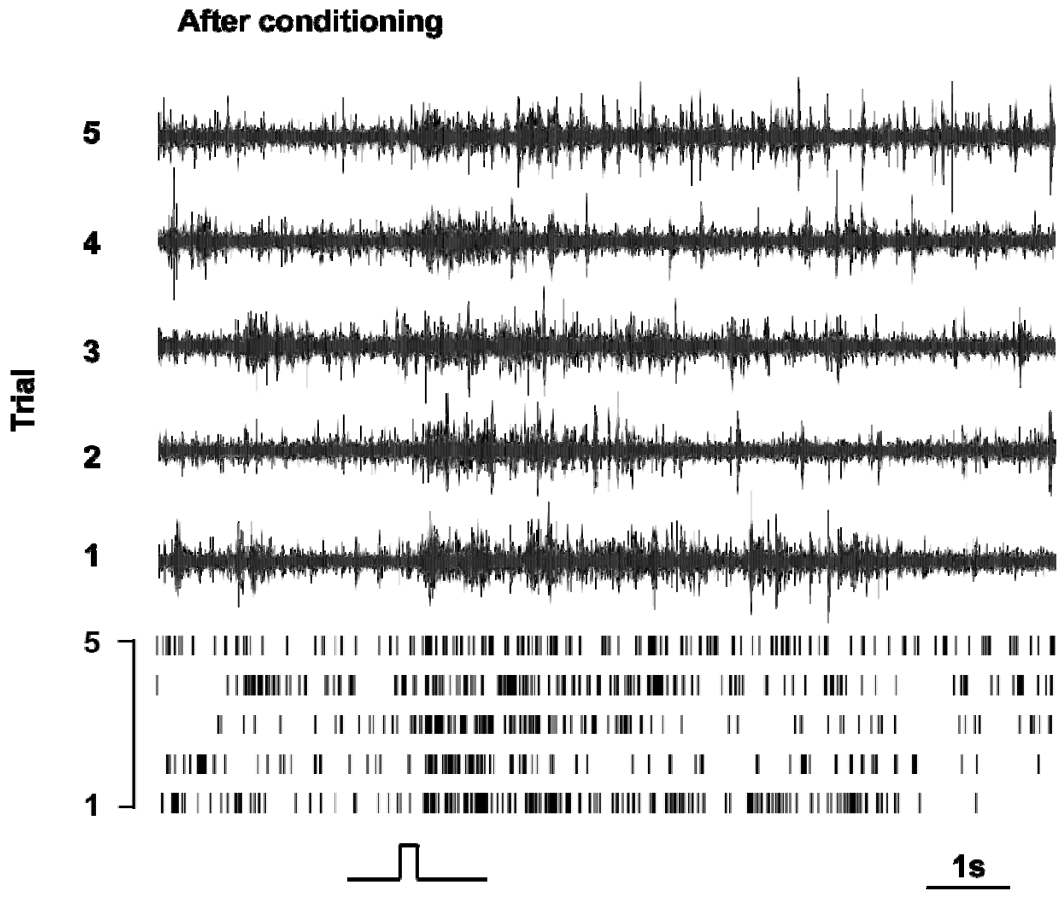

Figure 7 Raw data and detected spikes of neuronal responses to the first five trials of the visual stimulus at the stimulation site after 120 trials of conditioning between the visual stimulus and cortical stimulation.

Time course of the establishment of associative memory

Using the same protocol, we inserted testing trials at different times during the conditioning sessions over two days to examine the time course of the association (Figure 8). Recordings were taken from two electrodes: one at the stimulation site and one at the control site. Before conditioning, neurons at both electrodes showed auditory responses (Figure 9), but did not respond to the visual stimulus (Figure 10, t0). The neuron at the stimulation site showed no significant responses to the testing visual stimulus after the first block of conditioning (15 trials, t1), but showed significant responses after the second block (t2) of the light-cortical stimulation paired with foot shock, and increased responses to the visual stimulus in the subsequent conditioning blocks (t3 to t6 of Figure 10). Both the Z-score and firing rate of responses to the visual stimulus were significant after 30 conditioning trials compared with preconditioning responses (Figure 10, * $P<0.05$, **, $P<0.01$; ANOVA). Responses to the visual stimulus in Z-scores increased from -0.29±0.42 to 2.12±0.79 after 30 trials, and to 3.45±0.87 after 90 trials of conditioning (Figure 11). In comparison, the control site neuron showed no responses to the visual stimulus during the same course of conditioning (Figure 10, right panels and Figure 11). In another rat, responses to the light stimulus in Z-score increased from 0.18±0.45 to 1.31±0.56 after conditioning of 20 trials in 10 min ($P<0.05$, t-test).

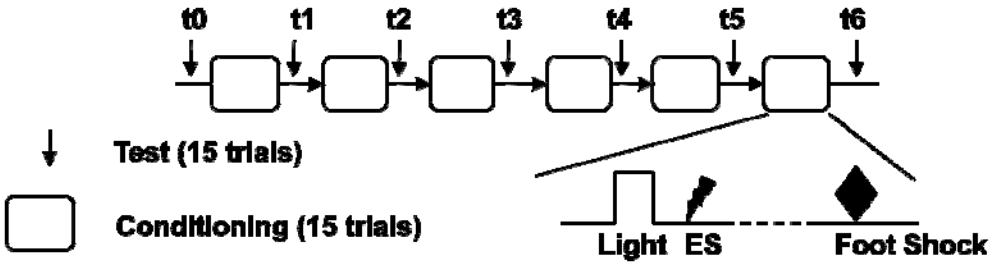

Figure 8 Conditioning and test paradigm to measure the time course of the associative memory traces in the auditory cortex. Each block consisted of 15-trial paring the visual stimulus and cortical stimulation with foot shock. Each histogram was based on testing data from 15 repeated trials.

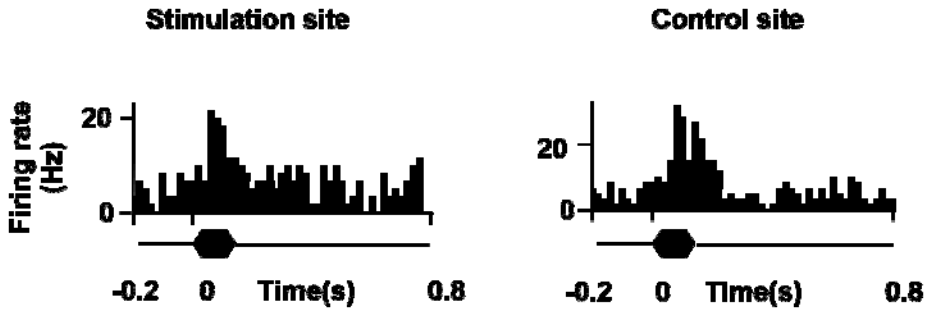

Figure 9 Auditory neuronal responses in both stimulation and control sites.

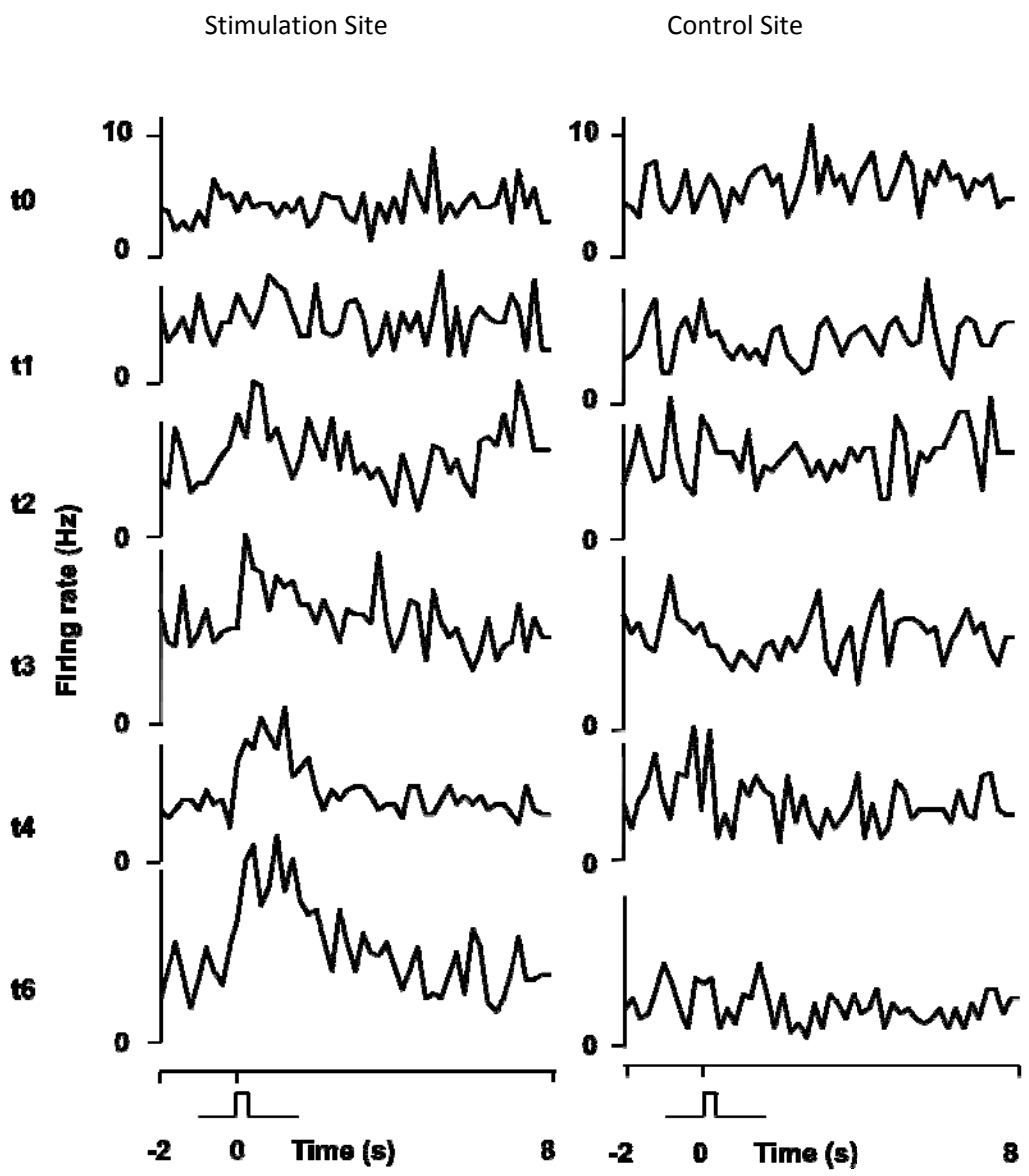

Figure 10 Neuronal responses to the visual stimulus before (t0) and at end of each conditioning block (t1-t6).

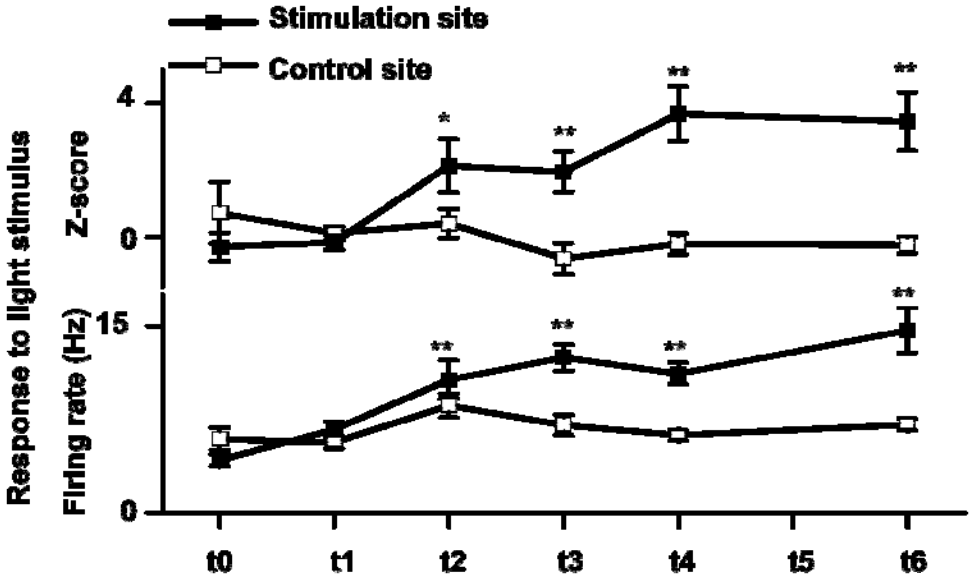

Figure 11 Changes in the responses during the 2-s period after the onset of the visual stimulus at different times during conditioning at the stimulation and control sites (compared to t0; *, P<0.05, **, P<0.01, ANOVA)

Behavioral confirmation of association

To examine whether a learned association is reflected in a behavioral context other than a freezing reflex, seven subjects were presented with a combined sound and light stimulus followed by foot shock. Control subjects (n=8) were presented with only the visual stimulus followed by the foot shock for 10 trials. The conditioning was confirmed in the experimental group by their withdrawal behavior when the combined sound and light stimulus was presented without foot shock and by the freezing response after the presentation of the combined sound and light.

Both groups were then trained to the subject-initiated sound-reward protocol. In order to be sure that the subjects used the sound cue to receive the reward they were required to poke and hold in the central hole for a varying delay interval of 100 – 1200 ms between the initiation and sound presentation. The training consisted of 5 stages. In stage 1, the subject was taught that water was delivered in the right-side hole for 50 trials. In stage 2, the subject was required to poke into the central hole, before moving to the right hole for the reward for 300 trials. In stage 3, the delay interval was inserted and changed randomly from 200 to 800 ms, and in stage 4, from 500 to 1500 ms, and at stage 5, from 100 to 1200 ms. All subjects successfully learnt stages 1 and 2 in the first day. Once the subject successfully moved to the right hole for the reward for 4 successive trials in stages 3 and 4, the training was promoted to the next stage, and 9 successive trials were given for the final stage. The slowest subject required 118 trials in stage 3, 141 trials in stage 4, and 149 trials in stage 5. The total trials of stages 3-5 ranged from 176 to 338 trials (Figure 12).

In the following testing session, the sound stimulus was replaced by the visual stimulus after the subject initiated the task. A successful trial was defined as one where the subject successfully approached the reward after the visual stimulus was triggered. Since the performance was to indicate the association between the light and sound stimuli, only trials in which the subject poked and waited long enough to trigger the visual stimulus were included in the statistics. In the experimental group, six subjects moved to the right hole for the reward at the first trial and all seven subjects at the second trial, indicating that they successfully associated the sound and visual stimuli using the visual stimulus to recall the sound cue before moving to the right hole for the reward (Figure 13). As in the subject-initiated sound-reward protocol, the interval between the initiation and the visual stimulus varied between 100 and 1200 ms.

The control group was designed to examine whether the reward-reception behavior after the sound was replaced by the visual stimulus in the experimental group was based on the utilization of the procedural sequence knowledge (1. poking into the central hole for initiation, 2. waiting for the cue signal i.e., auditory or visual, and 3. moving to the right hole for the reward) rather than via the association between the auditory and visual stimuli. In contrast to the experimental group, seven of eight control subjects failed to move to the right hole to receive the reward at the first trial after replacement of the sound stimulus with the visual stimulus (Figure 13). In other words, the control subjects failed to use the procedural sequential knowledge before receiving the reward. The difference between the experimental and control

groups clearly indicate that the experimental subjects used the light-sound associative memory before reward reception.

It was interesting to note that the subject in the experimental group paused before showing a fast reaction moving for the reward when the sound stimulus was replaced by the visual stimulus. For the experimental group, the reaction time, defined as the time interval after onset of light stimulus and the subject's leaving the central hole, was significantly lengthened from 420±28.3 ms before stimulus replacement to 882±96.6 ms (n=6) in the first trial and 804±203.3 ms (n=7) in the second trial after replacement (both $P < 0.05$, AVOVA, Figure 14). The reaction time, however, decreased to 452±36.8 ms in trials 72-80 ($P < 0.01$ compared to trial 1, t-test, Figure 14), indicating a learning process possibly involving the switch from the light-sound-reward procedure to the light-reward procedure. No significant difference in reaction time was detected between the experimental and control groups between trials 72 and 80 (452±36.8 ms vs 412±34.4 ms, P=0.42, t-test, Figure 14).

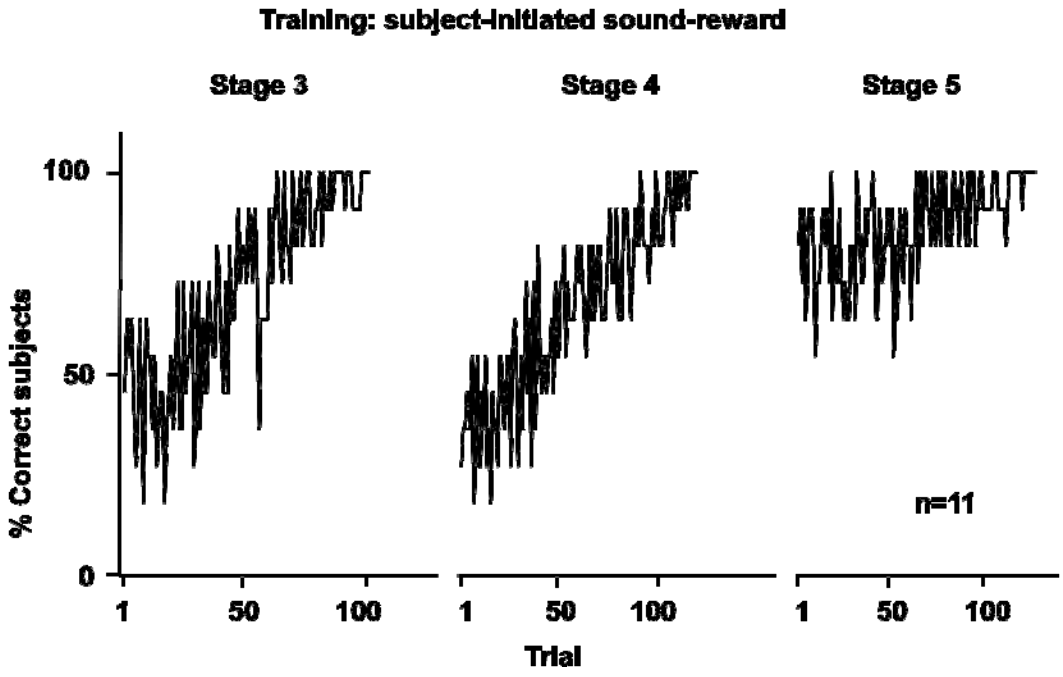

Figure 12 Training progress in terms of correct rate of reward reception in stages 3-5 of the subject-initiated sound-reward protocol. Once a subject succeeded in 4 successive trials in stages 3 and 4 or in 9 successive trials for stage 5, the training was promoted to the next stage or the end of the protocol. The performance for that subject would count as correct for the following trials at this stage after it was promoted to the next stage.

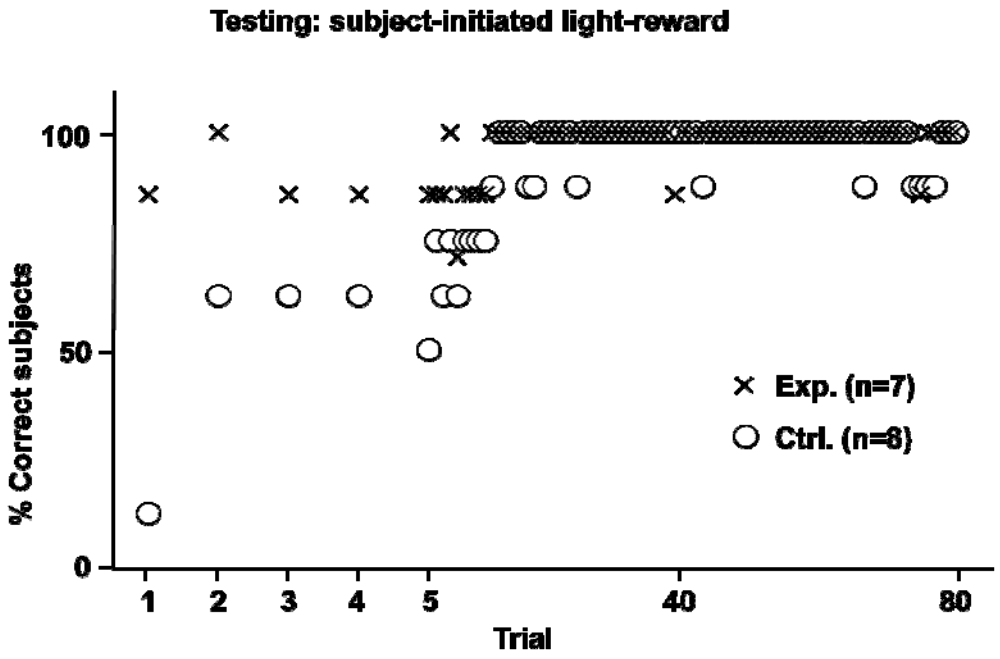

Figure 13 Performance of experimental (crosses) and control (open circles) subjects in testing trials in which the sound stimulus was replaced by the visual stimulus (i.e., subject-initiated light-reward protocol). A successful trial was defined as one where the subject successfully approached the reward after the visual stimulus was triggered. Since the performance was to indicate the association between the light and sound stimuli, only trials in which the subject poked and waited long enough to trigger the visual stimulus were included in the statistics.

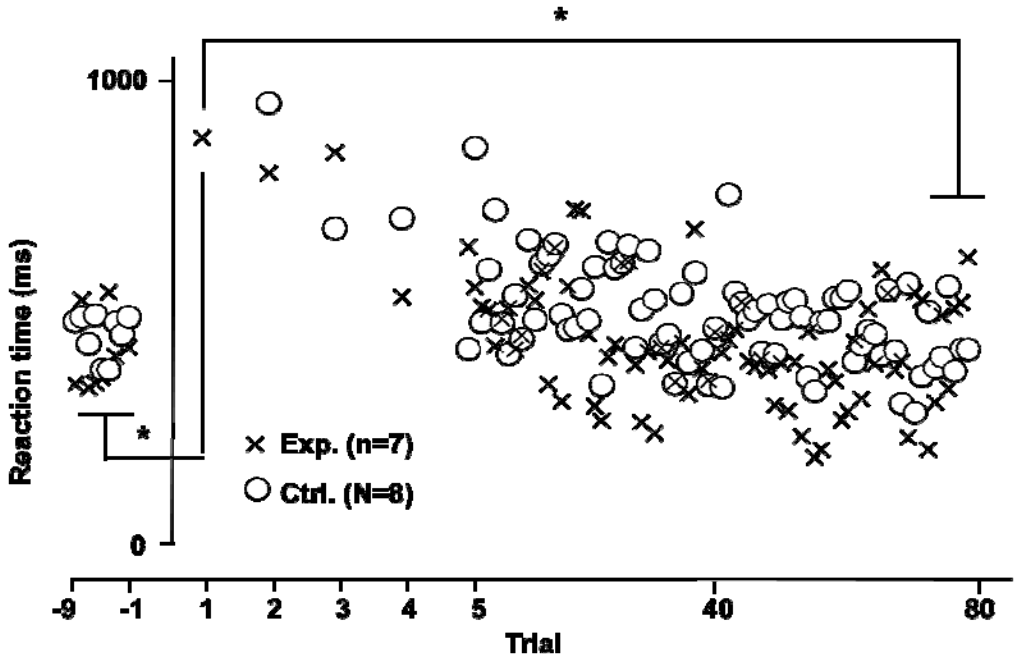

Figure 14 Reaction times to approach the reward after the light was triggered for both groups. Only subjects that succeeded approaching the reward in the corresponding trials were included in the statistics. Comparisons were made between the mean for trial 1 and mean for trials 72-80, and between the means of trial 1 and the last 9 trials in the subject-initiated sound-reward trials. * $P<0.05$, t-test.

Physiology of behavioral experiment

To examine whether the auditory cortex was activated by the visual stimulus during the above behavioral experiment, we replaced the sound stimulus with electrical stimulation of the auditory cortex in a separated group of subjects (n=4). The behavioral experiment was repeated after the change from sound to cortical stimulation. To ensure the association between the visual stimulus and cortical stimulation could be physiologically measured, we paired the visual stimulus with cortical stimulation for 120 trials (6 blocks) rather than the 10 trials used in the pure behavioral experiment. The subject learned to initiate the cortical stimulation – reward protocol and receive the water reward. The successful training with cortical stimulation reflected the effectiveness of the electrical stimulation. The training was similar to the pure behavioral experiment. The holding time between initiation and delivery of electrical pulses was randomly changed between 100 and 1200 ms. All four subjects successfully moved to the right hole for the reward at the first trial after cortical stimulation was replaced by the visual stimulus.

Subjects with cortical stimulation had similar performances in the subject-initiated light-reward protocol as compared to with those in the experimental group in the pure behavioral experiment (Figure 15 vs. Figure 13). In the example subject, we targeted the association auditory cortex to implant the stimulation-recording electrodes at the stimulation site and a recording electrode at the control site (Figure 16). Both neurons at the stimulation and control sites responded to auditory stimuli (Figure 17) and none responded to the visual stimulus before conditioning (Figure

18). After conditioning, neurons at the stimulation site responded to the visual stimulus while neurons at the control site did not (Figure 19). Only trials, in which the subject successfully triggered the visual stimulus and approached the reward, were included. Arrows show the time at which the subject initiated the trials by poking into the central hole, while square waves show the triggered visual stimulus. Comparisons were made between Z-scores before and after the visual stimulus over 1-s periods. * $P<0.05$, ANOVA.

The visual stimulus was then spontaneously produced by the experimenter, all subjects moved to the right hole for reward, indicating that they utilized the association of light and electrical activation of the auditory cortex in leading to the reward. The neuron at the stimulation site responded to the visual stimulus, while the neuron at the control site did not (Figure 20).

Occasionally the subject failed to trigger the visual stimulus before moving to the right hole for reward reception. These trials were called subject-initiated premature trials, and neuronal activities after sorting are listed in Figure 21. The arrows show the time when the subject poked into the central hole. Both neurons at the stimulation and control sites showed no obvious responses in the first 2-s after initiation. Another neuron at the stimulation site recorded by another recording electrode is showed from Figure 22 to 26. This neuron showed auditory responses (Figure 22) but no light responses before the conditioning (Figure 23). After the conditioning and training, the neuron showed obvious responses to the visual stimulus during the subject-initiated light-reward protocol (Figure 24). The two recording electrodes attached to the stimulation electrode were separated by 1 mm

in horizontal dimension. In other words, the region that was activated by the visual stimulus in the subject-initiated light-reward protocol was larger than the gap between the two recording electrodes as neurons in both electrodes responded to the visual stimulus (Figure 19 and Figure 24). However, the neuron showed no obvious response to the visual stimulus in the experiment-initiated light-reward protocol, unlike the neuron recorded by the other recording electrode in Figure 20 (Figure 25). In other words, the neuron showed a better response to the visual stimulus during the subject-initiated light-reward protocol than during the experimenter-initiated light-reward protocol. Similarly to the neuron in Figure 21, the neuron here showed no responses in those premature trials (Figure 26).

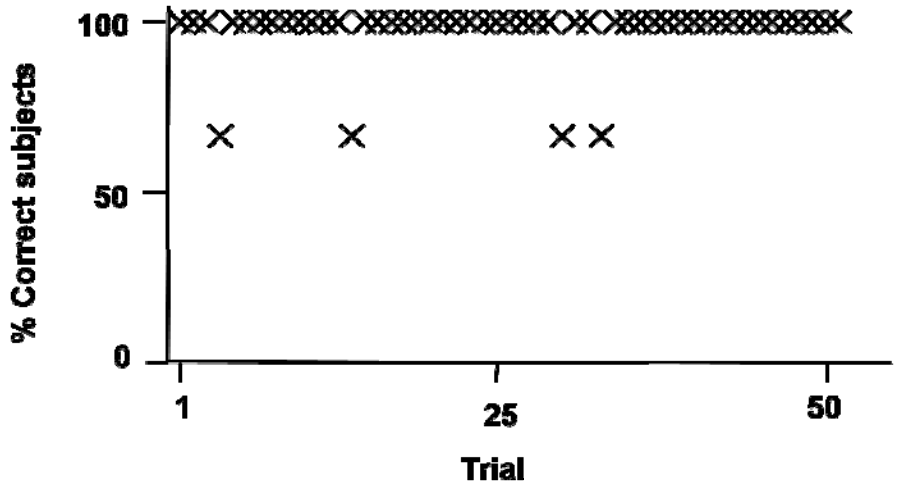

Figure 15 Behavioral results of subject-initiated light-reward protocol. Percentage of correct subjects shows the success rate of reward reception after the visual stimulus was triggered (data of one subject were not complete).

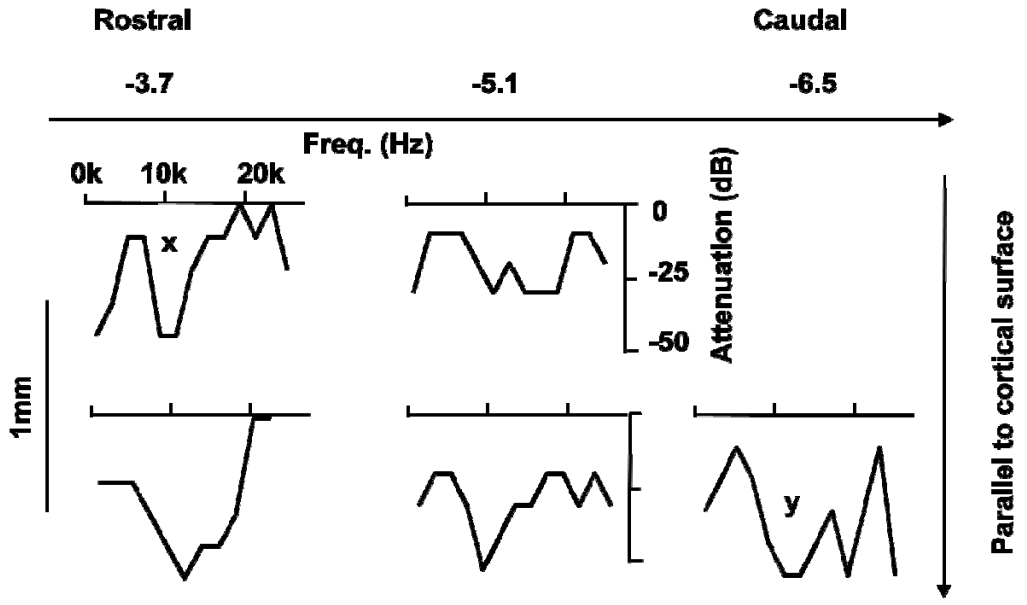

Figure 16 Tuning curves were obtained before implantation of the stimulation (x) and recording (y) electrodes in anesthetized condition.

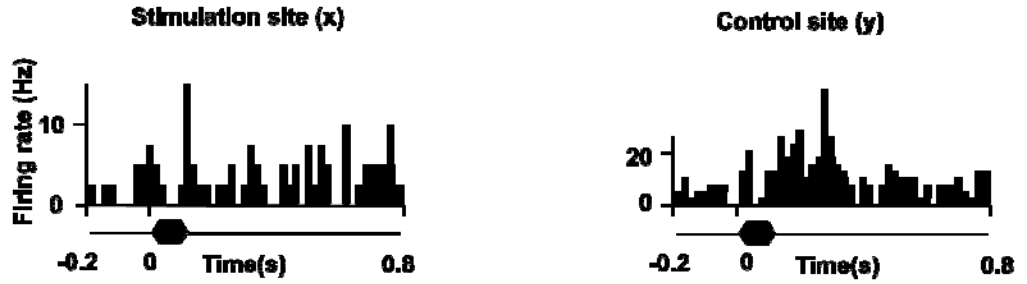

Figure 17 Auditory responses in the stimulation and control sites before association.

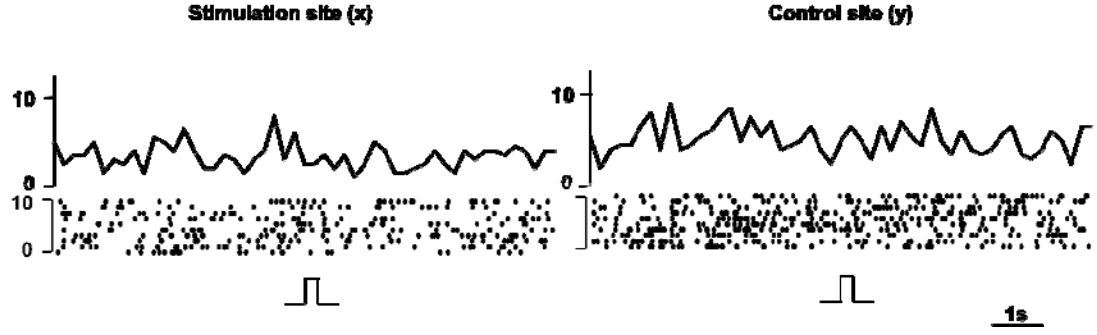

Figure 18 Neuronal responses to the visual stimulus before association.

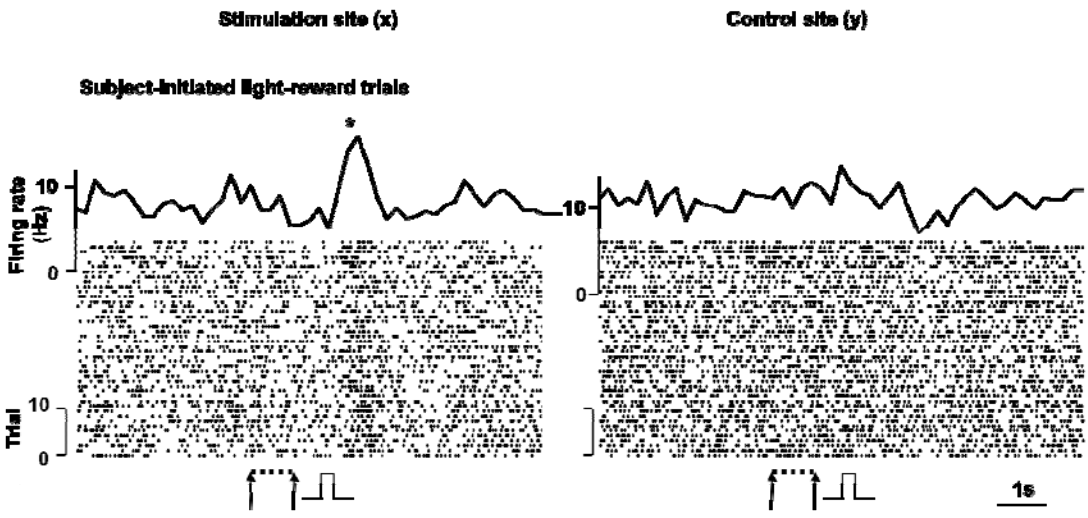

Figure 19 Neuronal responses during the subject-initiated light-reward protocol after the association of the visual stimulus and cortical stimulation and association of cortical stimulation and reward. Only trials, in which the subject successfully triggered the visual stimulus and approached the reward, were included. Arrows show the time on which the subject initiated the trials by poking into the central hole, while square waves show the triggered visual stimulus. Comparisons were made between Z-scores before and after the visual stimulus over 1-s periods. * $P<0.05$, ANOVA.

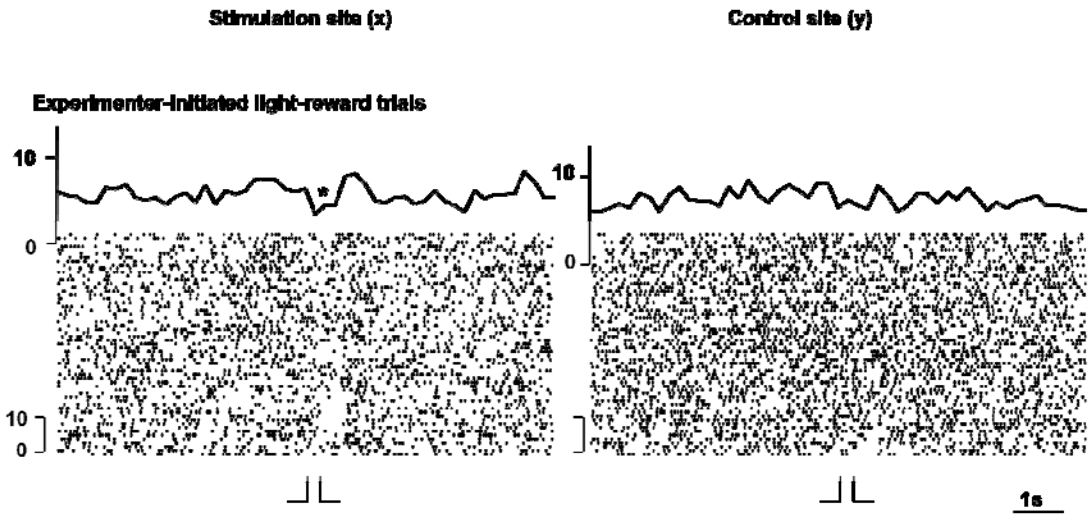

Figure 20 Neuronal responses during experimenter-initiated light-reward protocol.

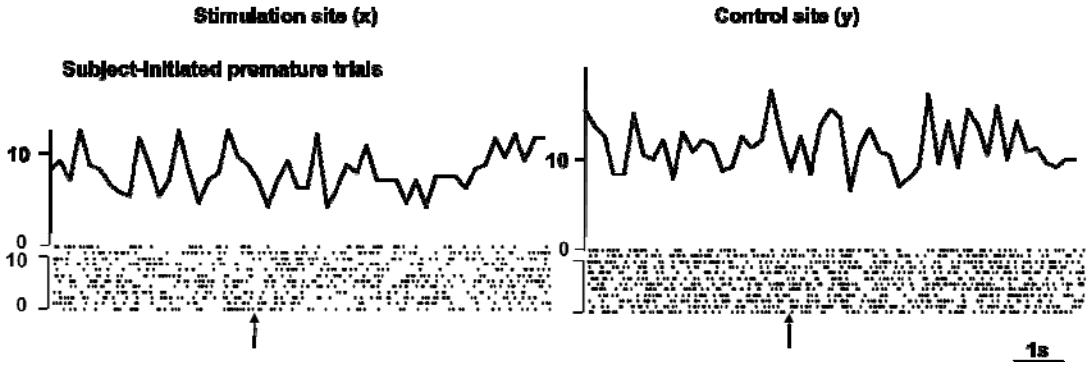

Figure 21 Neuronal activities sampled from trials in which the subject tried to initiate but failed to trigger the visual stimulus. The arrows show the time when the subject poked into the central hole.

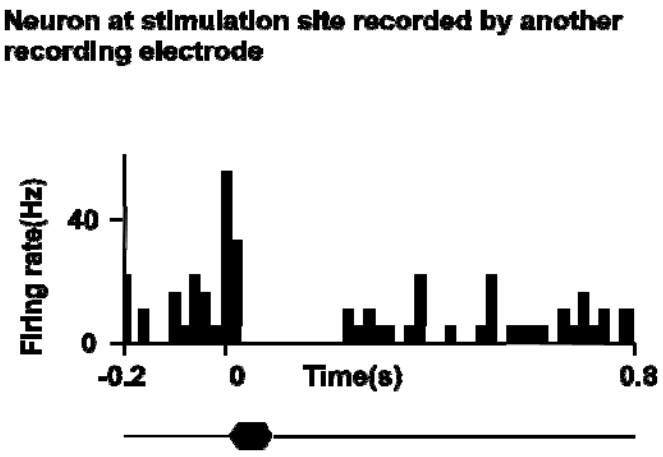

Figure 22 The histogram shows neuronal responses to the auditory stimulus before the association in the stimulation site (x) recorded by the second recording electrode attached to the stimulation electrode.

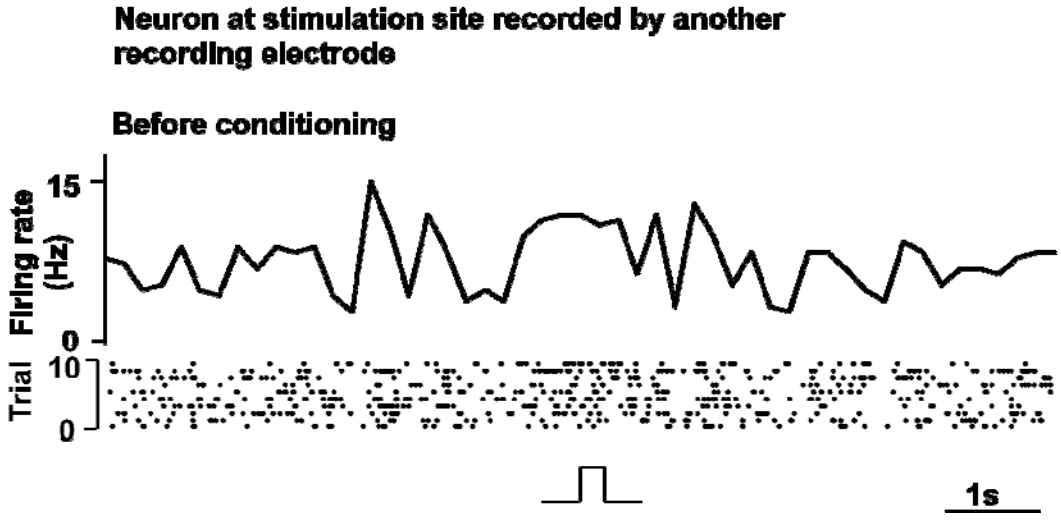

Figure 23 Neuronal responses in raster display (10 trials) and histogram to the visual stimulus before the association of the visual stimulus and cortical stimulation

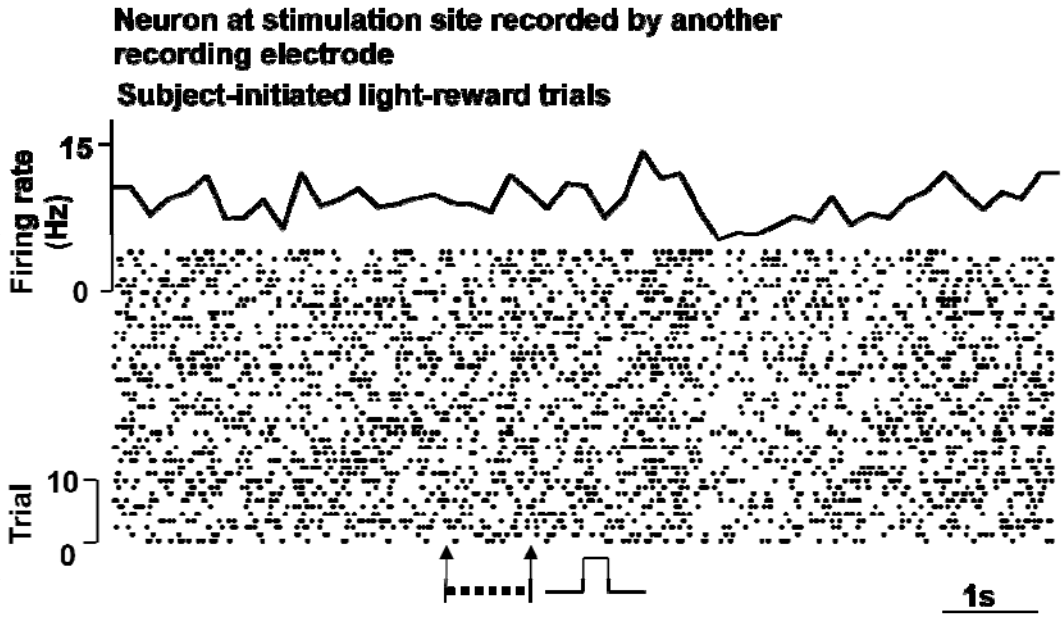

Figure 24 The raster display and histogram show neuronal responses during repeated subject initiated light-reward trials.

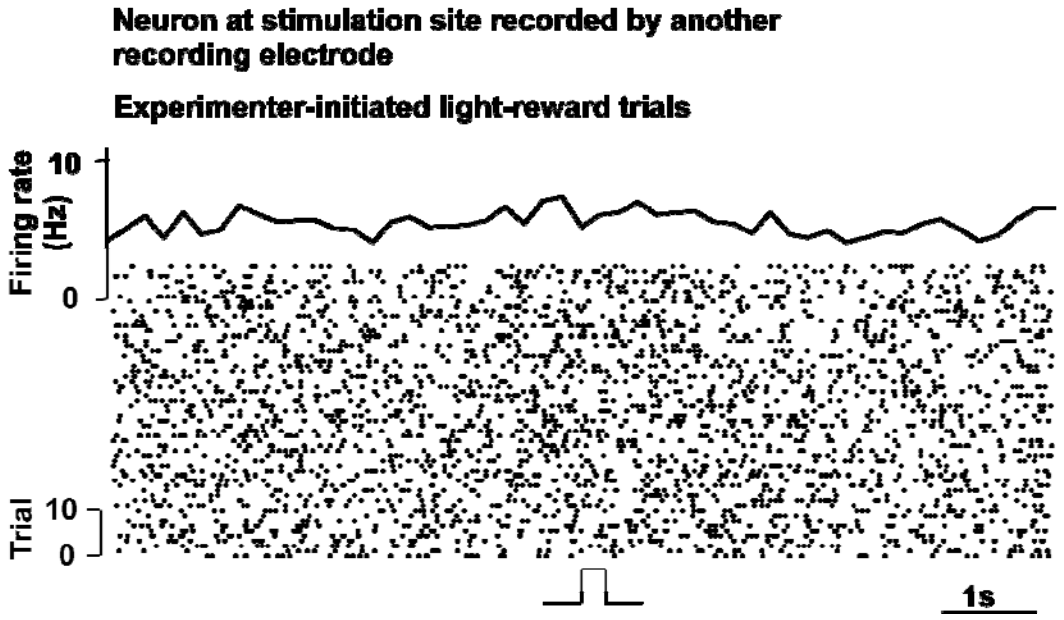

Figure 25 The raster display and histogram show neuronal responses during repeated experimenter-initiated light-reward trials.

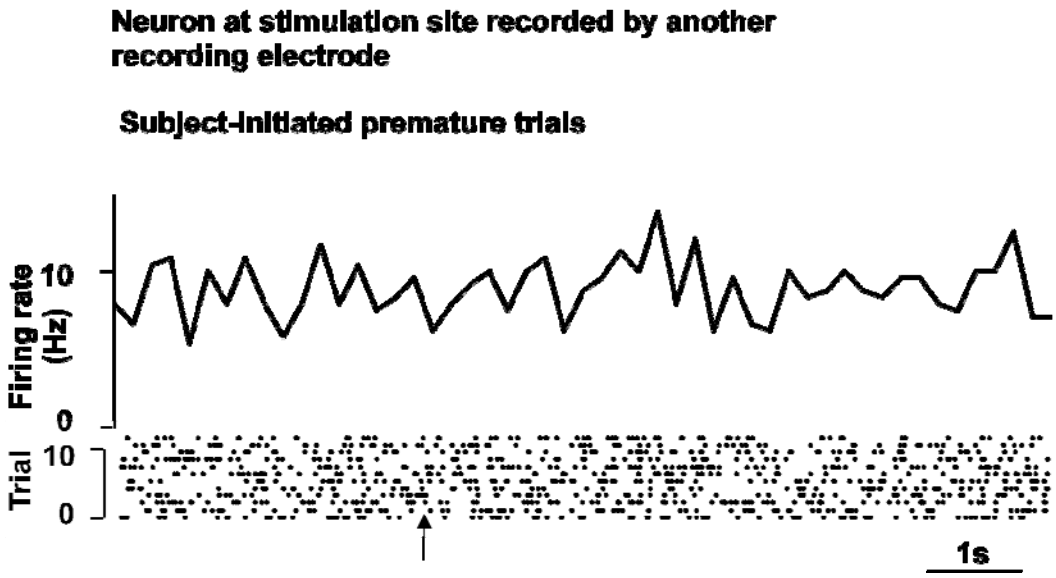

Figure 26 The raster display and histogram show neuronal activities sampled from those trials, in which the subject tried to initiate but did not hold long enough to trigger the visual stimulus (i.e., premature trials).

Extinction and relearning of the association

Neuronal responses to the repeated visual stimulus at different times after the conditioned visual stimulus-cortical stimulation association showed the extinction process (Figure 27). The auditory neuron showed no response to the visual stimulus before conditioning (t0), but maximal responses in the first measurement (t1) after 120 conditioning trials and 4 days of reward-reception training (Figure 28 and Figure 29 for raster plot). The responses to the visual stimulus showed a gradual decrease over the following 4 days (t2-t5), and decreased to near the preconditioning level (t0) 11 days after 587 repeated testing trials at t5 (Figure 28 and Figure 29 for raster plot). Notably, the association was still detectable even after 359 trials (t4) of distinctive testing and 8 days after conditioning. A suppression of neuronal responses after the subject poked into the central hole and before the delivery of the visual stimulus was observed, which can best be seen in the raster plots (Figure 29). This result supports a recent finding that engagement in a behavioral task suppresses the spontaneous activities in the auditory cortex[55]. A short inhibition could be detected when the visual stimulus was spontaneously provided by the experimenter in t4 and t5. It was more obvious in the early 20 trials of t4 than in the late ones. An increased activity could be seen after the inhibition in t4 but not in t5. The results were also confirmed by analysis of the Z-score (Figure 30).

After the association had decayed, 20 reconditioning trials re-established the association between the visual stimulus and cortical activation as demonstrated by

neuronal responses (Figure 31). Recordings were taken from another subject. Left panel: upper row shows the responses to the visual stimulus for 10 trials 7 days after the 140 conditioning trials and 2175 trials of extinction exposure. The neuron showed no significant responses to the visual stimulus (Z-score). The lower row shows responses to the visual stimulus for 10 trials immediately after a 20-trial reconditioning of the combined light and cortical stimulation with the foot shock. Right panel: comparison between Z-scores of neuronal responses to the visual stimulus before and after reconditioning indicated in the left panel (*, P<0.05, t-test).

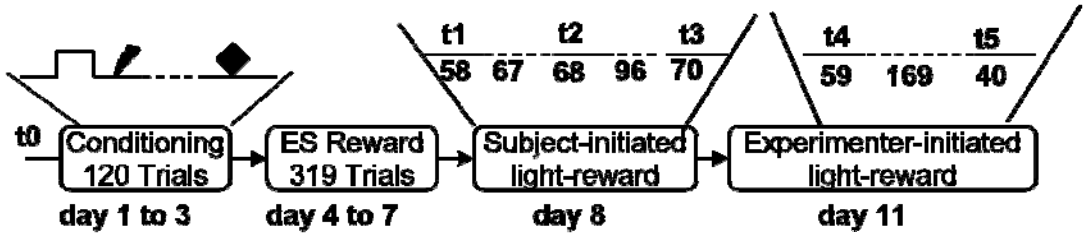

Figure 27 Experiment paradigm of extinction process.

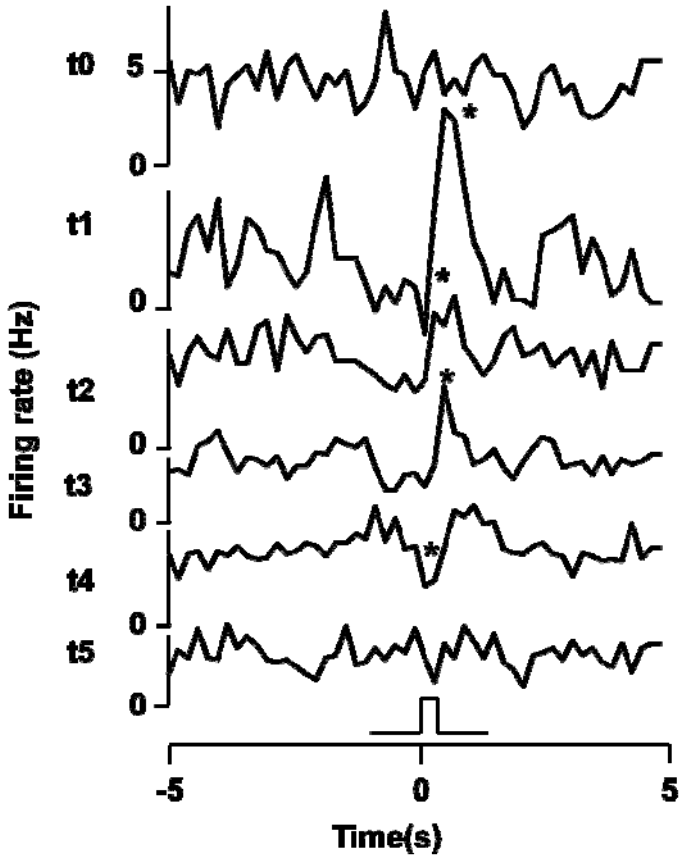

Figure 28 Neuronal responses to the visual stimulus before and at different times after the conditioning. Samples were taken before the conditioning (t0), immediately after the conditioning (t1, 120 trials of conditioning of the visual stimulus and electrical stimulation in the auditory cortex with foot shock), and at different times thereafter (t2 - t5, see Figure 27).

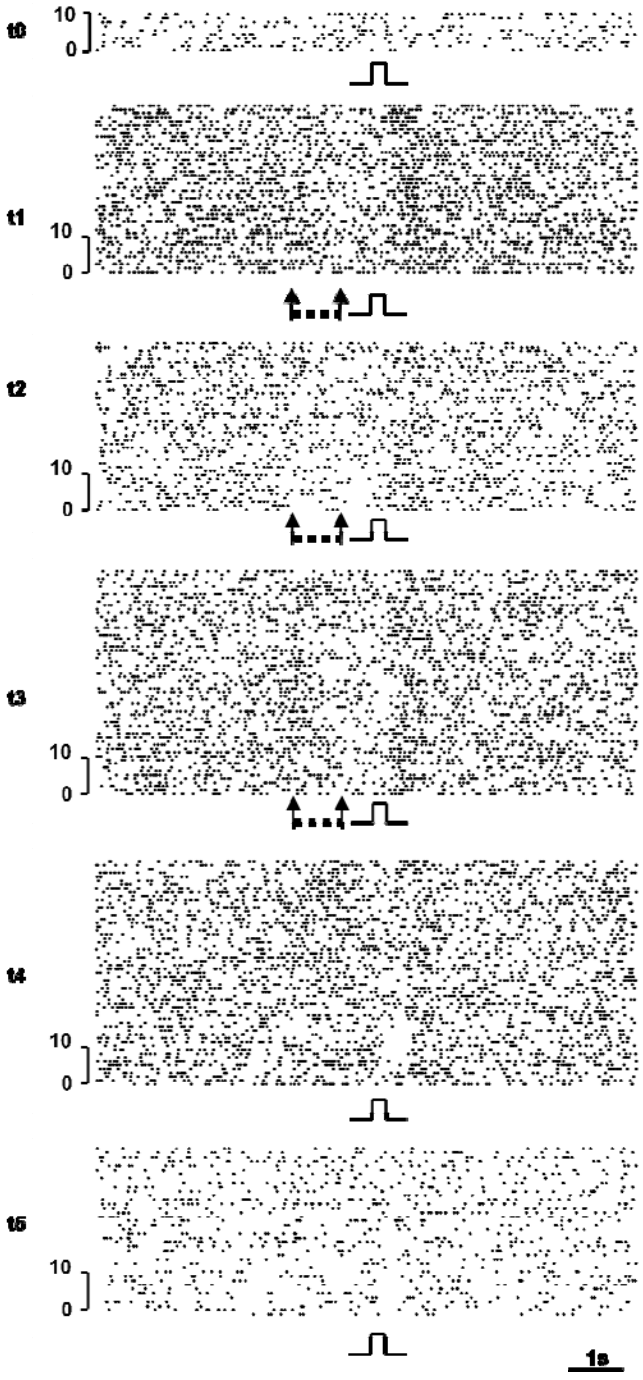

Figure 29 Raster displays show neuronal responses to the repeated light stimuli before and at different times after conditioning.

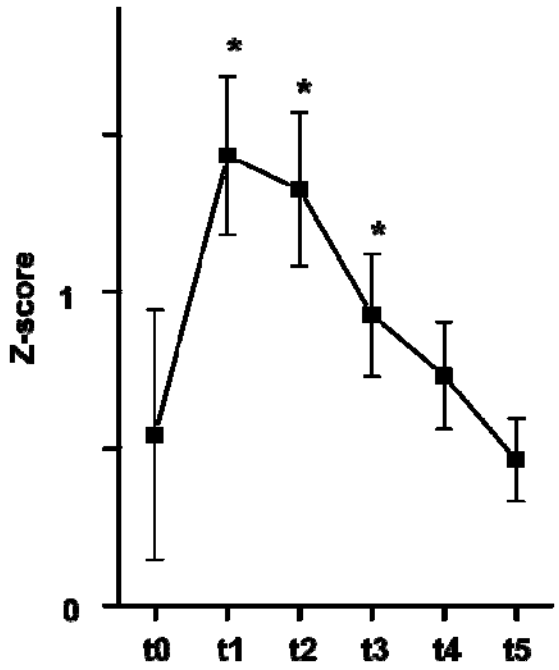

Figure 30 Z-scores to show the neuronal responses to the visual stimulus before and at different times after the conditioning (* $P < 0.05$, compared with t0, ANOVA).

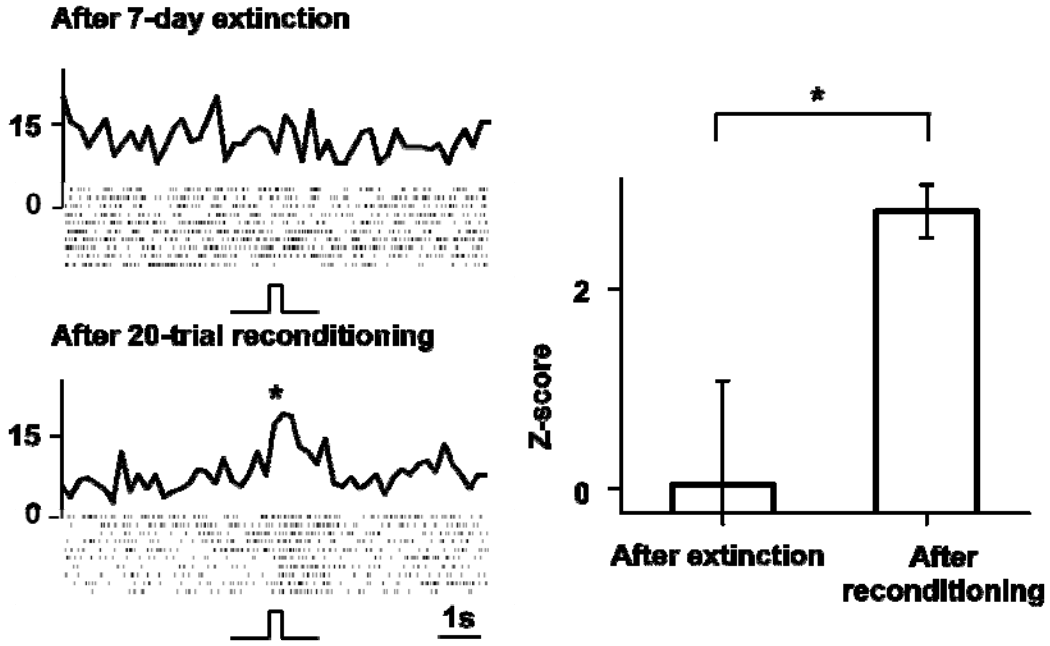

Figure 31 Neuronal responses to the visual stimulus after extinction and after re-conditioning. Recordings were taken from another subject. Left panel: upper row shows the responses to the visual stimulus for 10 trials 7 days after the 140 conditioning trials and 2175 trials of extinction exposure. The neuron showed no significant responses to the visual stimulus (Z-score). The lower row shows responses to the visual stimulus for 10 trials immediately after a 20-trial reconditioning of the combined light and cortical stimulation with the foot shock. Right panel: comparison between Z-scores of neuronal responses to the visual stimulus before and after reconditioning indicated in the left panel (*, $P<0.05$, t-test).

No new associative memory establishment after inactivation of entorhinal cortex (EC)

The most important question of the present study was to examine how the hippocampal system is potentially involved in the establishment of the above association. We targeted the gateway of the hippocampal system, the entorhinal cortex (Figure 32). The entorhinal cortex was inactivated after drug injection, reflecting from the neuronal activities picked up by the monitoring electrodes near the implanted injection cannula (Figure 33). We used classical condition paradigm to associate the visual stimulus and the electrical activation of the auditory cortex. Two electrode arrays, each of which included stimulation and recording electrodes, were implanted in the auditory cortex bilaterally, and neurons at both recording electrodes responded to auditory stimuli (Figure 34). The implanted sites were not strongly connected as the electrical stimulation in left auditory cortex evoked responses in the left side but not in the right auditory cortex (Figure 36). Figure 37 shows the experiment protocol, and the cylinder symbols show either DNQX or lidocaine injection in the entorhinal cortex at 15 min before the 60 conditioning trials.

Unilateral inactivation of the entorhinal cortex greatly affected the establishment of the associative memory both behaviorally and electrophysiologically in bilateral auditory cortex. All subjects after unilateral injection of either DNQX or lidocaine showed behavioral deficit in fear conditioning (n=4, Figure 38). Of four subjects examined physiologically, the establishment of associative memory in classical

conditioning was greatly affected bilaterally (Figure 38). No significant association was established after 120 trials of conditioning in any subject when the entorhinal cortex was unilaterally inactivated (Z-score in Figure 38; raster displays show the example of subject #3 in Figures 39-41). This result was in a great contrast to the result in Figure 5 where all subjects succeeded in establishing the association within 20-60 trials. No significant difference in Z-score could be detected even after 180 trials of conditioning in subjects #3 and #4. It was confirmed that effect of entorhinal cortex inactivation to the association establishment was bilaterally in all 4 subjects.

The association could be easily established on the above subjects in the following session of 60-trial conditioning without drug injection to the entorhinal cortex (n=3, Figure 38; see raster displays for subject #3). All three available subjects showed significant changes in Z-score in their responses to the light stimulus (*, $P < 0.05$; **, $P<0.001$, t-test). A further 40-trial conditioning enhanced the association as reflected in the Z-score in subject #1. Again, the association of the light stimulus was established to bilateral cortices where electrical stimulation electrodes were implanted. The establishment of the association was also reflected in the freezing time that reached to about 100% in t4 and t5 (Figure 38).

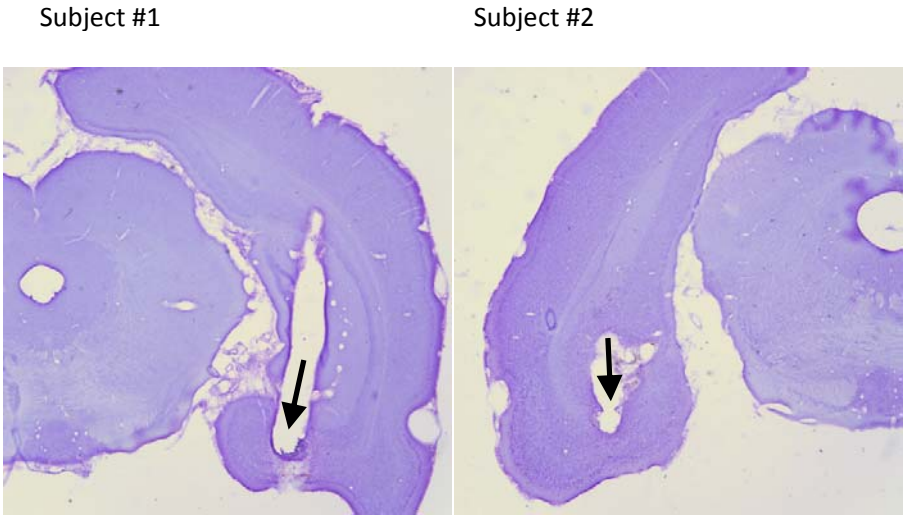

Figure 32 Coronal brain sections show the trajectory (indicated by the arrow) of the cannula of two subjects.

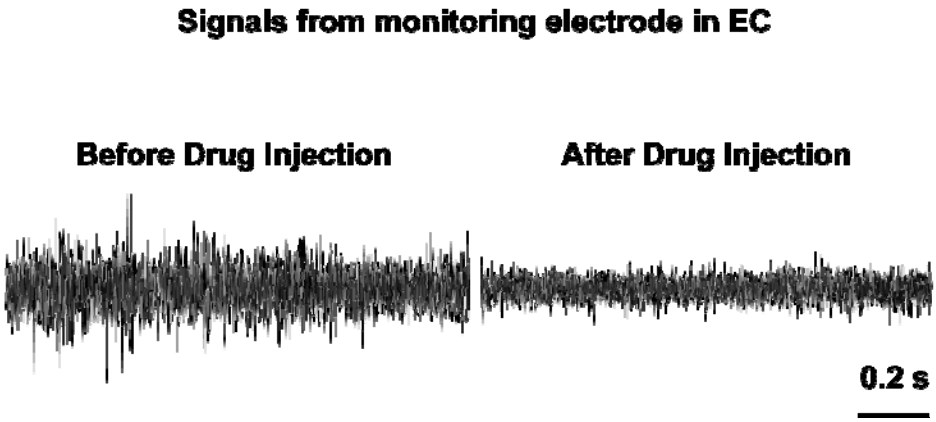

Figure 33 Neuronal activities in the entorhinal cortex before and at 15 min after drug injection

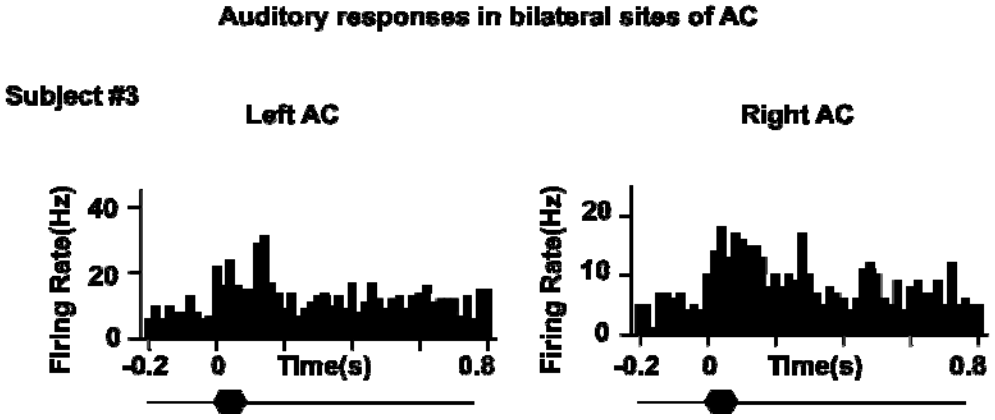

Figure 34 PSTHs show neuronal responses in both the left and right auditory cortices to acoustic stimuli of subject #3

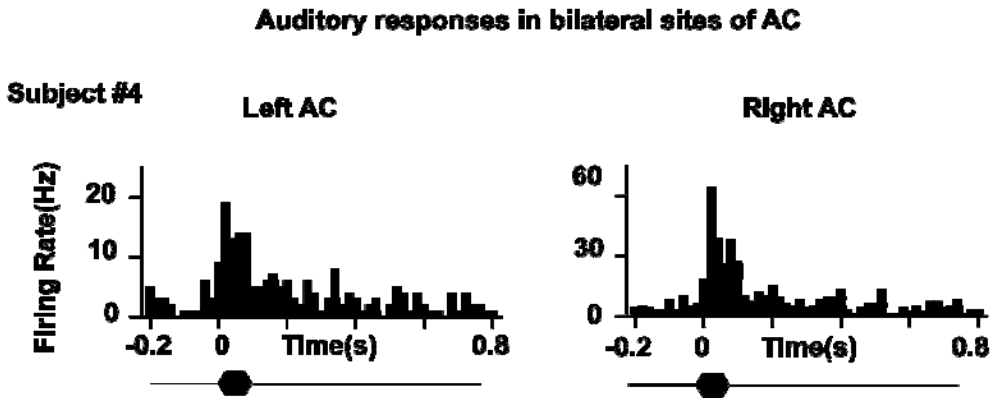

Figure 35 PSTHs show neuronal responses in both the left and right auditory cortices to acoustic stimuli of subject #4

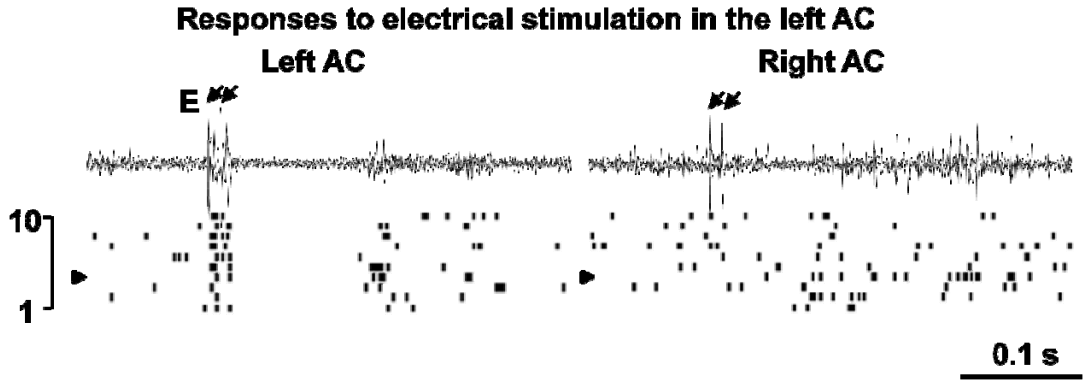

Figure 36 Raw traces and raster displays show neuronal responses in both left and right auditory cortices to two electrical pulses delivered to the left auditory cortex. Arrowheads indicate the trials that the sampled raw data were taken from. Arrows indicate the artifacts of the stimulation pulses.

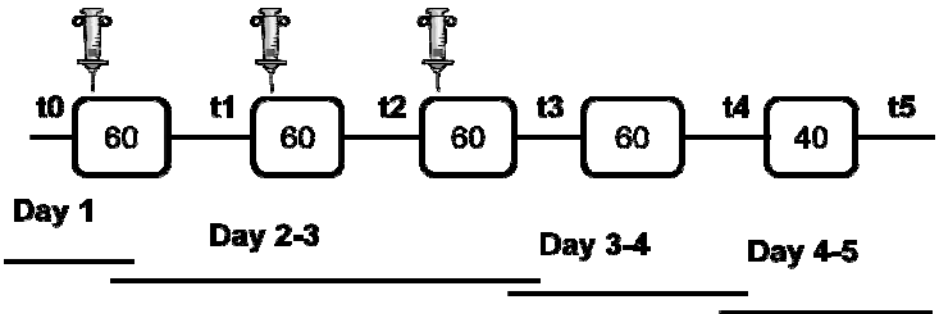

Figure 37 Experiment protocol for training. Cylinder symbols show either DNQX or lidocaine injection in the entorhinal cortex at 15 min before the conditioning of 60 trials. The measuring points, t1-t3, were taken at 4 to 24 h after the drug injection.

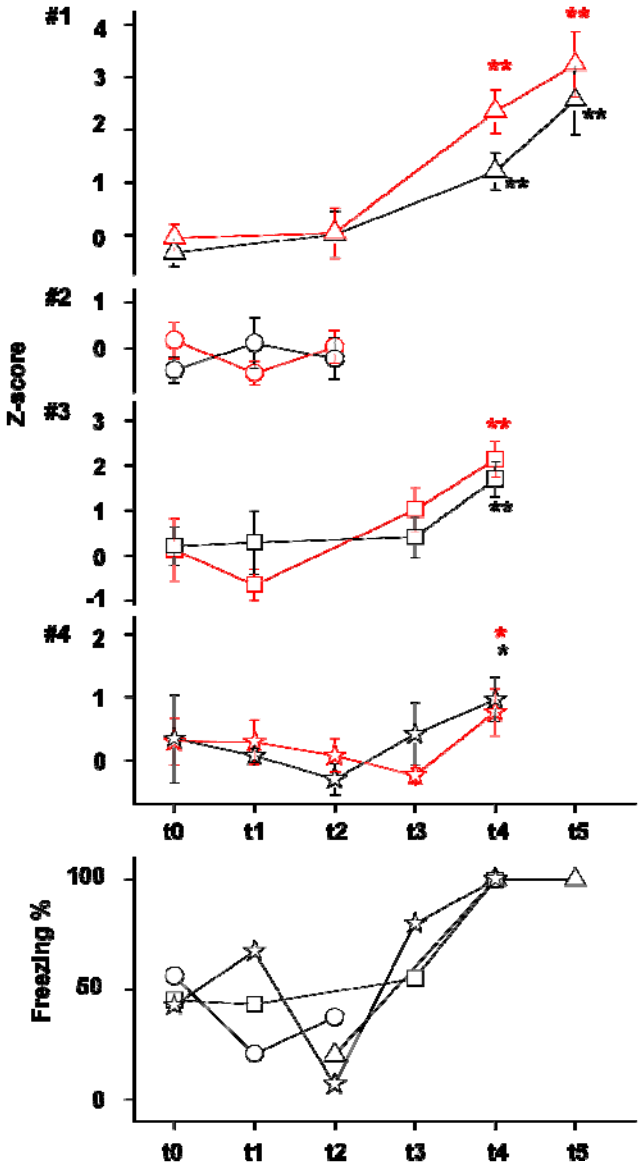

Figure 38 Z-scores of neuronal responses and freezing percentage to the visual stimulus before (t0), after conditioning with the entorhinal cortex inactivated (t1-t3) and further conditioning with entorhinal cortex intact (t4-t5). Red lines and symbols shows activities of auditory cortex ipsilateral to the inactivated EC, and black lines and symbols shows activities of auditory cortex contralateral to the inactivated EC. Four symbols were used to represent four subjects.

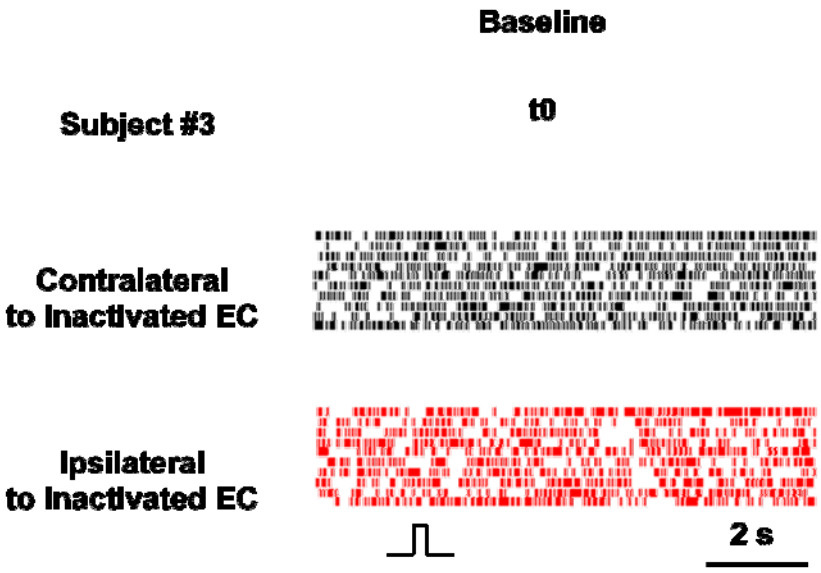

Figure 39 Raster plot at t0 of subject #3. Red one shows activities of auditory cortex ipsilateral to the inactivated EC, and black one shows activities of auditory cortex contralateral to the inactivated EC.

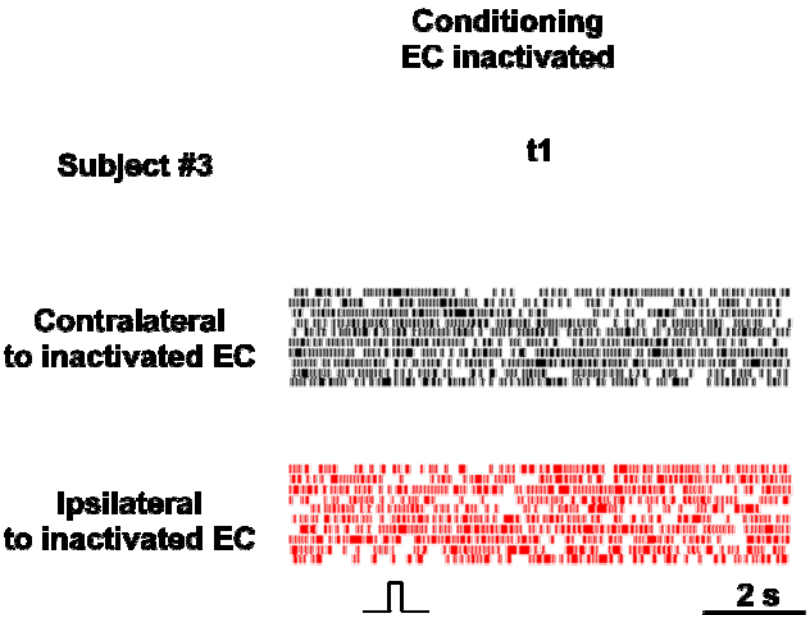

Figure 40 Raster plot at t1 of subject #3

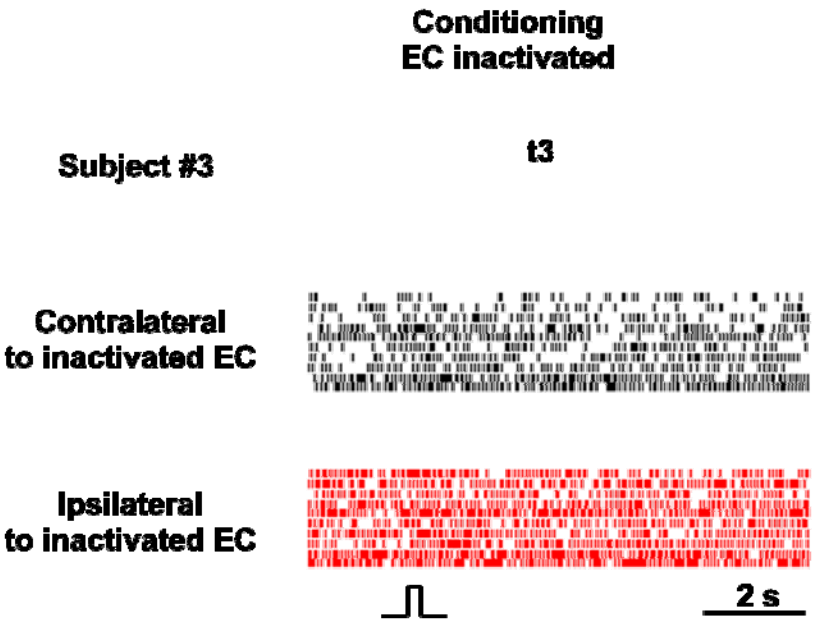

Figure 41 Raster plot at t3 of subject #3

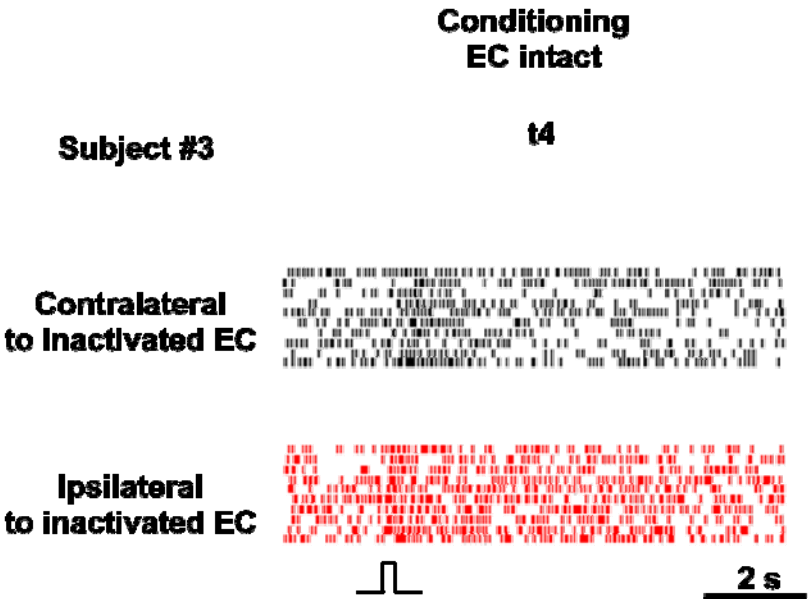

Figure 42 Raster plot at t4 of subject #3

Ipsilateral memory was affected with unilateral inactivation of entorhinal cortex

A total of 120 trials of conditioning with the intact entorhinal cortex were carried out on subjects #1 and #4 to make a strong association before the retrieval test. Figure 43 shows the schedule of the measuring points of retrieval experiment with date information. Date count started from the day when the first baseline data was taken and the conditioning was made, as indicated in Figure 37. The cylinder symbols show either DNQX or lidocaine injection in the entorhinal cortex. The measuring points, t1, t2, and t4, were taken at 15 min after the drug injection.

The inactivation of the entorhinal cortex affected the ipsilateral cortex greatly, but not in the contralateral cortex (Figure 44). The neuron near the stimulation electrode in the ipsilateral auditory cortex to the inactivated entorhinal cortex showed no obvious responses to the light stimulus in the testing trials (Raster displays Figure 45, 46 and 48; Figure 44). Statistically there were no significant responses to the light stimulus in the Z-score (Figure 44). However, the retrieval in the contralateral auditory cortex was not affected (Figure 44). An insertion of a test session in which no drug was injected to the entorhinal cortex showed that the both the ipsi- and contralateral cortices were activated by the testing trials and their Z-scores were significantly higher than 0. Comparing the Z-scores over the time periods of 6-9 days, the extinction process of the associations was observed over the 6-9 days and repeated testing. The Z-scores became not significant at t4 at both hemispheres and both subjects. Their extinction processes were comparable to the subject in Figure

30. The freezing time was kept at a high level of > 75% in t1 and t2 (Figure 44) and showed a fading trend, similar to the extinction process.

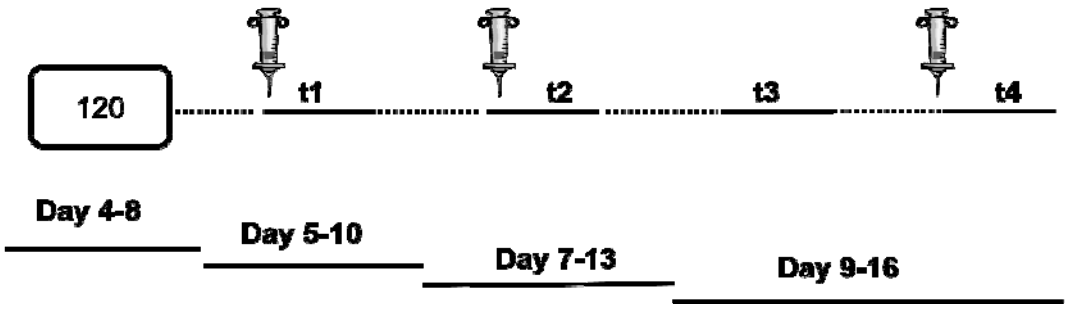

Figure 43 The schedule of the measuring points of retrieval experiment with date information The cylinder symbols show either DNQX or lidocaine injection in the entorhinal cortex. The measuring points, t1, t2, and t4, were taken at15 min after the drug injection.

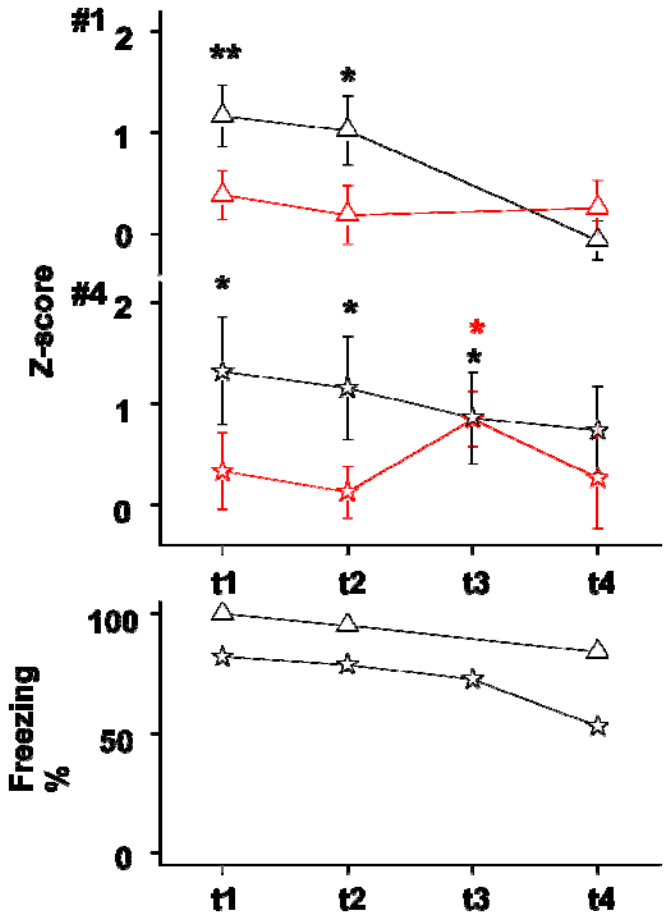

Figure 44 Z-scores of neuronal responses and freezing percentage to visual stimuli in different data points in the retrieval paradigm as shown in the Figure 43. Data points, t1, t2, and t4 were acquired at 15 min after drug injection to the entorhinal cortex, while data point, t3, was acquired when the entorhinal cortex was intact (*, $P < 0.05$, t-test). Red lines and symbols shows activities of auditory cortex ipsilateral to the inactivated EC, and black lines and symbols shows activities of auditory cortex contralateral to the inactivated EC. Two symbols were used to represent two subjects.

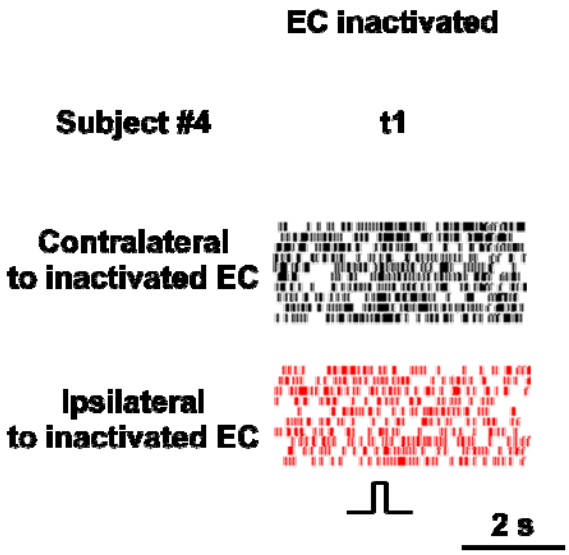

Figure 45 Raster plot at t1 of subject #4. Red one shows activities of auditory cortex ipsilateral to the inactivated EC, and black one shows activities of auditory cortex contralateral to the inactivated EC.

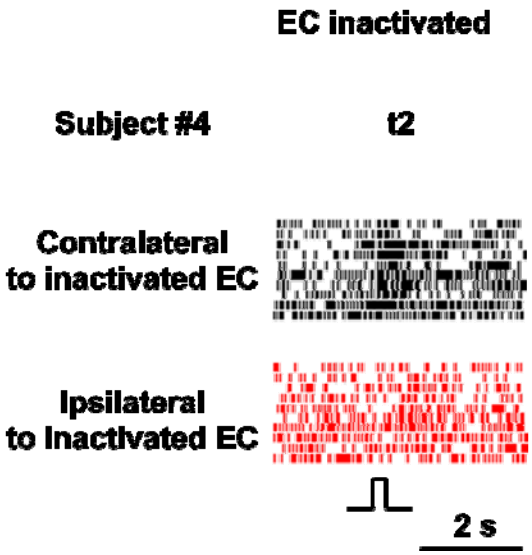

Figure 46 Raster plot at t2 of subject #4

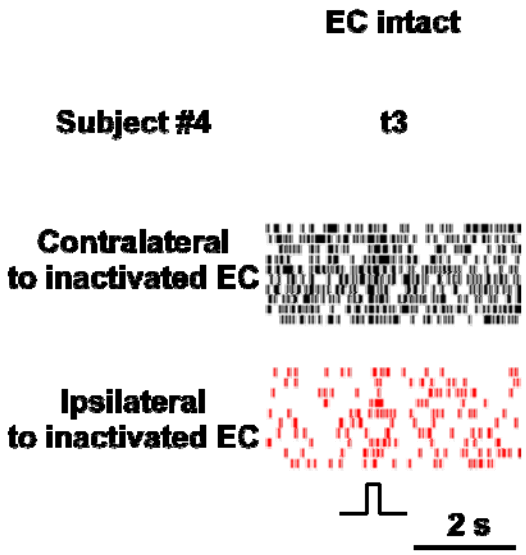

Figure 47 Raster plot at t3 of subject #4

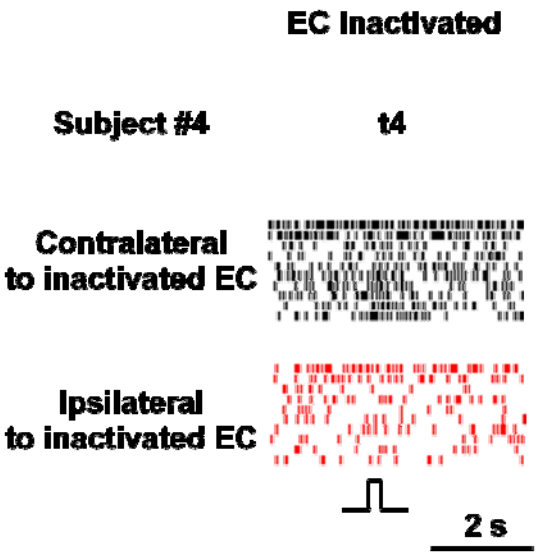

Figure 48 Raster plot at t4 of subject #4

Chapter 4 Conclusion and Discussion

The crucial finding of this study is that cross-modal associative memory between the visual and auditory systems (i.e., the association between the visual stimulus and cortical stimulation) could be established in 20 trials through conditionings (Figure 4). With the combined stimulation and recording electrode array, the establishment of the association between the cortical stimulation and the visual stimulus could be monitored through the recording electrodes in the array when the visual stimulus was presented. The physiological result that the associative memory could be established within 20 trials was further supported by the behavioral experiments, in which only 10 trials of conditioning established the associative memory (Figures 12 - 14). Unilateral inactivation of the entorhinal cortex totally abolished the establishment of the associations bilaterally between the electrical activation of the auditory cortices and the light stimulus after 60 to 180 trials of conditioning. As a control, the association was established in bilateral cortices without the inactivation of the entorhinal cortex on the same subjects after 60 trials of conditioning. The comparison could also be made between the subjects with and without injection. Once the association was established, the inactivation of the entorhinal cortex affected the retrieval of association in the ipsilateral cortex, but not the contralateral cortex.

In humans, an auditory stimulus alone could activate the visual cortex after paired auditory-visual stimuli conditioning[18, 19, 37]. The auditory cortex is activated by silent lip-reading and a cue light stimulus[14, 26, 57] and the visual cortex is activated by

auditory stimuli[19, 58, 59]. In trained monkeys, somatosensory neurons respond to auditory and/or visual stimuli[24, 25] and prefrontal cortical neurons responded to both auditory and visual stimuli1[8, 10, 12, 60]. The present results show for the first time the process where two physically separated sensory cortices are associated after brief training in a behavioral situation, extending our understanding from human to animal model that the auditory cortex can respond to a purely visual stimulus after a brief exposure to bimodal audiovisual stimuli[18, 61]. Recent studies have shown that cross-modal association and emotional memory are more likely to occur in the association auditory cortex compared to the primary auditory cortex[61, 62]. We confirmed that the association auditory cortex could be crossmodally linked to the visual stimulus (Figure 16). However, we cannot exclude the possible involvement of primary auditory cortex in the cross-modal association, since the association in six other subjects was made without distinction between primary and association cortices (see also ref. 63[63]). The auditory and visual cortices have direct connections to each other[23, 32, 64] and are both connected to the hippocampal system[38, 65-67]. The present preparation and protocol open new avenues for future investigation of learning and memory. We will be able to address issues such as how the amygdala is involved in the learning process[68], and how does the dorsal thalamus communicate the association of the visual and auditory signals with the hippocampal system including the amygdala with simultaneous recordings from different regions while the animal is performing a conditioned association[69]. Experiments in culture and slice preparations have shown stimulus-dependent changes in activity after repeated paired stimuli, and these changes depend on the

interval between the paired stimuli[29, 70, 71]. The present results extend this finding to an *in vivo* preparation with a longer time interval of 200 ms between the sound and visual stimuli, possibly indicating a difference between a limited network with *in-vitro* preparation and the whole brain with *in-vivo* preparation.

It was critical to confirm that the rat recalled the auditory memory by approaching the reward at the first trial after replacement of the sound stimulus with the visual stimulus. The positive result indicates that the associative memory between the sound and visual stimuli was strong enough in leading to the reward, as the light was only related to earlier foot shock experience. The physiological result in the reward experiment, in which the sound stimulus was replaced with cortical stimulation, shows that neurons at the stimulation site respond to the visual stimulus. The reward-reception part of the experiment is linked to memory retrieval processes. The association between the sound and visual stimuli was firmly made within 10 trials in the behavioral experiments, whereas that between cortical stimulation and the visual stimulus needed up to 20 trials to be detectable at the single neuron level. The pause-fast reaction for reward reception after replacement of the sound cue with the light cue in the experimental subjects (Figure 14), is probably linked to the recall process, (i.e., the visual stimulus triggered the recall of the sound stimulus through the early association with foot shock, and the sound memory led the subject to approach the reward as they were associated). The difference of response times between the subject-initiated sound-reward and the first trial of subject-initiated light-reward probably reflects the time needed for the recalling of sound memory by the visual stimulus.

Though the behavioral experiment in reward-reception would not inform us about the memory extinction as the subject would soon learn to utilize the light cue before approaching the reward, the change in the light-triggered responses at the auditory cortex stimulation site would provide a clue about the course of the association extinction. The shortening of reaction time after repeated trials may indicate a learning process involving a switch to the light→reward procedure from the light→sound→reward procedure.

The hippocampus-neocortex-transfer model proposed that the recent memory activates almost purely the hippocampus and the remote memory activates purely the neocortex[50, 72]. An argument for the slow involvement/consolidation of the cortex is that new memories need to be linked into existing memories in the cortex through a gradual, interleaving process to avoid damaging the existing memories[72]. The present result that the fast establishment of the associative memory in the auditory cortex within 10 min indicates that memory is stored in the cortex from the beginning, suggesting a revised model in which the neocortex is involved/activated strongly even for the recent memory (Fig. 2 of ref. 72[72]). The result that the establishment of the associative memory in the cortex needs the involvement of the hippocampal system in an animal model substantiates the previous observation in the human that no new enduring associative memory cannot be established without the involvement of the hippocampal system[40, 41, 44]. Most of the hippocampal-cortical connections are mediated by way of entorhinal-perirhinal-cortical connections and entorhinal-cortical connections[38, 73]. Both of the connections need to go through the entorhinal cortex. A unilateral inactivation of

the entorhinal cortex would affect the establishment of the association of the cortex in both hemispheres with our experimental conditions. The memory retrieval after establishment needed the involvement only the ipsilateral entorhinal cortex. The memory retrieval of either hemisphere would likely correlate with behavioral context, i.e., freezing time (Fig. 44).

This study presents most direct physiological evidence of the establishment of memory traces in the auditory cortex of behaving rats, showing the association between the auditory and visual modalities. The results fill the gap of the process that neurons of one modality respond to other modality stimulus in highly trained monkeys. The straightforward physiological results from behavioral subjects confirmed these findings and provided four further steps concerning the cross-modal associative memory. First, associative memory can be established at single neuron level after 20 trials of conditioning. Second, neuronal responses reflect the recalling of the established memory. Third, the associative memory could be confirmed behaviorally, and only 10 trials of conditioning were needed. Finally, the extinction process lasts for hundreds trials of testing in several days and reestablishment of the extincted memory happens after a brief reconditioning. The establishment of the associative memory in the auditory cortex needed the involvement of the entorhinal cortex. It was interesting to note that a unilateral inactivation of the entorhinal cortex affected the establishment of the association bilaterally in the cortex. That was also confirmed behaviorally as the animal showed no context learning. This result may reflect the bilateral projection of the entorhinal cortical neurons to the hippocampus (48). However, the retrieval of the memory was only affected in the

ipsilateral cortex to the inactivated entorhinal cortex, suggesting a less dependence of the hippocampal system in the retrieval than in the formation of associative memory. The present results imply that the hippocampal system is necessary for the formation of associative memories in the auditory cortex from the beginning. Memory retrieval still needs the involvement of the hippocampal system at least at early stage within 10 days, though its dependence on the hippocampal system would be less than that of the formation of associative memory.

Reference

1. Sumby, W.H. & Pollack, I. Visual contribution to speech intelligibility in noise. *Journal of the Acoustical Society of America* **26**, 212-215 (1954).

2. McGurk, H. & MacDonald, J. Hearing lips and seeing voices. *Nature* **264**, 746-748 (1976).

3. Pandya, D.N., Barnes, C.L. & Perecman, E. Architecture and connections of the frontal lobe. in *The frontal lobes revisited.* 41-72 (New York, NY, US: The IRBN Press, 1987).

4. Cusick, C.G., Seltzer, B., Cola, M. & Griggs, E. Chemoarchitectonics and corticocortical terminations within the superior temporal sulcus of the rhesus monkey: evidence for subdivisions of superior temporal polysensory cortex. *J Comp Neurol* **360**, 513-535 (1995).

5. Seltzer, B., *et al.* Overlapping and nonoverlapping cortical projections to cortex of the superior temporal sulcus in the rhesus monkey: double anterograde tracer studies. *J Comp Neurol* **370**, 173-190 (1996).

6. Sakai, K. & Miyashita, Y. Neural organization for the long-term memory of paired associates. *Nature* **354**, 152-155 (1991).

7. Lipton, P.A., Alvarez, P. & Eichenbaum, H. Crossmodal associative memory representations in rodent orbitofrontal cortex. *Neuron* **22**, 349-359 (1999).

8. Fuster, J.M., Bodner, M. & Kroger, J.K. Cross-modal and cross-temporal association in neurons of frontal cortex. *Nature* **405**, 347-351 (2000).

9. Schlack, A., Sterbing-D'Angelo, S.J., Hartung, K., Hoffmann, K.P. & Bremmer, F. Multisensory space representations in the macaque ventral intraparietal area. *J Neurosci* **25**, 4616-4625 (2005).

10. Sugihara, T., Diltz, M.D., Averbeck, B.B. & Romanski, L.M. Integration of auditory and visual communication information in the primate ventrolateral prefrontal cortex. *J Neurosci* **26**, 11138-11147 (2006).

11. Arno, P., *et al.* Occipital activation by pattern recognition in the early blind using auditory substitution for vision. *Neuroimage* **13**, 632-645 (2001).

12. Sadato, N., *et al.* Activation of the primary visual cortex by Braille reading in blind subjects. *Nature* **380**, 526-528 (1996).

13. Finney, E.M., Fine, I. & Dobkins, K.R. Visual stimuli activate auditory cortex in the deaf. *Nat Neurosci* **4**, 1171-1173 (2001).

14. Calvert, G.A., *et al.* Activation of auditory cortex during silent lipreading. *Science* **276**, 593-596 (1997).

15. Bernstein, L.E., *et al.* Visual speech perception without primary auditory cortex activation. *Neuroreport* **13**, 311-315 (2002).

16. Pekkola, J., *et al.* Attention to visual speech gestures enhances hemodynamic activity in the left planum temporale. *Hum Brain Mapp* **27**, 471-477 (2006).

17. Foxe, J.J., *et al.* Auditory-somatosensory multisensory processing in auditory association cortex: an fMRI study. *J Neurophysiol* **88**, 540-543 (2002).

18. Zangenehpour, S. & Zatorre, R.J. Crossmodal recruitment of primary visual cortex following brief exposure to bimodal audiovisual stimuli. *Neuropsychologia* **48**, 591-600 (2010).

19. McIntosh, A.R., Cabeza, R.E. & Lobaugh, N.J. Analysis of neural interactions explains the activation of occipital cortex by an auditory stimulus. *J Neurophysiol* **80**, 2790-2796 (1998).

20. Kayser, C. & Logothetis, N.K. Do early sensory cortices integrate cross-modal information? *Brain Struct Funct* **212**, 121-132 (2007).

21. Kayser, C., Petkov, C.I. & Logothetis, N.K. Multisensory interactions in primate auditory cortex: fMRI and electrophysiology. *Hear Res* **258**, 80-88 (2009).

22. Kayser, C., Petkov, C.I. & Logothetis, N.K. Visual modulation of neurons in auditory cortex. *Cereb Cortex* **18**, 1560-1574 (2008).

23. Bizley, J.K., Nodal, F.R., Bajo, V.M., Nelken, I. & King, A.J. Physiological and anatomical evidence for multisensory interactions in auditory cortex. *Cereb Cortex* **17**, 2172-2189 (2007).

24. Zhou, Y.D. & Fuster, J.M. Visuo-tactile cross-modal associations in cortical somatosensory cells. *Proc Natl Acad Sci U S A* **97**, 9777-9782 (2000).

25. Zhou, Y.D. & Fuster, J.M. Somatosensory cell response to an auditory cue in a haptic memory task. *Behav Brain Res* **153**, 573-578 (2004).

26. Brosch, M., Selezneva, E. & Scheich, H. Nonauditory events of a behavioral procedure activate auditory cortex of highly trained monkeys. *J Neurosci* **25**, 6797-6806 (2005).

27. Lemus, L., Hernandez, A., Luna, R., Zainos, A. & Romo, R. Do sensory cortices process more than one sensory modality during perceptual judgments? *Neuron* **67**, 335-348 (2010).

28. Maren, S. Neurobiology of Pavlovian fear conditioning. *Annu Rev Neurosci* **24**, 897-931 (2001).

29. Johnson, H.A., Goel, A. & Buonomano, D.V. Neural dynamics of in vitro cortical networks reflects experienced temporal patterns. *Nat Neurosci* **13**, 917-919 (2010).

30. Buonomano, D.V. Timing of neural responses in cortical organotypic slices. *Proc Natl Acad Sci U S A* **100**, 4897-4902 (2003).

31. Cappe, C. & Barone, P. Heteromodal connections supporting multisensory integration at low levels of cortical processing in the monkey. *Eur J Neurosci* **22**, 2886-2902 (2005).

32. Falchier, A., Clavagnier, S., Barone, P. & Kennedy, H. Anatomical evidence of multimodal integration in primate striate cortex. *J Neurosci* **22**, 5749-5759 (2002).

33. Smiley, J.F., *et al.* Multisensory convergence in auditory cortex, I. Cortical connections of the caudal superior temporal plane in macaque monkeys. *J Comp Neurol* **502**, 894-923 (2007).

34. Schroeder, C.E., *et al.* Somatosensory input to auditory association cortex in the macaque monkey. *J Neurophysiol* **85**, 1322-1327 (2001).

35. Budinger, E., Heil, P., Hess, A. & Scheich, H. Multisensory processing via early cortical stages: Connections of the primary auditory cortical field with other sensory systems. *Neuroscience* **143**, 1065-1083 (2006).

36. Romanski, L.M. & LeDoux, J.E. Information cascade from primary auditory cortex to the amygdala: corticocortical and corticoamygdaloid projections of temporal cortex in the rat. *Cereb Cortex* **3**, 515-532 (1993).

37. McIntosh, A.R. & Gonzalez-Lima, F. Large-scale functional connectivity in associative learning: interrelations of the rat auditory, visual, and limbic systems. *J Neurophysiol* **80**, 3148-3162 (1998).

38. Insausti, R., Herrero, M.T. & Witter, M.P. Entorhinal cortex of the rat: cytoarchitectonic subdivisions and the origin and distribution of cortical efferents. *Hippocampus* **7**, 146-183 (1997).

39. Suzuki, W.A. The anatomy, physiology and functions of the perirhinal cortex. *Curr Opin Neurobiol* **6**, 179-186 (1996).

40. Scoville, W.B. & Milner, B. Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry* **20**, 11-21 (1957).

41. Corkin, S., Kosslyn, S.M. & Andersen, R.A. Lasting consequences of bilateral medial temporal lobectomy: Clinical course and experimental findings in H. M. in *Frontiers in cognitive neuroscience.* 516-526 (Cambridge, MA, US: The MIT Press, 1992).

42. Teng, E. & Squire, L.R. Memory for places learned long ago is intact after hippocampal damage. *Nature* **400**, 675-677 (1999).

43. Wang, S.H., Teixeira, C.M., Wheeler, A.L. & Frankland, P.W. The precision of remote context memories does not require the hippocampus. *Nat Neurosci* **12**, 253-255 (2009).

44. Lesburgueres, E.*, et al.* Early tagging of cortical networks is required for the formation of enduring associative memory. *Science* **331**, 924-928 (2011).

45. Witter, M.P. Organization of the entorhinal-hippocampal system: a review of current anatomical data. *Hippocampus* **3 Spec No**, 33-44 (1993).

46. van Groen, T., Miettinen, P. & Kadish, I. The entorhinal cortex of the mouse: organization of the projection to the hippocampal formation. *Hippocampus* **13**, 133-149 (2003).

47. Murray, E.A., Gaffan, D. & Mishkin, M. Neural substrates of visual stimulus-stimulus association in rhesus monkeys. *J Neurosci* **13**, 4549-4561 (1993).

48. Higuchi, S. & Miyashita, Y. Formation of mnemonic neuronal responses to visual paired associates in inferotemporal cortex is impaired by perirhinal and entorhinal lesions. *Proc Natl Acad Sci U S A* **93**, 739-743 (1996).

49. Graham, K.S. & Hodges, J.R. Differentiating the roles of the hippocampal complex and the neocortex in long-term memory storage: evidence from the study of semantic dementia and Alzheimer's disease. *Neuropsychology* **11**, 77-89 (1997).

50. Wiltgen, B.J., Brown, R.A., Talton, L.E. & Silva, A.J. New circuits for old memories: the role of the neocortex in consolidation. *Neuron* **44**, 101-108 (2004).

51. Alvarez, P. & Squire, L.R. Memory consolidation and the medial temporal lobe: a simple network model. *Proc Natl Acad Sci U S A* **91**, 7041-7045 (1994).

52. Squire, L.R., Clark, R.E. & Knowlton, B.J. Retrograde amnesia. *Hippocampus* **11**, 50-55 (2001).

53. Yu, Y.Q., Xiong, Y., Chan, Y.S. & He, J. Corticofugal gating of auditory information in the thalamus: an in vivo intracellular recording study. *J Neurosci* **24**, 3060-3069 (2004).

54. Yu, X.J., Xu, X.X., He, S. & He, J. Change detection by thalamic reticular neurons. *Nat Neurosci* **12**, 1165-1170 (2009).

55. Otazu, G.H., Tai, L.H., Yang, Y. & Zador, A.M. Engaging in an auditory task suppresses responses in auditory cortex. *Nat Neurosci* **12**, 646-654 (2009).

56. Jaramillo, S. & Zador, A.M. The auditory cortex mediates the perceptual effects of acoustic temporal expectation. *Nat Neurosci* **14**, 246-251 (2010).

57. Brosch, M., Selezneva, E. & Scheich, H. Formation of associations in auditory cortex by slow changes of tonic firing. *Hear Res* **271**, 66-73 (2011).

58. Crottaz-Herbette, S., Anagnoson, R.T. & Menon, V. Modality effects in verbal working memory: differential prefrontal and parietal responses to auditory and visual stimuli. *Neuroimage* **21**, 340-351 (2004).

59. Martuzzi, R.*, et al.* Multisensory interactions within human primary cortices revealed by BOLD dynamics. *Cereb Cortex* **17**, 1672-1679 (2007).

60. Romanski, L.M. Representation and integration of auditory and visual stimuli in the primate ventral lateral prefrontal cortex. *Cereb Cortex* **17 Suppl 1**, i61-69 (2007).

61. Meyer, M., Baumann, S., Marchina, S. & Jancke, L. Hemodynamic responses in human multisensory and auditory association cortex to purely visual stimulation. *BMC Neurosci* **8**, 14 (2007).

62. Sacco, T. & Sacchetti, B. Role of secondary sensory cortices in emotional memory storage and retrieval in rats. *Science* **329**, 649-656.

63. Weinberger, N.M. Specific long-term memory traces in primary auditory cortex. *Nat Rev Neurosci* **5**, 279-290 (2004).

64. Rockland, K.S. & Ojima, H. Multisensory convergence in calcarine visual areas in macaque monkey. *Int J Psychophysiol* **50**, 19-26 (2003).

65. Witter, M.P. & Groenewegen, H.J. Connections of the parahippocampal cortex in the cat. III. Cortical and thalamic efferents. *J Comp Neurol* **252**, 1-31 (1986).

66. Brown, M.W. & Aggleton, J.P. Recognition memory: what are the roles of the perirhinal cortex and hippocampus? *Nat Rev Neurosci* **2**, 51-61 (2001).

67. Mohedano-Moriano, A., *et al.* Topographical and laminar distribution of cortical input to the monkey entorhinal cortex. *J Anat* **211**, 250-260 (2007).

68. Romanski, L.M. & LeDoux, J.E. Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. *J Neurosci* **12**, 4501-4509 (1992).

69. Komura, Y., *et al.* Retrospective and prospective coding for predicted reward in the sensory thalamus. *Nature* **412**, 546-549 (2001).

70. Bi, G. & Poo, M. Distributed synaptic modification in neural networks induced by patterned stimulation. *Nature* **401**, 792-796 (1999).

71. Shahaf, G. & Marom, S. Learning in networks of cortical neurons. *J Neurosci* **21**, 8782-8788 (2001).

72. McClelland, J.L., McNaughton, B.L. & O'Reilly, R.C. Why there are complementary learning systems in the hippocampus and neocortex: insights from

the successes and failures of connectionist models of learning and memory. *Psychol Rev* **102**, 419-457 (1995).

73. Swanson, L.W. & Kohler, C. Anatomical evidence for direct projections from the entorhinal area to the entire cortical mantle in the rat. *J Neurosci* **6**, 3010-3023 (1986).