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**The Hong Kong Polytechnic University**

**Department of Rehabilitation Sciences**

**UNCONSCIOUSLY IMPLANTED MEMORY IN THE  
PRESENCE OF CHOLECYSTOKININ RETRIEVED IN  
A BEHAVIORALLY RELEVANT CONTEXT**

**ZICONG ZHANG**

**A thesis submitted in partial fulfillment of the requirements for the  
degree of Doctor of Philosophy**

**August 2014**

# CERTIFICATION OF ORIGINALITY

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## **Abstract**

Previous studies demonstrated that the perirhinal and entorhinal cortices in the hippocampus serve as the gateway for relaying information from the hippocampus system to the neocortex. Specifically, it was found that Cholecystokinin (CCK), a neuropeptide which is abundant in the perirhinal and entorhinal cortices, enabled neuroplasticity in the auditory cortex through cortical projections of the hippocampus. Another study showed that the cross-modal association can be established in the auditory cortex, which enabled auditory neurons to respond to the visual stimulus. This visuo-auditory association drove animal's behavior in an auditory rewarding task, addressing the influence of the neuronal changes on the behavioral outcomes. More importantly, the application of CCK antagonist in the auditory cortex prevented the establishment of visuo-auditory association, suggesting that the neuroplasticity in the cortex is CCK dependent.

Together with these findings, we postulate that the CCK-induced neuroplasticity in the auditory cortex leads to relative behavioral changes. A series of experiments were conducted to address this hypothesis. Rats implanted bilaterally with electrodes and injection cannula in the auditory cortex were trained to approach the left or right hole of a behavioral apparatus to retrieve a physical reward depending on whether the right or left auditory cortex was electrically stimulated. Next, under

anesthesia, a previously irrelevant light stimulus was repeatedly paired with electrical stimulation at one side of the auditory cortex in the presence of CCK, after which auditory neurons started to respond to the light stimulus in both anesthetized and awake states. In the following behavioral testing, when the light stimulus was presented instead of electrical stimulation, rats approached the hole that was “engineered” to be associated with reward availability. Besides, several control experiments were performed to validate the results and eliminate the non-specific effects of drug application and other manipulations.

Finally we found that the visuo-auditory association induced by CCK was reflected in behavioral context, supporting the hypothesis arisen from the previous studies.

These findings provide a scientific foundation for “memory implantation”.

**Keywords:** Auditory cortex, neural plasticity, operant conditioning, entorhinal cortex, memory encoding, cholecystokinin.

## **Publications arise from the thesis**

### **Journal publications**

Li, X., Yu, K., **Zhang, Z.**, Sun, W., Yang, Z., Feng, J., Chen, X., Liu, C.H., Wang, H, Guo, Y.P., and He, J. Cholecystokinin from the entorhinal cortex enables neural plasticity in the auditory cortex. *Cell Research* 2014; **24**: 307-330.

**Zhang, Z.**, Lu, D., Li, X., Chen, X., Sun, W., Guo, Y., Ng, GYF., and He, J. Unconsciously implanted memory in the presence of cholecystokinin retrieved in a behaviorally relevant context. (In preparation).

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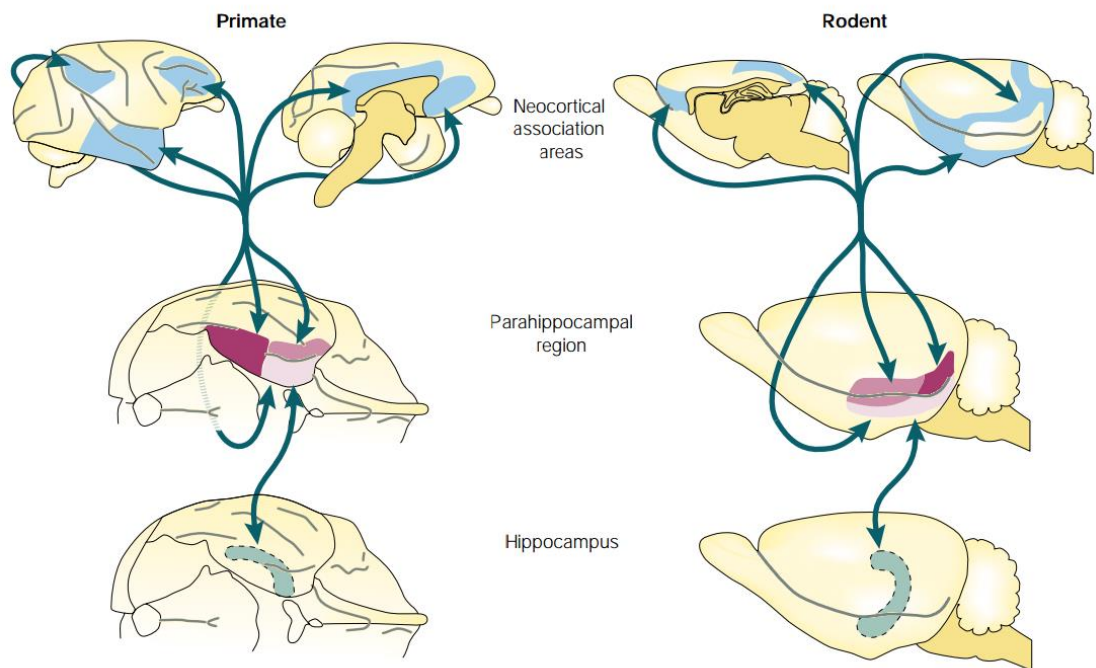


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# Chapter 1: Introduction

## *The hippocampus system in the brain*

The hippocampus system consists of the hippocampus and the adjacent entorhinal and perirhinal cortices (Squire and Zola-Morgan, 1991). These structures connect reciprocally with the neocortex. The parahippocampal region, including perirhinal, entorhinal and postrhinal cortices, serves as a convergence site for cortical input and mediates the distribution of cortical afferents to the hippocampus. The outcome of hippocampal processing is directed back to the parahippocampal region, the output of which is directed back to the same areas of the cerebral cortex that were the source of input to this region (Brown and Aggleton, 2001; Eichenbaum, 2000) (Fig. 1). The entorhinal cortex is a major input and output of the hippocampal system, playing the role of nodal point of cortico-hippocampal circuits. Superficial layers of entorhinal cortex receive cortical information, which is relayed to structures in the hippocampus, and hippocampal output reaches deep layers of entorhinal cortex, that project back to the cortex (Canto et al., 2008). The entorhinal area is the most highly differentiated cortical field in the hippocampal system. The perforant pathway originates from the entorhinal cortex to the dentate gyrus and Ammon's horn is thought to form the basic unit of information processing in the hippocampal system (Swanson and Kohler, 1986).

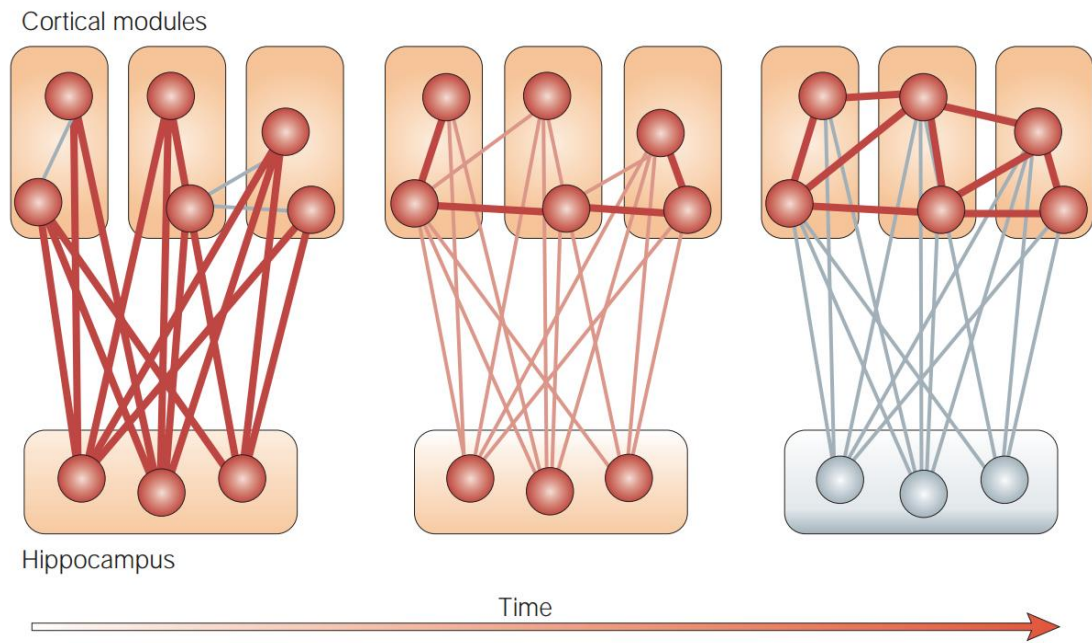


**Figure 1.** The anatomy and connectivity of the hippocampal memory system. Blue, neocortical areas; purple, perirhinal cortex; dark purple, the parahippocampal (or postrhinal) cortex; light purple, the entorhinal cortex. (Eichenbaum, 2000)

## *The role of hippocampus system in memory establishment and consolidation*

The understanding on the functions of hippocampus system in memory comes from the clinical observations in patients with amnesia. Patients with bilateral hippocampal resections showed deficits in forming new long-term memories (Corkin, 1984; Scoville and Milner, 1957), suggesting that hippocampus is essential for memory establishment. These patients however, could still recall their remote memories, such as the memories of the childhood and the old places they used to live (Corkin, 1984; Teng and Squire, 1999). Animal behavioral study showed that remote context memories spared in mice with hippocampal lesion (Wang et al., 2009). These findings suggest that remote memories are stored elsewhere from the hippocampus, most likely in the neocortex (Lesburgueres et al., 2011; Squire et al., 2001). In the standard memory model, the early stage of memory establishment is hippocampus-dependent. The sensory, motor and cognitive information encoded in cortical areas are integrated in the hippocampus and fused into a coherent memory trace. The reactivation of hippocampal-cortical network during memory consolidation strengthens the cortico-cortical connections. As memory ages, the cortico-cortical connections become strong enough to be hippocampal independent (Frankland and Bontempi, 2005) (Fig. 2). The hippocampal-cortical network

integrates new information into the existing memory and forms the episodic memories.



**Figure 2.** The standard model for hippocampus-dependent memory formation.

(Frankland and Bontempi, 2005)

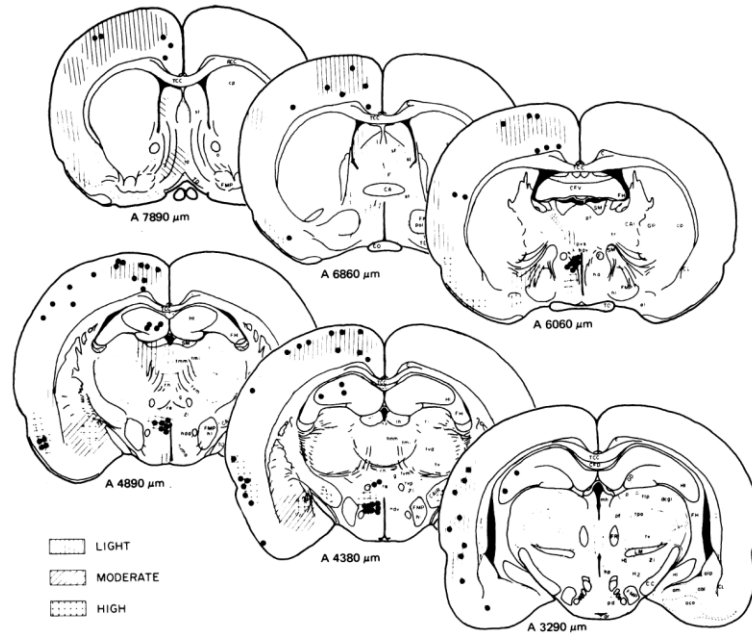
### ***Neuroplasticity induced by hippocampal-cortical projections***

The memory establishment involves a series of neuroplastic changes in the neocortical areas. It remains largely unknown as to how the hippocampus enables neuroplasticity in the neocortex. One of the candidates involved in this process is a neuropeptide named cholecystokinin, or CCK in brief. Previous studies showed that the entorhinal and perirhinal cortices are heavily labeled by CCK (Fig. 3)

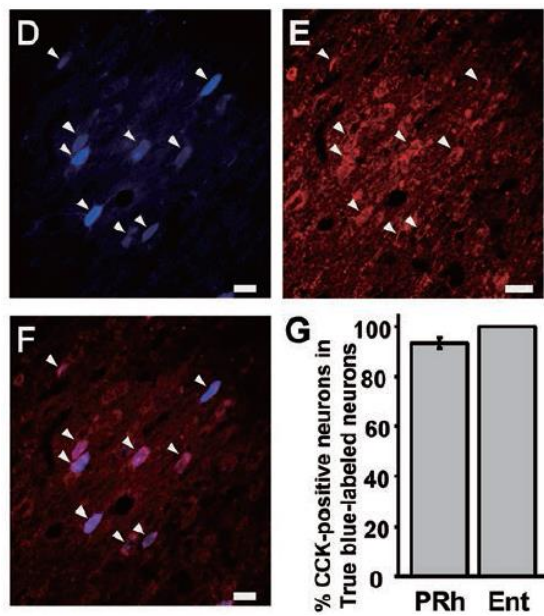
(Greenwood et al., 1981; Innis et al., 1979; Kohler and Chan-Palay, 1982). A recent study showed that the auditory cortical projection neurons in the perirhinal and entorhinal cortices are mostly CCK-positive neurons (Fig. 4) (Li et al., 2014). These immunohistological results serve as the anatomical basis for CCK-involved hippocampus functions.

CCK is the most abundant of all neuropeptides in the brain (Rehfeld, 1978). It has been reported that application of CCK receptor antagonist suppressed conditioned fear (Tsutsumi et al., 1999), and knocking out CCK receptor gene reduced anxious behavior in rodents (Horinouchi et al., 2004). These findings suggest that CCK is involved in memory-related functions. Further studies showed that CCK is involved in long-term potentiation and long-term depression in hippocampal neurons (Dahl and Li, 1994), and mice lacking CCK gene exhibit poor performance in passive avoidance task and impaired spatial memory (Lo et al., 2008). These findings evidenced that CCK is one of the functional chemicals in hippocampus-dependent memory formation.

A recent study demonstrated that CCK potentiated synaptic strength *in vivo* and resulted in long-lasting excitatory postsynaptic potentials (EPSPs) (Li et al., 2014), supporting that CCK participates in the process of synaptic changes, which is thought to be the fundamental process of memory formation.



**Figure 3.** Distribution of CCK octapeptide in the neocortex and hippocampal system (Innis et al., 1979). Black dots indicate CCK-positive neurons.



**Figure 4.** Entorhinal and perirhinal neurons projecting to auditory cortex are mainly CCK-positive neurons (Li et al., 2014). Cortical projection neurons in entorhinal

cortex were labeled by retrograde tracer True Blue (blue). CCK was labeled in red by immuno-staining.

### ***Cross-modal association in the brain***

It is known that the experience of one sensory modality can influence the activity of cortical areas of other sensory modalities. This is defined as cross-modal association.

For example, auditory stimuli can evoke neural response in visual cortex (McIntosh et al., 1998; McIntosh and Gonzalez-Lima, 1998; Zangenehpour and Zatorre, 2010).

Visual stimuli can evoke and modulate neural response in the auditory cortex (Bizley et al., 2007; Kayser et al., 2007; Kayser et al., 2008; Meyer et al., 2007).

The cross-modal responses in both visual and auditory cortex would increase after brief exposure to bi-modal audiovisual stimuli (Zangenehpour and Zatorre, 2010).

The association also occurred in other cortices. In monkeys, somatosensory and prefrontal cortical neurons respond to both visual and auditory stimuli (Fuster et al.,

2000; Watanabe, 1992). Visual, somatosensory and even motor inputs can evoke auditory neuronal response after associative task training (Brosch et al., 2005, 2011;

Scheich et al., 2011). Moreover, the association between different modalities has been found to occur at the single neuronal level (Sugihara et al., 2006). Taken

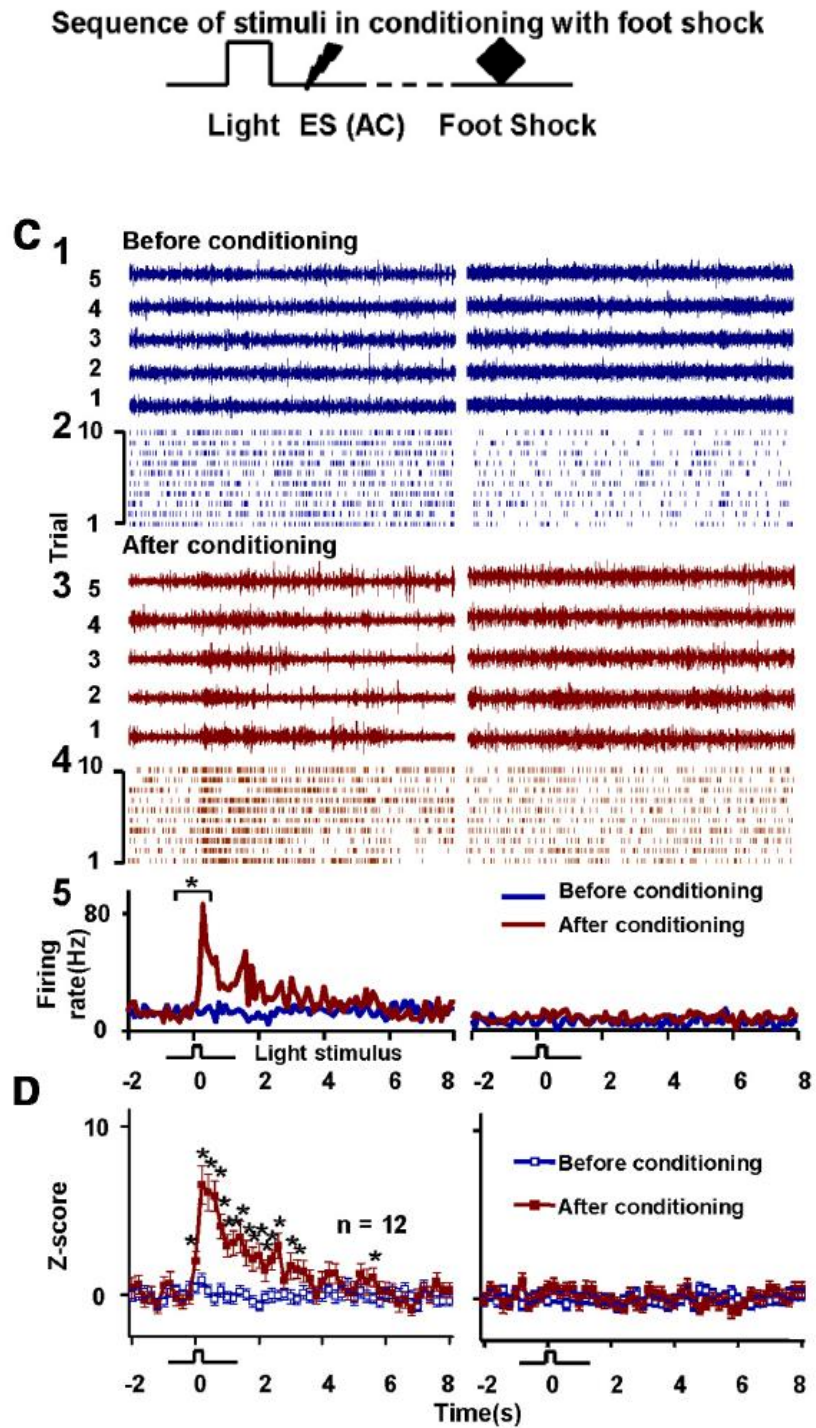


together, the studies suggest that a cross-modal associative memory could be established using appropriate training protocol.

Anatomical connections between visual and auditory cortices serve as the basis for integrating cross-modal sensory information. Connections occur both at subcortical and cortical levels. At subcortical level, for example, visual information is transmitted from superior colliculus to the medial geniculate nucleus of the auditory pathway (Melchner et al., 2000). At cortical level, there are direct reciprocal projections between visual and auditory cortices (Vaudano et al., 1991; Falchier et al., 2010).

In a recent study, visuo-auditory associative memory was established in the auditory cortex (Chen et al., 2013). Visual stimulus can be associated with electrical stimulation in rat's auditory cortex by classical fear conditioning. This associative memory is reflected as the visual response in the auditory cortex (Fig. 5), and also reflected in the behavioral context in the subsequent rewarding task. In the task, rats were trained to use electrical stimulation of the auditory cortex as the cue for water reward. Electrical stimulation was then replaced by light stimulus in the testing trials. With the existence of visuo-auditory association, all trained animals were able to correctly respond to the light stimulus and retrieve the water reward. These results

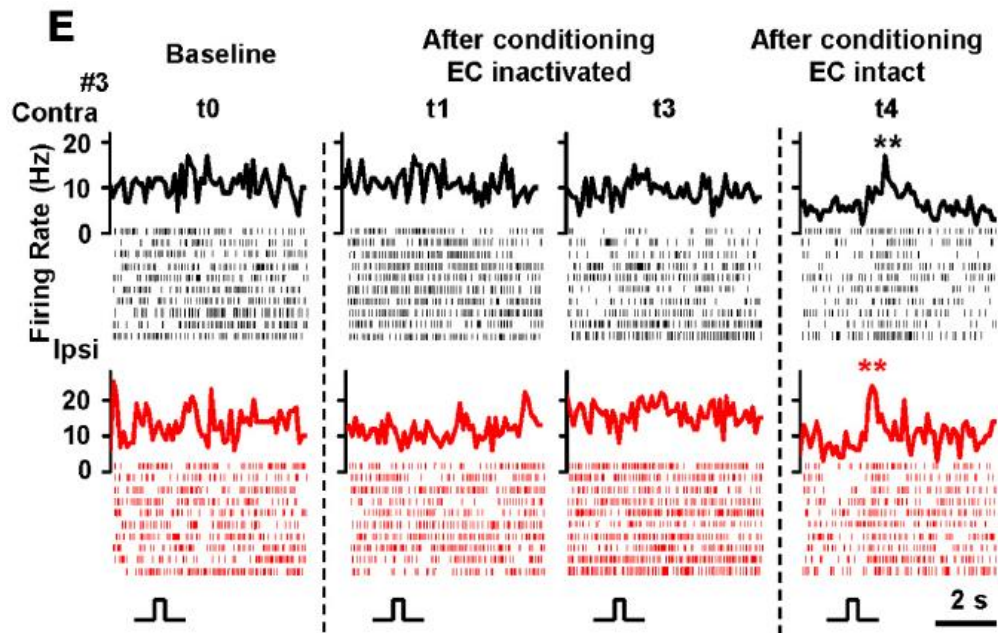
indicate that the visuo-auditory associative memory can be related to animal's behavior and expressed under certain condition.



**Figure 5.** Visual responses in the auditory cortex induced by fear conditioning. The upper panel shows the sequence of stimulus presentations during conditioning. Raw data, raster plots, PSTHs and Z-scores in the lower panel demonstrate neuronal

responses at the stimulation and control sites to repeated visual stimuli before (blue) and after (red) conditioning. (Chen et al., 2013)

Further experiments showed that unilateral and temporal inactivation of the entorhinal cortex prevented the encoding of such associative memory (Fig. 6), as well as the retrieval of previously established associative memory (Chen et al., 2013). It indicates that entorhinal cortex is necessary for the establishment of visuo-auditory associative memory. The findings further support the view mentioned above that entorhinal cortex functions as the node for gating integrated information transferring from the hippocampus to the neocortex, and the integration of different sensory information is the essential process of cross-modal association.



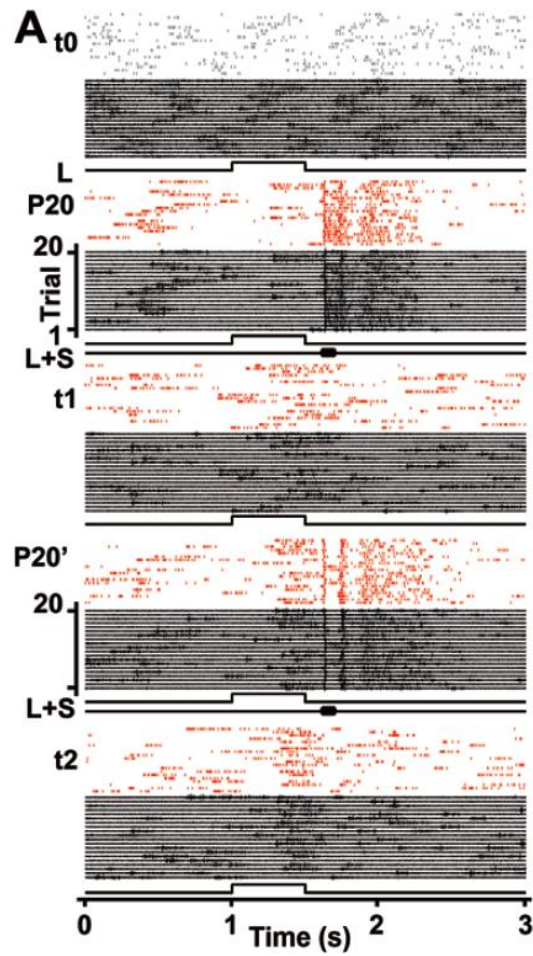
**Figure 6.** Unilateral inactivation of entorhinal cortex prevents visuo-auditory association establishment. Red, inactivation site; black, contralateral site. (Chen et al., 2013)

### *CCK enables neural plasticity in the cortex*

A recent study took these two areas together and found that CCK is actually involved in the hippocampus-dependent memory formation. Activation of entorhinal cortex potentiated neuronal responses in the auditory cortex, which can be suppressed by CCK receptor antagonist. Infusion of CCK in the auditory cortex enabled auditory neurons to respond to a light stimulus that was repeatedly paired with acoustic stimuli for only 20 trials (Fig. 7) (Li et al., 2014). This result is similar

to the previous findings (Chen et al., 2013), but it further highlights the direct impact of CCK in the establishment of cross-modal association.

All together, these findings indicate that the CCK positive cortical projections from the entorhinal cortex play an essential role in the cross-modal associative memory formation. The underlying process of neural plasticity is CCK dependent, and it can be simulated under the influence of endogenous CCK application. These cutting edge discoveries led us to think of a way to artificially influence the associative memory to certain extend, and examine the behavioral outcome using appropriate measurements.



**Figure 7.** Visual responses in the auditory cortex after pairing visuo-auditory stimuli in the presence of CCK. Raster plots and raw traces show neuronal responses to the light stimulus (L; t0, t1 and t2) and the combined light and noise burst stimulus (L+S; P20 and P20') at different times before (grey) and after (red) CCK infusion. (Li et al., 2014)

### *Aims of this study*

It remains unknown whether such associative memory established in the cortex is dependent on CCK on site. In the present study, CCK antagonist was infused into rat's auditory cortex, when the visual stimuli and electrical stimulations in the auditory cortex were associated by classical fear conditioning. We examined the electrophysiological changes of the auditory neurons in response to visual stimuli after the fear conditioning. This result will tell us whether the CCK in the auditory cortex is involved in the cross-modal association established by associative learning in the awake animal.

To further test our hypothesis that CCK is a memory-writing chemical in the neocortex, we tested whether the presence of CCK would allow us to implant a memory in the cortex of anesthetized rats that could be retrieved in a behaviorally relevant context. Rats with electrodes implanted in the bilateral auditory cortex were trained to retrieve water reward from the left or right hole of a behavioral apparatus depending on whether the right or left auditory cortex was stimulated. An initially irrelevant light stimulus was then repeatedly paired with electrical stimulation of the auditory cortex in one hemisphere after infusion of CCK under anesthesia, potentially establishing an artificial link between the light stimulus and stimulation of the CCK-infused hemisphere. Finally, we tested whether the light stimulus would



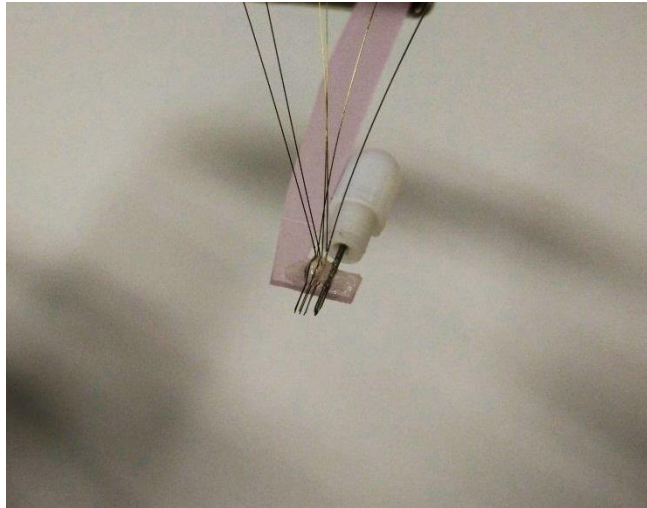
activate the visuo-auditory association and lead the rats to approach the hole that was “engineered” to be associated with the reward.

## **Chapter 2: Methods**

The methods in this study mainly consists of training apparatus design and behavioral training, micro-surgery and electrode implantation, multi-unit extracellular recording in anesthetized and behaving states, micro-injection, histological observation and off-line data analysis. Most experimental designs are described in detail in this chapter while some additional information will be introduced in the following chapters.

### ***Electrode array assembling***

The electrode array for chronic implantation was custom made. The 2x3 electrode array composed of four tungsten recording electrodes (0.5-1.0 MOhm, California Fine Wire Company, CA), one stainless steel stimulation electrode (<100 kOhm, A-M Systems, WA) and one reference electrode with the same material as recording electrode except for the 1 mm tip exposure in order to have low impedance. The tip-sharpened guidance cannula with dummy tube (6 mm, RWD Life Science, China) was fixed near the stimulation electrode. The distance between the adjacent electrodes was 500  $\mu\text{m}$ , and the guidance cannula was around 800  $\mu\text{m}$  to the stimulation electrode (Fig. 8).



**Figure 8.** Electrode array and injection guide cannula.



**Figure 9.** The training box decorated with poking holes and stainless steel grid, for both discrimination task and fear conditioning.

### ***Training apparatus for rewarding discrimination task and fear conditioning***

A training apparatus was designed for conducting both rewarding discrimination task and fear conditioning (Fig. 9). The size of the acrylic training cage was 30 (L) × 30 (W) × 40 (H) (cm). A stainless steel grid was placed at the bottom of the cage. The front panel of the cage was decorated with three holes in a horizontal direction. All the holes were equipped with infrared sensors for detecting nose poke of the animal. The two side holes were also equipped with water dropping tubes for delivering water reward. Nose poke detected by the infrared sensors was transferred to input signal and sent to RX7 Stimulator Base Station of the TDT system (Tucker-Davis Technologies, FL). An OpenEx program was created to convert and calculate the input signal, analyze animal's behavior and determine whether the water reward should be delivered. The command for reward delivery was sent to control the opening time of the magnetic pumps placed outside the training chamber, which would deliver water drops to the side hole to the animal through a polyvinyl chloride tube.

For fear conditioning training, another acrylic cage of the same size but without holes and sensors was used instead. Stainless steel grid at the bottom of the cage

was connected to Grass S48 Stimulator (Grass Technologies, USA) for foot-shock delivery.

### ***Subject preparation and electrode array implantation***

Male Sprague-Dawley rats with clean ears and normal auditory function served as the subjects in this study. The rats were 8 to 12 weeks in age and 250 to 350 g in weight. The rats were provided by the Centralized Animal Facilities in the Hong Kong Polytechnic University. All the experimental procedures were approved by the Animal Subjects Ethics Sub-Committee of the Hong Kong Polytechnic University and City University of Hong Kong. License to conduct experiments on rats was issued by the Department of Health in Hong Kong SAR and renewed annually.

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Ceva Sante Animale Co., France). Anesthesia was maintained during surgery with supplemental dose of 15 mg/kg/h. Atropine sulphate (0.05 mg/kg, i.m.; Sigma) was administered 15 minutes before anesthesia to inhibit tracheal secretions. The subject was mounted on the stereotaxic device (SR-6N, Narishige, Japan) following the induction of anesthesia, and a middle incision was made in the scalp after sterilization by 75% alcohol and the local application of anesthetic (2% xylocaine, s.c.). Craniotomies were performed over the temporal lobe (-3.0 to -6.0 mm posterior to bregma and -

3.7 to -4.7 mm from the top) to access the primary auditory cortex, and the dura matter of the opening window was removed. Body temperature of the subject was maintained at 37.5 °C with a heat blanket placed under the abdomen of the subject.

The custom-made electrode array was held by micro-manipulator (SM-15 and MO-10, Narishige, Japan) and lower into either ipsi- or bi-lateral auditory cortex. Ground electrodes for recording and stimulation were separately connected to the screws on the skull. Auditory response to the noise and pure tone was recorded during insertion of the electrodes to identify and confirm the area of the auditory cortex.

When the electrode tips reached the layer 4 or 5 of the auditory cortex (1000-1100 µm in depth) and recordings showed acceptable signal-noise ratio and clear auditory response, the skull opening window was covered by silicone elastomer (World Precision Instruments, USA). The injection cannula and the connection sockets for recording and stimulation were cemented by dental cement (megadental GmbH, Germany) to the skull. After surgery, the rats were held in their home cages for recovery. Antibiotic medications (Erythromycin Ointment) were administered regularly to protect the wound against infection.

### ***Sensory stimuli and electrical stimulation***

All stimuli were generated from the computer-controlled RX7 integrated stimulation station (Tucker-Davis Technologies, FL). Acoustic stimuli were generated as analog signals, transmitted to PA5 attenuator and delivered through the open- or close-field electrostatic speaker (ED1, TDT). The sound pressure level of the acoustic stimuli was controlled by the OpenEx program and calibrated with sound level calibrator (Tenmars, Taiwan). Pure tones (1000~20000 Hz, <80 dB SPL) and white noise (<80 dB SPL) were used to identify the neuronal properties of the auditory cortex during electrode array implantation. Acoustic stimuli were also used to train the subject to retrieve water reward in the early stage of behavioral training. Visual stimuli were generated as analog signals and delivered through the white LED array. For fear conditioning, the light was delivered from the LED array placed 40 cm above the training box, and the illumination of the box was calibrated at 100 Lux (Tenmars, Taiwan). For rewarding discrimination task, the LED array was placed just above the middle hole of the front panel, and the illumination of the light perceived by the subject was 100 Lux. The electrical foot-shock were controlled by the analog output from RX-7 and generated from the stimulator (Grass Technologies, USA), and then delivered through the stainless steel grid under the training box. The intensity of electrical foot-shock was measured by digital multimeter (Fluke 17B, USA) and

controlled in the range of 0.5-0.9 mA, and was adjusted according to the animal's avoidance and freezing responses. Electrical stimulation for activating neurons was controlled by digital signals and generated from the ISO-Flex isolator (A.M.P.I., Israel). The electrical stimulation was 80~150  $\mu$ A, 20 Hz at frequency and consists of 2-10 pulses (0.5 ms pulse width) (He, 1997; Yu et al., 2009; Yu et al., 2004).

### ***Association of auditory and visual stimuli by fear conditioning***

Electrode array and drug injection cannula were implanted in the left auditory cortex of the rats. Before fear conditioning, either CCK-B receptor antagonist (L-365,260, 50  $\mu$ g/ml, 2  $\mu$ l, 0.2  $\mu$ l/min, Tocris; n=5) or saline (0.9% NaCl, Baxter; n=4) was infused into the auditory cortex through the implanted injection cannula. At 15 minutes after infusion, the rat was allowed to stay in the training box to habituate for 5 minutes, and the exploring behavior was recorded as the control baseline. In each of the conditioning trials, a light stimulus (100 ms) was delivered, followed by a two-pulse electrical stimulation in the auditory cortex (200 ms delayed from the onset of light), and a train pulse of foot-shock (100 Hz, 600 ms, 500 ms delayed from the onset of light). The complete fear conditioning session consists of 30 trials of this sequential stimulation, with the inter-trial-interval of 30 seconds. In the single subject, drug infusion and fear conditioning sessions were repeated for three times. For the rats that were treated with CCK-B receptor antagonist, infusion of



saline and fear conditioning were conducted 3 days after the antagonist intervention as control. Test trials, in which only light was given, were conducted after each conditioning session in the same length and inter-trial-interval to examine the multi-unit activities of auditory neurons in response to the conditioned visual stimulation. In some subjects, glutamate AMPA receptor antagonist (DNQX, 15 mM, 2  $\mu$ l, 0.2  $\mu$ l/min, Tocris) was infused after all interventions to examine the effectiveness of the infusion.

### ***Auditory cortex stimulation and reward retrieval***

Before the rat was trained to discriminate electrical stimulation in the auditory cortex and retrieve the water reward, the feasibility of this discrimination task was tested with auditory and visual stimulation as the rewarding cue.

Before training, the daily water intake of the animal was restricted to 50% of the normal amount for two days. The rat was then put into the training cage and allowed to habituate for 5 minutes before training. At the first stage of training, the naïve animal was trained to get the idea that there was water drop that can be obtained from the side holes of the front panel. When the rat poked into either the left or right hole, a drop of water (15-20  $\mu$ l, same in the subsequent condition) was delivered from the same hole. A complete session of stage 1 consisted of 100 trials with each

of which being a single poke. After completion of two sessions of stage 1, the training moved on to stage 2. In stage 2, the rat was trained to poke into the middle hole of the front panel to initiate an acoustic cue (pure tone, 1.6 kHz or 8 kHz, 100 ms), and then poke to either the left or right hole to retrieve the water reward. The time that the rat waited in the middle hole until stimulation was given was considered as “hold time”, which was set at 100, 200 and 500 ms from session 1 to 3 of this stage. The prolonged hold time trained the animal to expect the coming stimulation cue, and allowed the researcher to observe the improvement of task performance in response to the given stimulation. In stage 3, the nose-poke in the middle hole triggered a high- or low-frequency tone stimulus, which indicated the corresponding hole for water reward (for 5 rats, 8 kHz high frequency indicated left hole and 1.6 kHz low frequency indicated right hole; vice versa for the other rats). The trial was considered as “left trial” and “right trial” when the reward retrieval was on the left and right hole, respectively. Each training session contained 100 trials. The beginning session of this stage contained only the left or the right trials.

After the rat had finished three consecutive sessions of single direction and achieved a correct rate of higher than 80 %, the trials were assigned as 10 left trials followed by 10 right trials, alternatively. After the rat had achieved the correct rate of higher than 80 % for three consecutive sessions, the trials were assigned to 5 left and 5

right trials alternatively. After the rat had achieved 80 % correct rate, the trials were finally assigned to pseudo-random sequence, where the left or right trial was assigned randomly to the animals when they triggered the stimulation.

To train the rat to discriminate the electrical stimulation in the left or right auditory cortex and retrieve water reward, the rat underwent similar training process. In stage 1, rat was trained to use a pure tone of high (8 kHz) or low (1.6 kHz) frequency as cue for left or right water reward. In stage 2, the triggered tone was replaced with electrical stimulation (80~150  $\mu$ A, 0.5 ms pulse width, 5 pulses, 20 Hz) in the auditory cortex. Electrical stimulation in the auditory cortex of left hemisphere indicated reward retrieval on the right hole, and vice versa. The animal was required to perform over 80 % correct rate in left- and right-only sessions. In stage 3, left and right hemispheres were stimulated alternatively between sessions with each of which containing 10 trials. Finally in stage 4, the animal was trained in the session where the left and right stimulations were randomly assigned to each trial, and the correct rate of over 90 % was considered as well trained.

***Pairing the light stimulus with electrical stimulation of the auditory cortex in anesthetized rats in the presence of CCK***

After the rat was well trained to perform the electrical stimulation discrimination task, a baseline testing was performed to examine the rat's initial response to the irrelevant light stimulation. In each testing session, electrical stimulation was normally presented as those in training session, while a light stimulus (500 ms, 100 Lux) was randomly inserted at a very low frequency (1 in 100 trials). In response to the light stimulus, animals either approached the left or right hole, or re-poked into the middle hole to trigger next trial. The animal's behavioral responses and neuronal responses were recorded. There was no water reward after the presentation of the light stimulus no matter which hole the animal approached. Behavioral responses to 10 presentations of the light stimulus were recorded, except for one rat that had only received 3 light stimulations. The few number of the light presentations in the baseline test was to avoid learning effect that the animal might learn that the light was a meaningless cue and it would then show passive response to the light consequently. Neuronal activities were also recorded when the animals rested in the training box without performing any task and light stimuli were repetitively presented.

Rats were then anesthetized with sodium pentobarbital (50 mg/kg, ip., Ceva Sante Animale Co., France) and placed in front of the hole panel, similar to the position where they performed the task. The neuronal responses to light and noise stimulus were recorded. Cholecystinin octapeptide (CCK-8, abbreviated as CCK, 10 ng/ $\mu$ l in ACSF, 0.5~1.0  $\mu$ l, 0.1  $\mu$ l/min, Tocris) was infused into the left or right auditory cortex through the implanted cannula. The light stimulus was then paired with electrical stimulation in the CCK-infused auditory cortex. In each trial, a 500-ms light was presented, followed by electrical stimulation (two pulses, 80~150  $\mu$ A, 20 Hz) at the offset of light. The inter-trial-interval was 10 seconds. 40~60 trials of pairings were conducted in each day for 5~6 days. Neuronal responses to the light stimulus in both the CCK-infused and intact hemisphere were examined after pairing.

After the rats had fully recovered from anesthesia, the post-intervention behavioral responses to the light stimulus were examined in the context of discrimination task. The light stimulus was randomly presented at the same frequency as that in the baseline test, while the rat was engaging in the task. Animal either tended to approach the certain hole for water reward, or ignored the light cue and re-poked into middle hole to initiate another trial. To evaluate these behaviors in a quantifiable manner, a "Decision Index" was created to transfer the animal's

behavioral decision into numeric output. When the left hole was the hole that was “engineered” to be associated with the light stimulation, approaching the left hole would be given a score of 1; showing a tendency to approach the left hole but stopping before poking nose into the hole would be given a score of 0.75; showing no clear tendency to approach either hole and re-poked the middle would score 0.5; showing a tendency to approach the right hole but stopping before poking nose into the hole would score 0.25; and approaching to the right hole would have a 0 score. When the right hole was “engineered” to associate with the light stimulation, an opposite scoring system was used. The scores were marked by the experimenter when testing was monitored by real-time video camera, and reviewed by naïve observer examining the offline video records. Neuronal responses to the light stimulus were also examined to see whether visuo-auditory association was established in the auditory cortex and to check the connectivity between the two hemispheres.

In three rats, reversal pairings of the light stimulus and the electrical stimulation of the opposite hemisphere were conducted at least 3 weeks later in the presence of CCK, and the behavioral and neuronal responses to the light stimulus were examined in the following weeks.

### ***Vehicle controls***

To verify that the behavioral and neuronal changes after CCK infusion and pairings were not due to non-specific effects of fluid infusion or other manipulations during anesthesia, three control experiments were conducted. Neuronal and behavioral responses to the light during anesthesia and the task were recorded in all situations.

In the first control experiment, CCK and artificial cerebrospinal fluid (ACSF) were simultaneously infused into the target and control hemisphere respectively when the rat was anesthetized, and the light stimulus was paired with simultaneous electrical stimulation in both hemispheres.

In the second control, ACSF or CCK was firstly infused into one hemisphere and the light stimulus was paired with electrical stimulation of the same hemisphere. Two weeks later, the other substance (i.e., CCK or ACSF) was infused into the same hemisphere, and the pairings were repeated.

In the third control, ACSF was firstly infused into one hemisphere and stimulus pairings were performed in this hemisphere. One hour later, CCK was infused into the other hemisphere and stimulus pairings were performed. The two parts of infusion and pairing were not overlapped and the washout period was inserted in order to avoid interaction between the two hemispheres, so that time of the

experiment could be shortened since they were conducted within the same period of anesthesia.

### ***Data analysis***

Multi-unit neuronal activities of the auditory neurons were recorded by TDT system and digital oscilloscope (Axon Digidata 1440A, Molecular Devices, CA) which were controlled and monitored with computer. Animal's behavior were monitored and captured by an infrared camera connected to PC software (Ulead VideoStudio, Ulead Systems Inc., Taiwan). Single-unit spikes were identified using spike sorting software (OpenSorter, TDT; SPKTool, MATLAB open source software, Mathworks Inc., Natic, MA). Three standard deviations (SDs) above baseline were set as the threshold to distinguish the spikes. A template matching method in SPKTool or a K-means clustering method in OpenSorter was adopted to sort single-unit spikes. A unit with the largest amplitude and normal overlaid spike profile was chosen from each electrode. Another criterion was that the number of spikes with an inter-spike interval of  $< 2$  ms in the histogram should be  $< 0.2\%$  of the total number of spikes. The timing of spike occurrence relative to stimulus delivery was calculated using MATLAB software. Peristimulus time histograms (PSTHs) were calculated over a bin size of 20 ms for auditory cortex responses and 50 ms for visual responses.



In the chronic experiment which was conducted across many days, the recordings of neuronal activities were usually coming from different units. To compare neuronal responses across different units and sessions, the Z-scores (mean  $\pm$  standard error) were employed to standardize the neuronal changes (Otazu et al., 2009). Z-scores of neuronal responses to visual stimuli within a certain time period were calculated against the mean spontaneous firing rate within the same period, thereby representing the distance between the neuronal responses and the mean of spontaneous firing in units of SD ( $Z = (x-\mu)/\delta$ ; where  $x$  is the neuronal response in each trial, and  $\mu$  and  $\delta$  are the mean and SD, respectively, of spontaneous firing rates across all trials). Higher Z-scores typically indicate a larger neuronal response. To avoid the difference of Z-scores resulting from the different total number of testing trials, the same number of trials (20 or 30) was extracted from each session. Changes in Z-score after each conditioning session were used to assess the effectiveness of conditioning to induce neuronal plasticity.

Paired student's  $t$ -tests were used to compare neuronal responses with spontaneous neuronal activity. One-way repeated measures analysis of variance (ANOVA) was used to test the differences in mean Z-scores before and at different time points after stimulus pairing sessions. Tukey's post-hoc tests were used for mean comparisons.

Chi-Square tests were used to find out the differences in Decision Index before and after interventions. Statistical significance was set at  $p < 0.05$ .

## Chapter 3: Results

### *CCK-B receptor antagonist prevent cross-modal neuroplasticity in the auditory cortex in awake rats*

In the previous study, cross-modal association in the auditory cortex can be induced by fear conditioning. After several blocks of aversive fear conditioning with sequential presentation of light stimulus and electrical stimulation in the auditory cortex, the auditory neurons which initially were not responsive to light stimuli became responsive to the light stimuli, indicating the establishment of visuo-auditory association at the stimulation site of the auditory cortex.

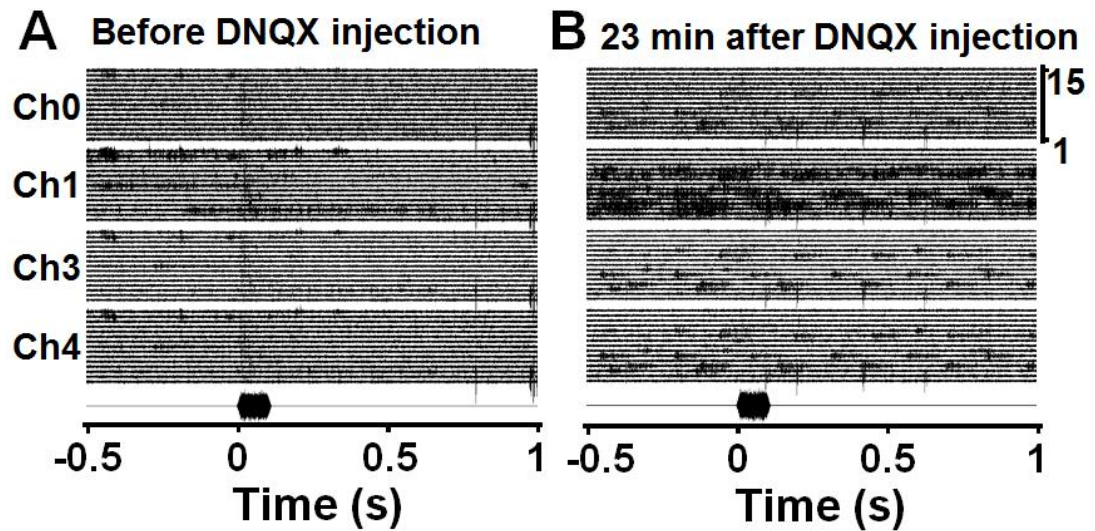
In the present experiment, the same fear conditioning protocol was adopted while CCK-B receptor antagonist was infused to the auditory cortex. Rats were surgically implanted with recording electrodes, stimulation electrode and injection cannula in the auditory cortex. When DNQX (Tocris), a glutamate receptor antagonist, was infused to the auditory cortex through the injection cannula, the spontaneous neuronal activities were diminished for up to 23 minutes, which was the effective time window of the drug (Fig. 10). This result indicated that the injection cannula implanted into the auditory cortex was effective to deliver liquid drug, without any tissue blockage inside the cannula.

Recorded neurons at the stimulation site were not responsive to the light stimulus ( $t_0$ , Fig. 11A). After CCK-B antagonist was infused into the auditory cortex and the rats had undergone three blocks of 30 trials of fear conditioning with the sequential presentation of light stimulus and electrical stimulation, no observable response to light stimulus was recorded in the auditory cortex ( $t_1\sim t_3$ , Fig. 11A). In contrast, the neurons became apparently responsive to light stimulus after one to two blocks of 30 trials of fear conditioning in the absence of the CCK-B antagonist three days afterwards in the same subject ( $t_5\sim t_6$ , Fig. 11A), which was consistent with the previous study that the visuo-auditory association was readily established by fear conditioning. In the control experiment where saline was infused into the stimulation site, the auditory neurons became responsive to the light stimulus after one block of fear conditioning, indicating that the prevention effect of CCK-B receptor antagonist in visuo-auditory association was not due to non-specific infusion effect (Fig. 11B). In summary, visuo-auditory association in the auditory cortex was not established after three blocks of 30 fear conditioning trials in the presence of CCK-B antagonist, but was observed after only one block of fear conditioning without drug infusion or in the presence of saline.

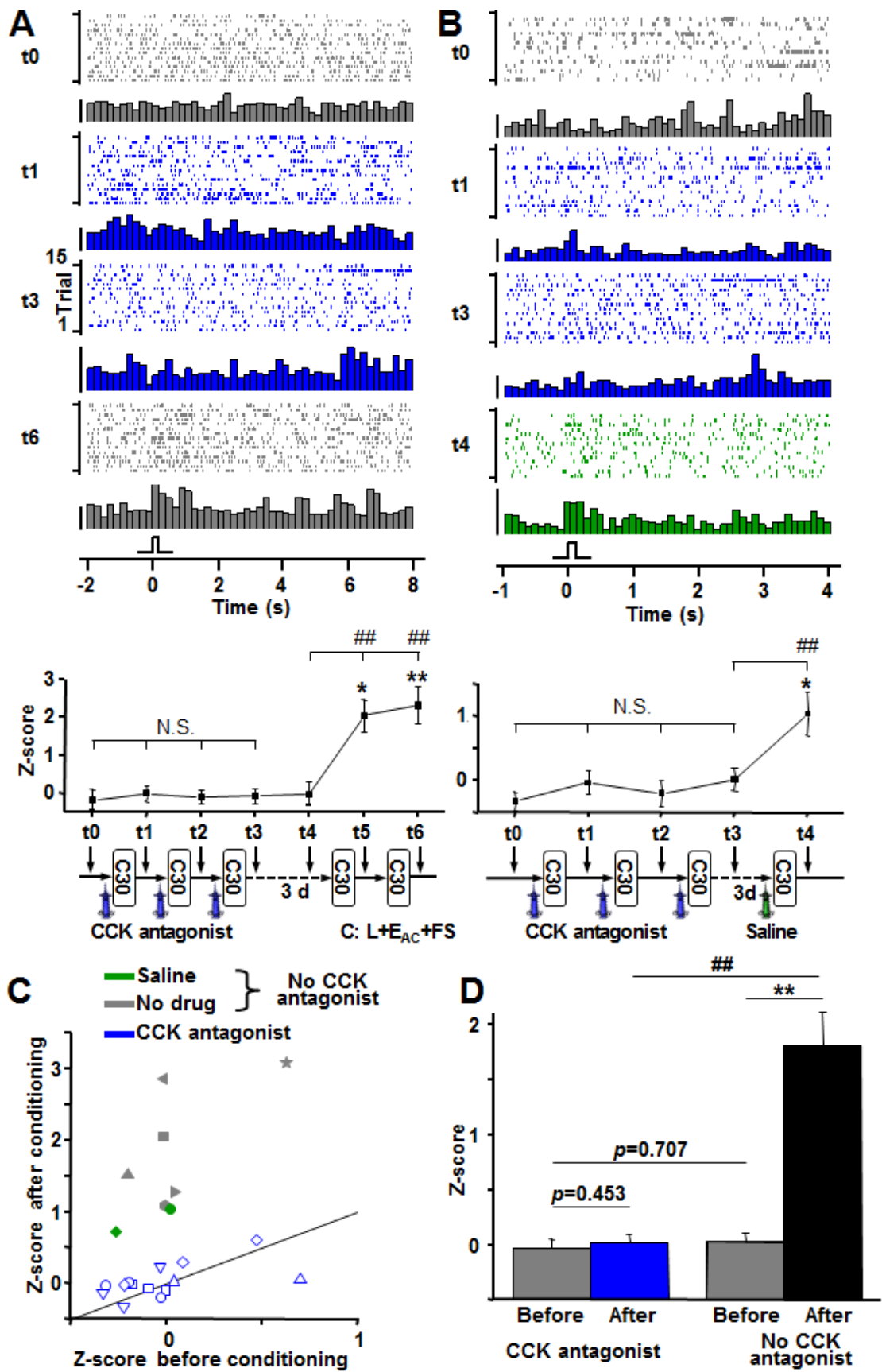
To summarize the experiment in awake rats, Z-scores of neuronal responses to the light stimulus after conditioning were plotted as a function of Z-scores before

conditioning (Fig. 11C). No neurons showed observable increase in Z-score after conditioning in the presence of CCK-B antagonist, but all neurons in 8 rats showed significant increase in Z-score in the absence of the antagonist. The neuronal changes after drug infusion and conditioning were analyzed using two-way repeated measures ANOVA. A significant main effect for both factors (before and after conditioning, and antagonist versus no antagonist,  $p < 0.001$ ) and a significant interaction between the two factors ( $p < 0.001$ ) were found.

Post-hoc analysis using *t*-test was further conducted. Mean Z-scores did not change after conditioning in the presence of the CCK-B receptor antagonist ( $0.026 \pm 0.062$  vs  $0.026 \pm 0.078$ ,  $n = 14$ ,  $p = 0.453$ , paired *t*-test), but increased significantly ( $0.022 \pm 0.095$  vs  $1.708 \pm 0.310$ ,  $p < 0.001$ ,  $n = 8$ , paired *t*-test) after conditioning in the absence of the CCK-B antagonist (Fig. 11D). Z-scores from the CCK-B antagonist and no CCK-B antagonist groups were equivalent before conditioning but significantly different after conditioning ( $p < 0.001$ , unpaired *t*-test, Fig. 11D).



**Figure 10.** Effectiveness of the implanted infusion cannula was tested using a glutamate receptor antagonist. Raw traces show neuronal responses of four channels to repeated auditory stimuli recorded by four separate electrodes in the auditory cortex. **(A)** Neuronal responses before DNQX injection. **(B)** Neuronal responses 23 min after DNQX injection. Noise recorded in the right panel was likely generated by animal movements.



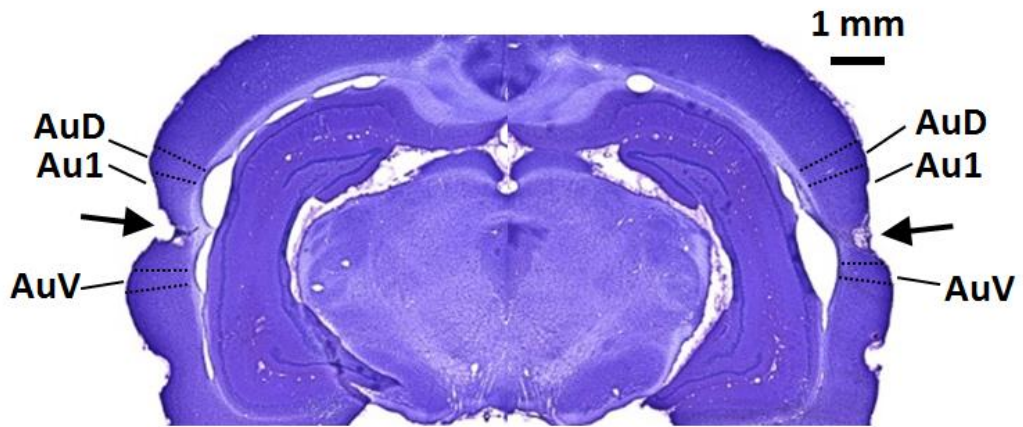
**Figure 11.** CCK antagonist blocked the encoding of an associative memory in the auditory cortex of awake rats. **(A)** Raster plots and PSTHs show neuronal responses to the light stimulus before (t0, gray) and after (t1 and t3, blue) local infusion of a CCK receptor antagonist in the auditory cortex as well as after (t6, gray) conditioning without the CCK receptor antagonist. For both (A) and (B), each conditioning trial consisted of sequential presentation of a light stimulus (L), electrical stimulation of the auditory cortex (E<sub>AC</sub>), and foot shock (FS). Conditioning sessions consisted of 30 trials (C30). Scale bar for PSTHs: 25 spikes/bin; bin: 200 ms. Z-scores were calculated based on the differences between average neuronal firing during a 1.2-s period after the onset of a light stimulus and spontaneous firing during the same duration before stimulus onset. Experimental timelines are shown below the graphs. \* $p < 0.05$ , \*\* $p < 0.01$ , paired  $t$ -test; N.S., not significant, ### $p < 0.01$ , one-way ANOVA. **(B)** Raster plots show neuronal responses to the light stimulus before (t0, gray) and after local infusion of a CCK receptor antagonist (t1 and t3, blue) or saline (t4, green) and conditioning. Scale bar for PSTHs: 25 spikes/bin; bin: 100 ms. Z-scores were calculated based on the differences between average neuronal firing during a 1-s period after the onset of a light stimulus and spontaneous firing during the same duration before stimulus onset. Experimental timelines are shown below the graphs. \* $p < 0.05$ , paired  $t$ -test; N.S.,



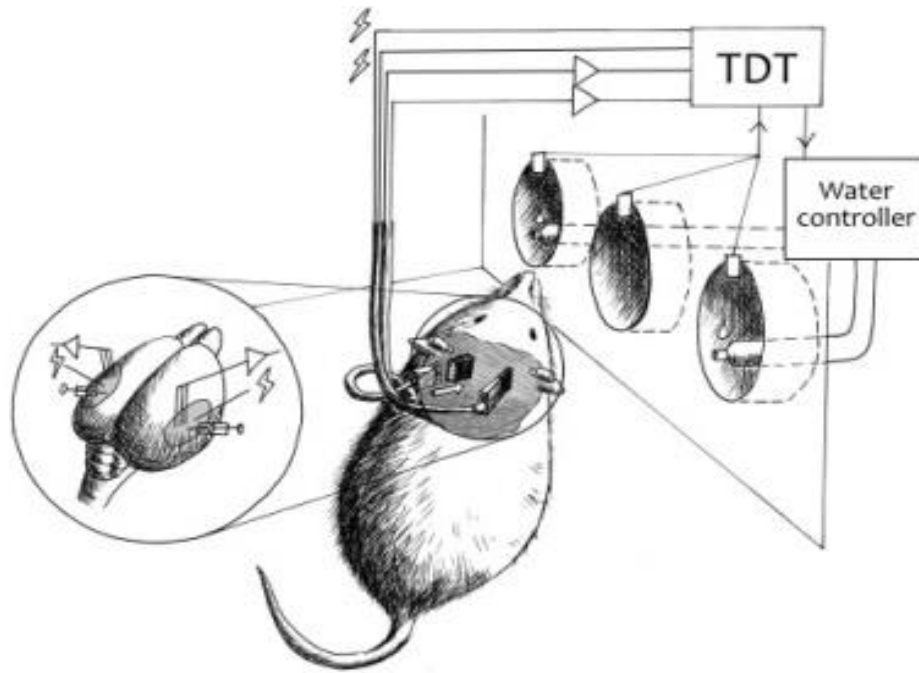
not significant,  $##p < 0.01$ , one-way ANOVA. **(C)** Z-scores of neuronal responses to the light stimulus after conditioning are plotted as a function of Z-scores before conditioning for all neurons in both the experimental condition (with infusion of CCK receptor antagonist) and control conditions (without infusion of CCK antagonist, i.e., no injection or injection of saline). Neurons showing statistically significant changes in Z-score after conditioning are shown as filled symbols; otherwise they are shown as open symbols. Different control conditions are indicated by different colors. **(D)** Mean Z-scores before and after conditioning with or without the CCK antagonist in the auditory cortex.  $**p < 0.01$ , paired  $t$ -test;  $##p < 0.01$ , unpaired  $t$ -test. Post-hoc  $t$ -tests were performed after two-way repeated measures ANOVA.

### *Electrical stimulation in the auditory cortex as the cue for water reward retrieval*

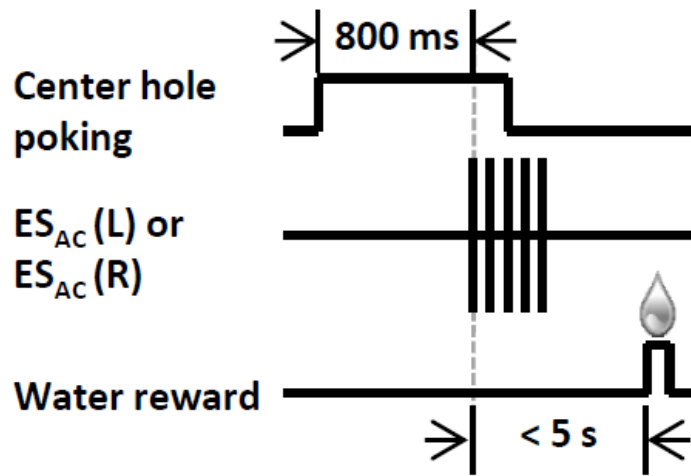
Rats were implanted with recording and stimulation electrode arrays and guide cannula in the bilateral auditory cortex (Fig. 12). After recovery from surgery, they were trained to nose-poke in the central hole of the training apparatus to initiate a training trial, in which electrical stimulation in the left or right auditory cortex was triggered (Fig. 13). Electrical stimulation of the right auditory cortex indicated the reward retrieval in the left hole, and vice versa (Video 1). There was a fixed latency of 800 ms from the onset of central hole poking before electrical stimulation was given, and the rats were allowed to retrieve reward within 5 seconds (Fig. 14). Across four stages of training, during which the rats were trained initially to discriminate pure tone and then the electrical stimulation in auditory cortex to retrieve water reward, the success rate in training session reached over 90% (Fig. 15). All 11 rats successfully learned to utilize electrical stimulation in auditory cortex as a cue to approach the correct hole for water reward retrieval.



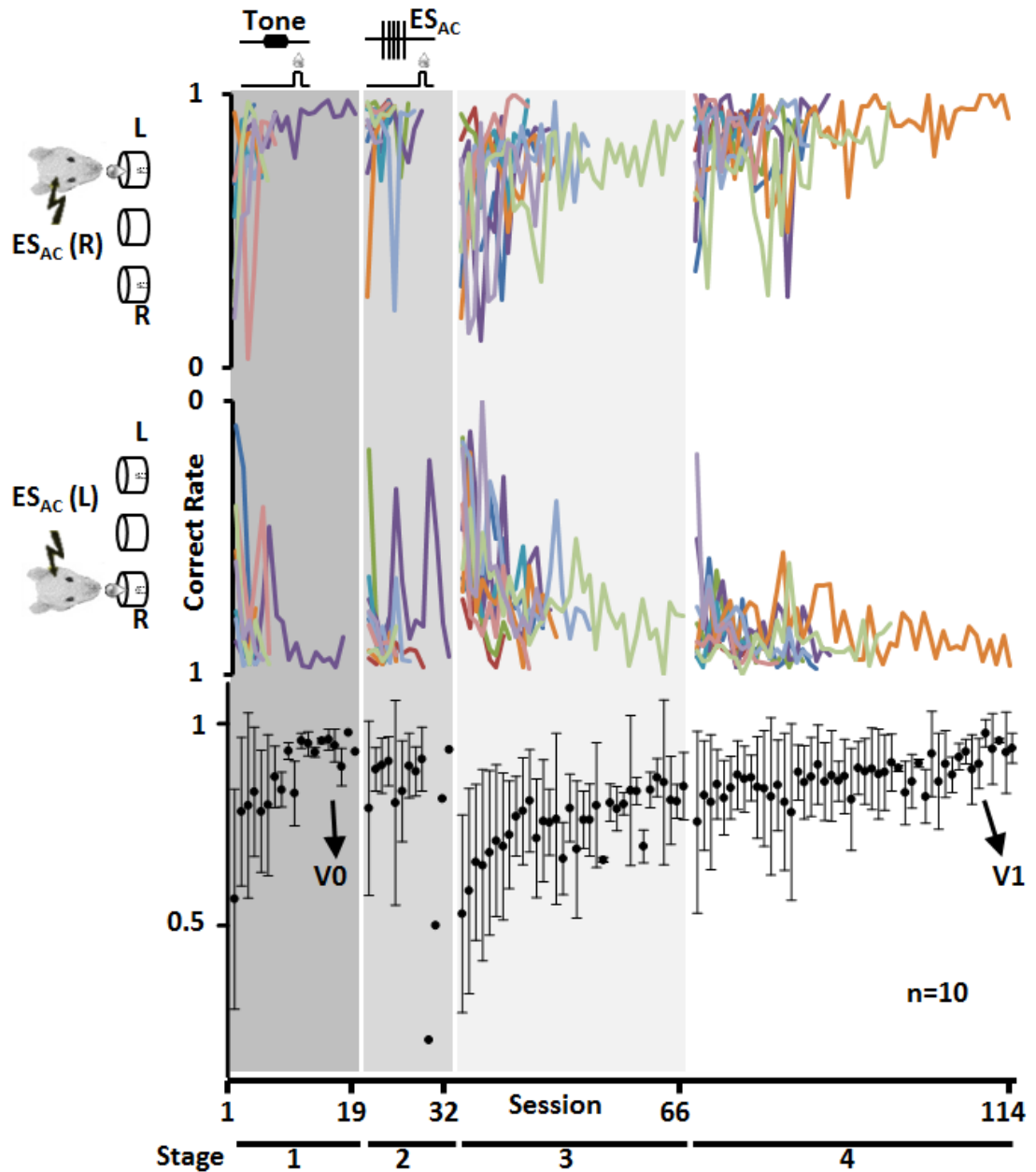
**Figure 12.** Nissl staining showing location of the bilateral electrodes and drug infusion cannulae (marked with arrows). Au1, primary auditory cortex; AuD, secondary auditory cortex, dorsal; AuV, secondary auditory cortex, ventral.



**Figure 13.** Schematic drawing demonstrates the training environment. Left, central, and right holes of a behavioral apparatus contained infrared sensors, and water dispensers were placed in the left and right holes. Rats were trained to nose-poke in the central hole and wait for electrical stimulation of the auditory cortex before approaching one of the holes for water reward.



**Figure 14.** Timing of nose-poke detection in the central hole, electrical stimulation of the auditory cortex, and reward delivery. The train of electrical stimulation was presented 800 ms after the onset of central hole poking. A drop of water reward was available in the corresponding hole within 5 seconds after the onset of electrical stimulation.

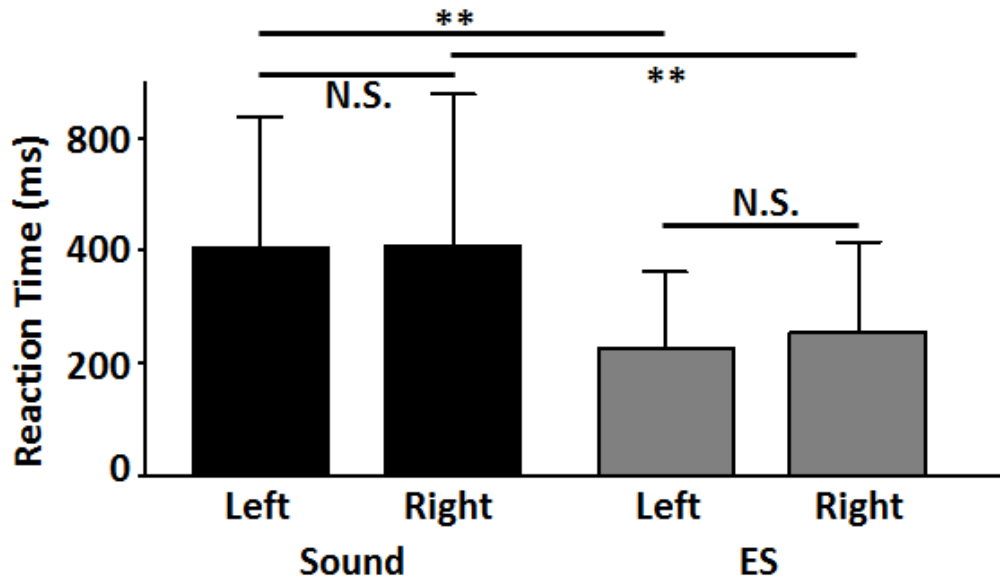


**Figure 15.** Correct rate in reward retrieval across four training stages. In stage 1, rats learned to use two different sounds as cues for reward availability in the right or left hole. In stage 2, rats learned to use electrical stimulation of the left or right auditory cortex as a cue for reward availability in the right or left hole, respectively. Hemispheres were alternately stimulated between sessions, with 100 trials per

session. In stage 3, hemispheres were alternately stimulated between sessions, with 10 trials per session. In stage 4, hemispheres were stimulated in a pseudo-random manner in individual trials. The correct rate across stages increased gradually and reached 90% on average. Four stages are shaded by four gradient colors from dark grey to white. Video 0 (V0) and V1 demonstrate the training process.

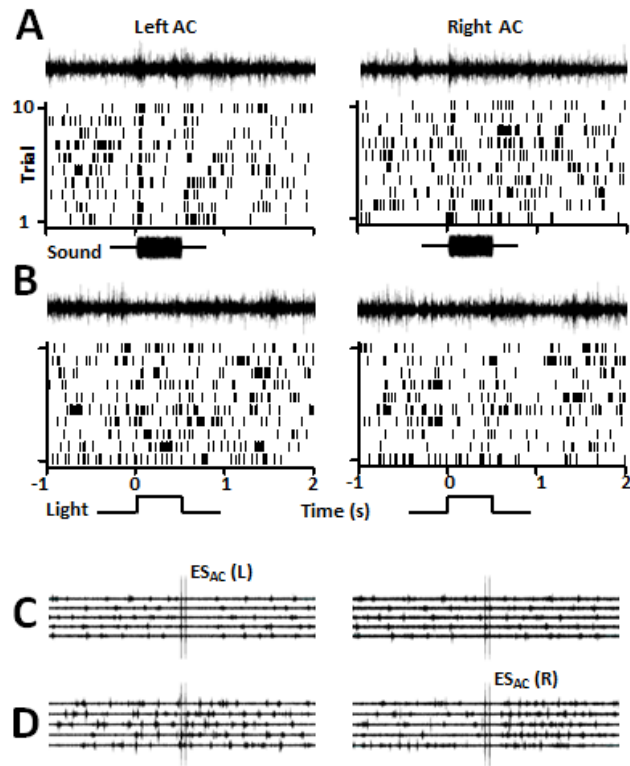
The time interval from the onset of cue to animal's withdrawal in middle hole was defined as reaction time. There were no significant differences between the two hemispheres in behavioral reaction times to the cue, regardless of whether the cue was pure tone or electrical stimulation of the auditory cortex (Fig. 16; Tukey's post-hoc  $p = 0.990$  for sound and  $p = 0.129$  for electrical stimulation). However, rats showed shorter reaction times when the cue was electrical stimulation than when it was pure tone (Fig. 16;  $F = 105.9$ ,  $p < 0.001$ , one-way ANOVA; post-hoc  $p < 0.001$  for both hemispheres). This reduction in reaction time may be due to better learning during advanced training stages and/or shortened signal transmission, with electrical stimulation of the auditory cortex being faster than an auditory signal traveling from the cochlea to the auditory cortex.



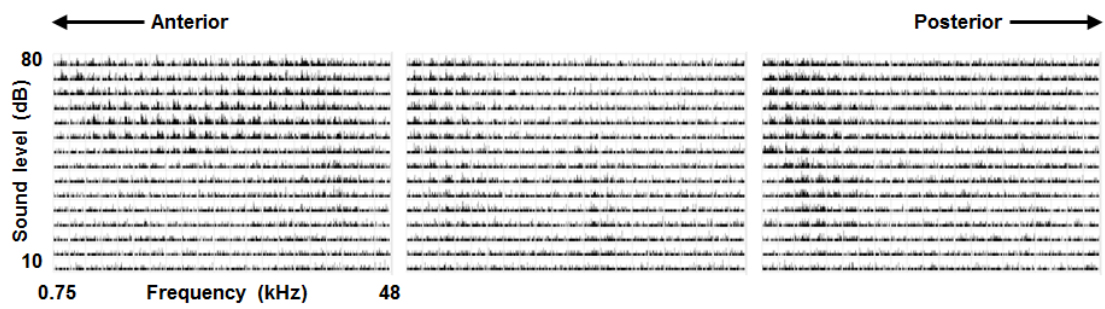


**Figure 16.** Means and SDs for behavioral reaction time. No significance was found in difference between two hemispheres despite of the cue types. Reaction time was faster when responding to cortical electrical stimulation in both hemispheres, than responding to sound stimulation.  $**p < 0.001$ , Tukey's post-hoc test. N.S., not significant.

Neuronal responses to light and sound stimuli were recorded from both hemispheres after recovery from surgery. Neurons in both hemispheres showed clear response to noise-burst stimulus (Fig. 17A), but no or weak responses to light stimulus (Fig. 17B). The recorded neurons showed fine tuning to the pure tone, indicated that the electrodes positioned in the primary or adjacent associative auditory cortex (Fig. 18). Electrodes in the two hemispheres were deliberately implanted symmetrically at slightly different locations to avoid invoking strong commissural connections. Electrical stimulation of the left auditory cortex activated neurons in the ipsilateral hemisphere (Fig. 17C, inhibitory response shown in left panel), but not in the contralateral hemisphere (Fig. 17C, right panel), and vice versa. (Fig. 17D, excitatory response in the ipsilateral hemisphere shown in right panel, no observable response in the contralateral hemisphere shown in left panel).



**Figure 17.** Neuronal activities recorded from auditory cortex after electrode implantation. Raw traces and raster displays show robust neuronal response to noise (A), no response to light (B), and local activation with electrical stimulation of the left and right auditory cortex (C and D).



**Figure 18.** PSTHs show auditory response to monofrequency tones at different levels from neuron samples. The tuning areas were tonotopically organized, showing that the recording sites were mostly in primary auditory cortex.

### ***Read out of unconsciously implanted memory in a behavioral context***

After the rats were well trained to utilize electrical stimulation in the auditory cortex as the cue for water reward retrieval, a baseline test was conducted to examine the animal's behavioral response to light stimulus during the task. In each session of baseline test, electrical stimulation was randomly replaced with a light stimulus for only one trial (Fig. 19). Totally, 10 light stimuli were randomly inserted into 1000 training trials (100 trials per session, 10 sessions), except for rat #2 and #11 which had been tested only for 3 and 5 trials of light stimulus, respectively. Some rats showed a preferred direction to go when the light stimulus was presented instead of electrical stimulation (e.g., rat #1, #6, #11: left hole = right auditory cortex; #7, #8, #9, #10: right hole = left auditory cortex), while some rats showed no preference (e.g., rat #2, #3, #4, #5) (Fig. 20, green dots). After baseline testing, the subject was anesthetized and the light stimulus was paired with electrical stimulation of the non-preferred hemisphere under CCK infusion (Fig. 19; Fig. 20, red arrow heads). Thus, the "engineered" direction was opposite to the preferred direction. Rats then underwent post-intervention test with the same procedures as the baseline test for the following weeks. Notably, no water reward was delivered after the light stimulus in all tests to prevent the independent association between the light and reward.

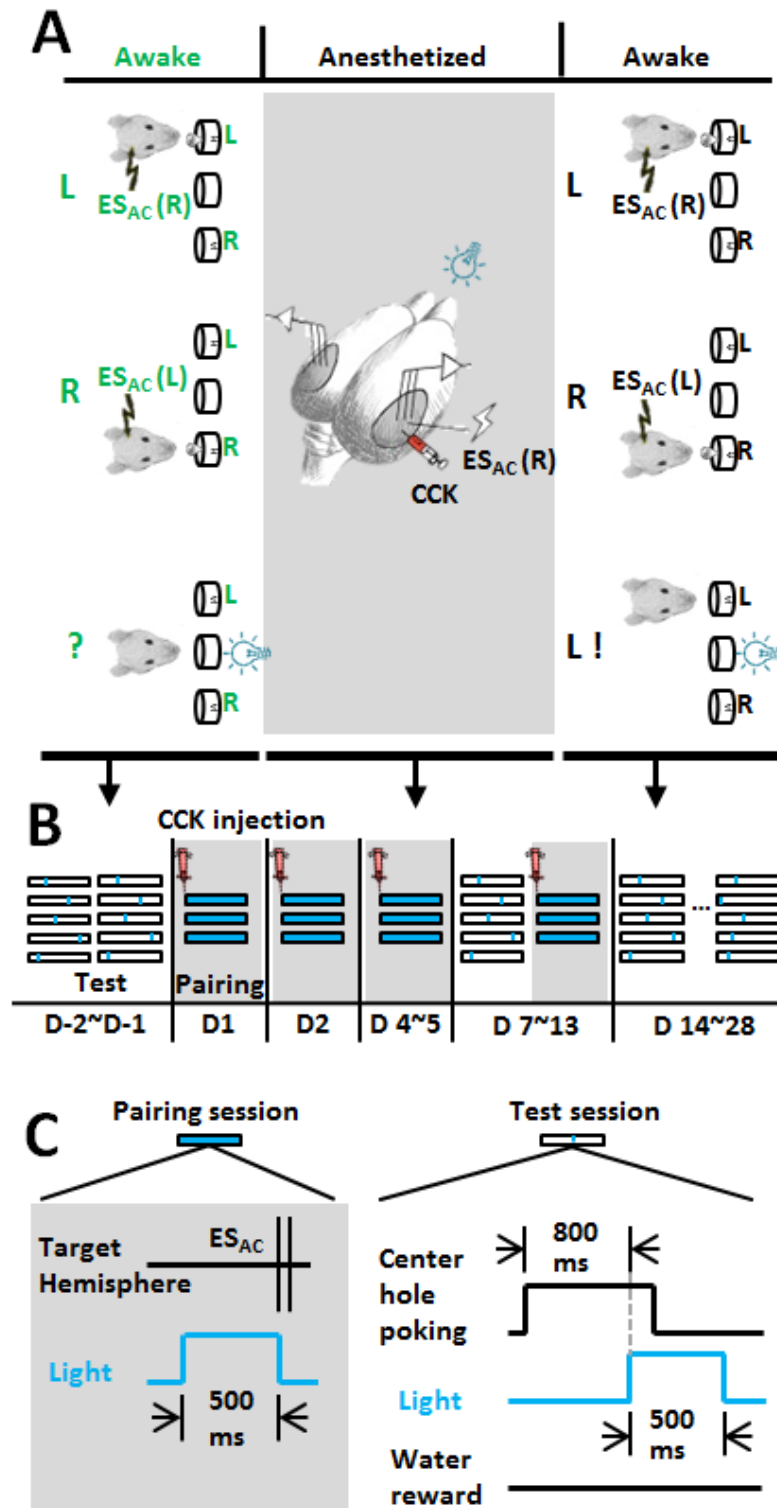
For rat #1, it tended to approach to the left hole when light stimulus was given during baseline testing. On days 1, 3, 5, 8, 9, and 12, the light stimulus was paired with the electrical stimulation in the left auditory cortex under anesthesia and CCK infusion, i.e., the rat was “engineered” to approach to the right hole in response to light stimulus (Fig. 20, red arrowheads). On days 3 and 5 where post intervention testing was conducted, the rat showed no clear tendency to approach either left or right hole (Fig. 20, black dots). On day 9, the rat approached the right (i.e., “engineered”) hole in four trials, and the previously preferred left hole for only one trial. On days 18 and 22, the rat approached only the right hole when the testing light stimulus was presented.

For rat #2, no clear preference was observed when the light stimulus was given during baseline test. The light stimulus was then paired with electrical stimulation in the right auditory cortex under anesthesia and CCK infusion on days 1, 2, 3, 4, and 8. In the post-intervention testing, the rat approached the right hole in response to the light stimulus on the first few days, but started to approach the left (i.e., “engineered”) hole on the subsequent days. On days 27 and 28, the rat approached the left hole in response to light stimulus in all testing trials.

For rats #3~5, stimulus pairings under anesthesia and CCK infusion were conducted between day 1 and 7, and the post-intervention testing started on day 6 and 7. All

rats consistently approached the “engineered” hole in response to the light stimulus after pairing.

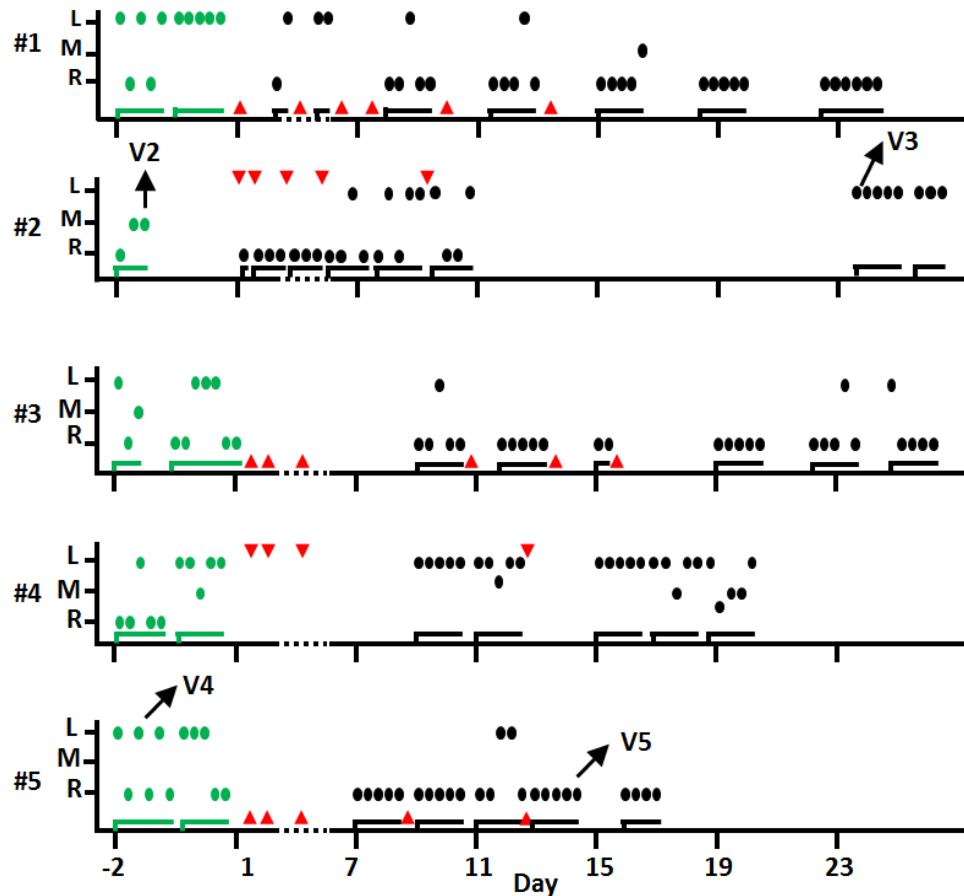
These “engineered” changes in behavior maintained for several days without further intervention. Although the lasting days for post-intervention testing varied, the behavioral changes maintained at least 4 days, and up to 10 days after the last stimulus pairing (10 days for rat #1, 20 days for rat #2, 12 days for rat #3, 7 days for rat #4, and 4 days for rat #5). The Decision Index increased significantly over testing weeks (Fig. 21; w0: baseline testing, w3-4: post-intervention testing;  $F = 25.0, p < 0.001$ ; w0 vs. w2: post-hoc  $p < 0.001$ ; w0 vs. w3-4: post-hoc  $p < 0.001$ ).



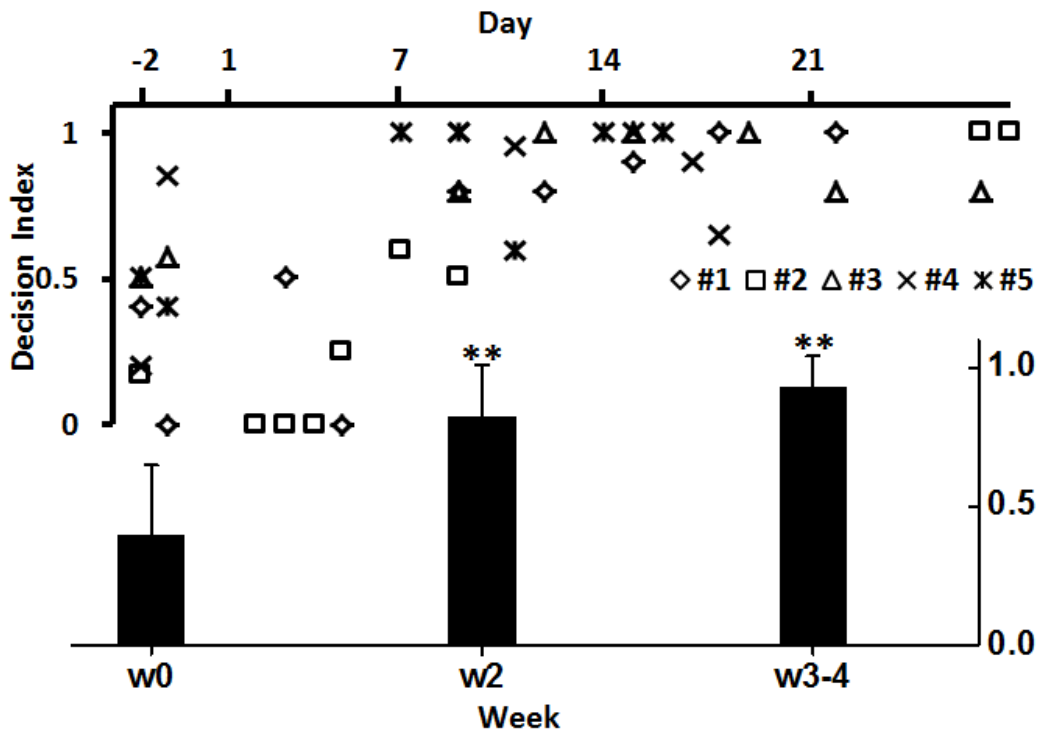
**Figure 19.** Experimental design. (A) After rats were trained to use cortical stimulation as a cue for reward availability, they underwent three experimental phases: baseline testing while awake, memory implantation under anesthesia



(shaded area, same in follows) and in the presence of CCK, and post-intervention testing while awake. During baseline testing, a light stimulus occasionally replaced electrical stimulation of the auditory cortex, and behavioral responses to the light (indicated by “?”) were recorded. **(B)** During the memory implantation phase, rats were under anesthesia, and CCK was infused into the auditory cortex of one hemisphere before pairings of the light stimulus with electrical stimulation of that hemisphere. Stimulus pairings occurred during 2-3 sessions (20 trials per sessions) each day on multiple days. Post-intervention testing was identical to baseline testing. Each empty bar indicates one testing session; blue line in empty bar indicates single testing trial with light; each blue filled bar indicates one stimulus pairing session. **(C)** Time relations between stimulations in pairing sessions, and between poking and light stimulation in testing sessions.

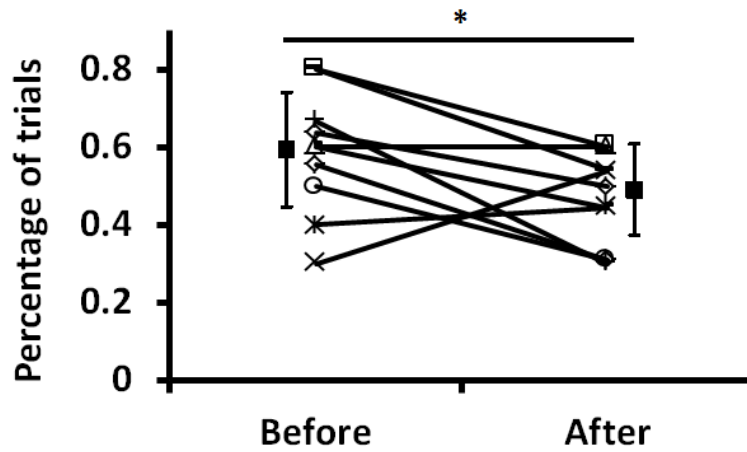


**Figure 20.** Behavioral responses to the light stimulus during baseline (green dots) and post-intervention (black dots) testing. Each dot represents animal’s directional decision when light was presented in a single testing trial. ‘L’ indicates left hole approach, ‘R’ indicates right hole approach, and ‘M’ indicates no preference. Stimulus pairings associated with one hemisphere under CCK infusion are marked by red arrowheads. Each bar under the dots corresponds to one day of behavioral testing. V2 and V4 videos demonstrate behavioral responses before stimulus pairings; V3 and V5 videos demonstrate behavioral responses after stimulus pairings.



**Figure 21.** Individual values (upper) and mean  $\pm$  SDs (lower) for Decision Index across days and weeks. Decision Index value for days was averaged from each subject. Decision Index was defined as ‘1’ when the rat approached the “engineered” hole, ‘0’ when it approached the other hole, and ‘0.5’ when it approached neither hole. w0, day -2 and -1, baseline; w2, day 7 to 13; w3-4, day 14 to 28. \*\* $p < 0.01$ , post-hoc tests versus w0.

The preference of animal's behavior in response to light stimulus during baseline testing was partially due to the difference of correct rates on two directions. There was an observable tendency (59.9 %) for rats to approach the direction which displayed higher correct rate during baseline testing. This tendency however, disappeared in the post-intervention testing after stimulus pairing (49.6 %;  $p = 0.049$ , paired  $t$ -test; Fig. 22). The result indicated that the rats learned to dissociate the light stimulus with the frequency of reward retrieval after trials of light testing. Therefore, animal's behavior in response to light during the post-intervention reflected mostly the unconsciously implanted association between the light stimulus and the electrical stimulation in the auditory cortex.

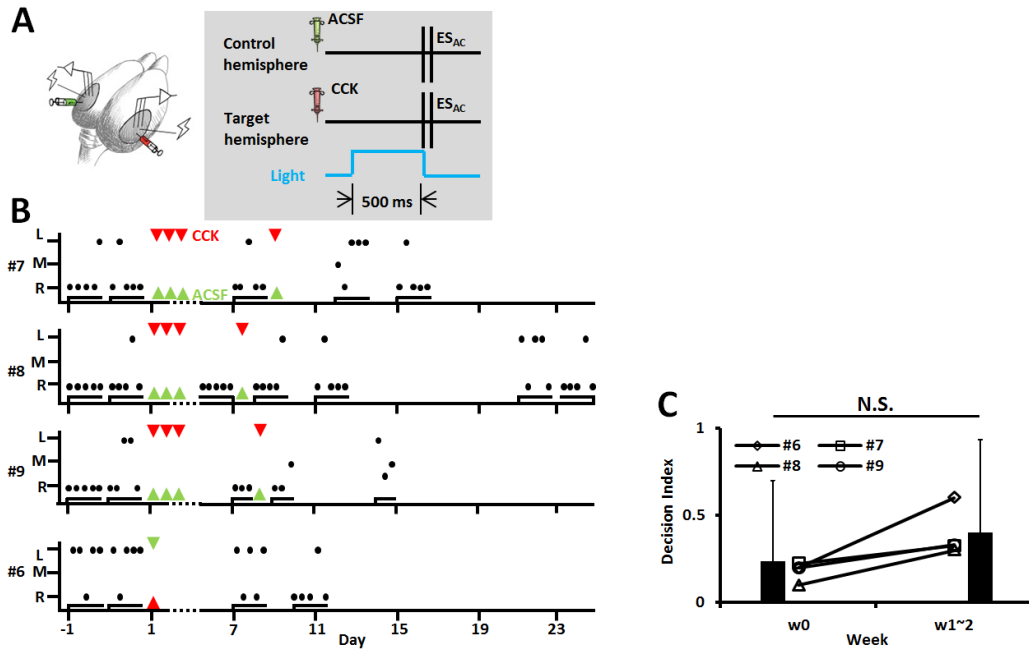


**Figure 22.** Tendency for rats to approach the hole associated with greater reward retrieval (higher correct rate) when the light stimulus was presented as test trial. Various symbols represent individual and mean (filled squares and error bars) percentages of trials that rats approached the side with higher correct rate before and after pairing.  $*p = 0.049$ , paired  $t$ -test.

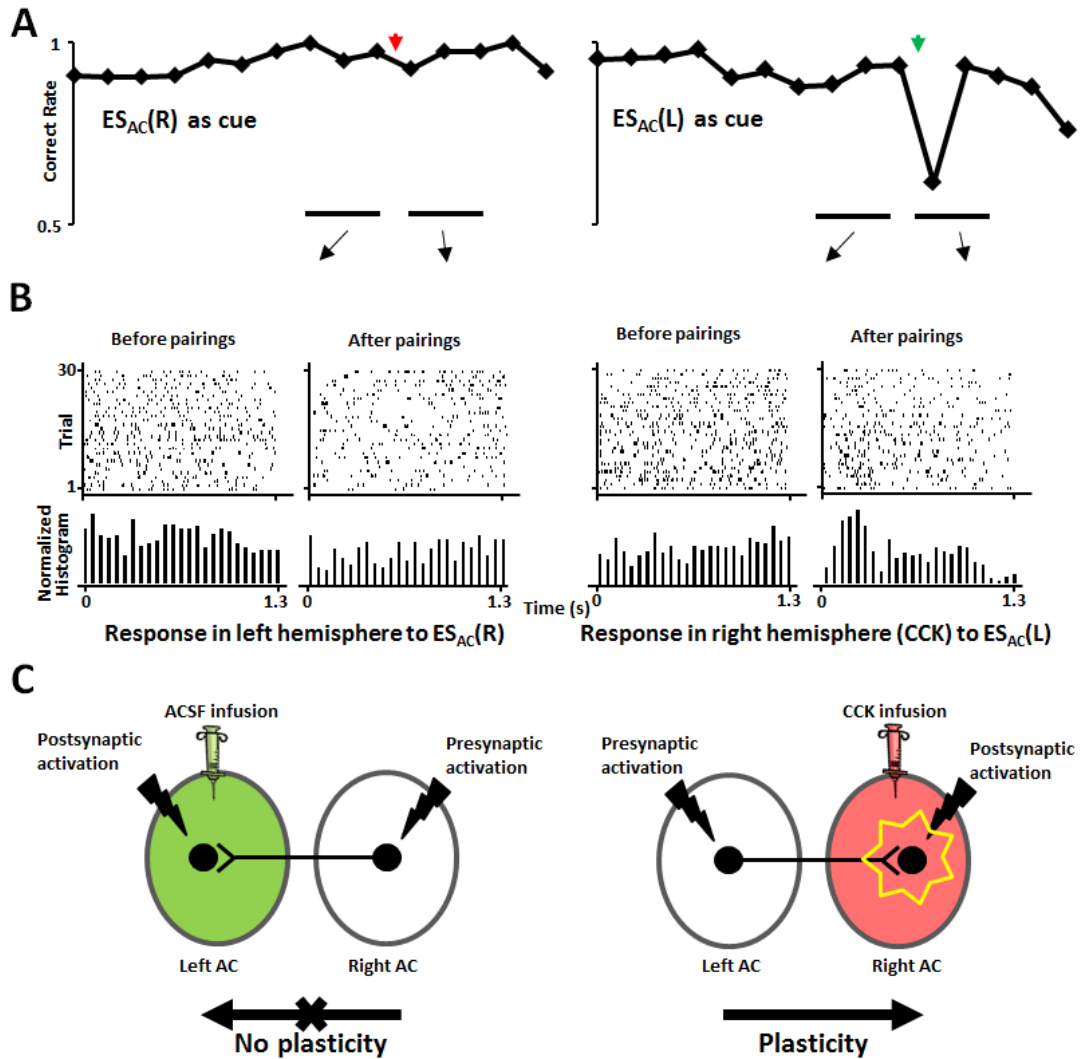
### ***Controls for non-specific effects of infusion and stimulus pairings***

To eliminate the possibility that the behavioral changes were due to non-specific effects of anesthesia, infusion manipulations or pairings, three control experiments were performed.

In the first control experiment, CCK was infused in one hemisphere and artificial cerebrospinal fluid (ACSF) was infused into the other hemisphere, and the light stimulus was paired with simultaneous electrical stimulation of both hemispheres under anesthesia (Fig. 23A). All four rats showed the tendency to approach the target direction in the two weeks after pairings (Fig. 23B). The Decision Index of all subjects increased after pairings (Fig. 23C). The relatively small increase in Decision Index ( $p = 0.119$ , Chi-Square test) compared to the experimental group may be due to the interactions between hemispheres during stimulus pairings. That is, the CCK-infused hemisphere started to respond to electrical stimulation of the ACSF-infused hemisphere after stimulus pairings (Fig. 24B, right). Neuroplasticity was possibly induced under the influence of CCK when auditory cortices of both hemispheres were co-activated (Fig. 24C). This activation of both hemispheres interfered animal's performance and biased the decision when light stimulus was given. Also, behavioral success rate with electrical stimulation of the ACSF-infused hemisphere dropped substantially the day after stimulus pairings (Fig. 24A, right).



**Figure 23.** (A) CCK and ACSF were simultaneously infused into the left and right hemispheres, respectively, prior to pairings of the light stimulus and electrical stimulation of both hemispheres. Shaded area, under anesthesia. (B) Behavioral responses to the light stimulus. Arrowheads indicate stimulus pairing sessions after infusion of CCK (red) or ACSF (green). (C) Decision Index for individual rats before (w0) and after stimulus pairings (w1-2). Mean  $\pm$  SDs of Decision Index are plotted in bar chart. N.S., not significant,  $p = 0.119$ , Chi-Square test.

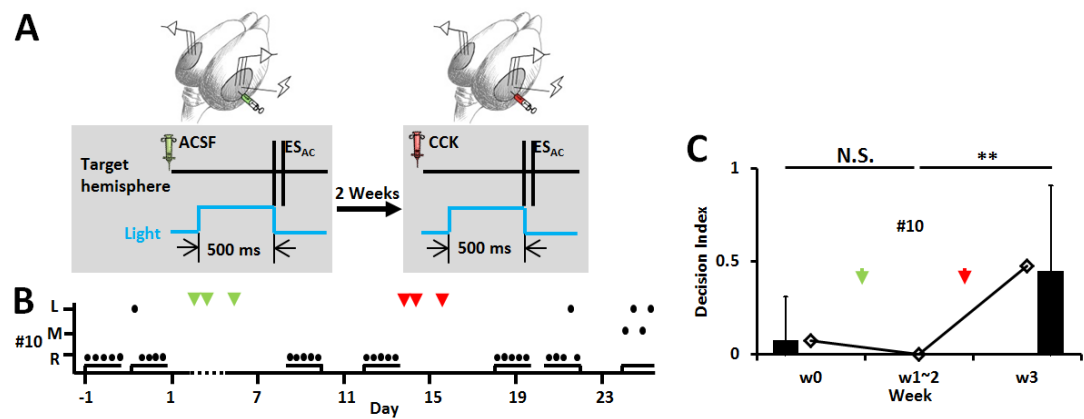


**Figure 24.** Hemispheric interference resulting from pairing the light stimulus with simultaneous activation of both hemispheres after infusion of CCK into one hemisphere and ACSF into the other hemisphere. **(A)** Rate of approaching the correct hole before and after simultaneous stimulus pairings in response to stimulation of the CCK-infused (left panel) or ACSF-infused (right panel) hemisphere. Arrows indicate simultaneous stimulus pairings (red: CCK-infused hemisphere, green: ACSF-infused hemisphere). Each data point corresponds to



correct rate of 100 trials. **(B)** Left: Raster plots and PSTHs showing neuronal responses in the ACSF-infused auditory cortex (i.e., the left hemisphere) to electrical stimulation of the CCK-infused auditory cortex (i.e., the right hemisphere) before and after simultaneous stimulus pairings. Right: vice versa. **(C)** Schematic drawing demonstrates plastic changes in the CCK-infused auditory cortex (red) induced by simultaneous activation of both hemispheres. Such plasticity was not observed in the ACSF-infused auditory cortex (green).

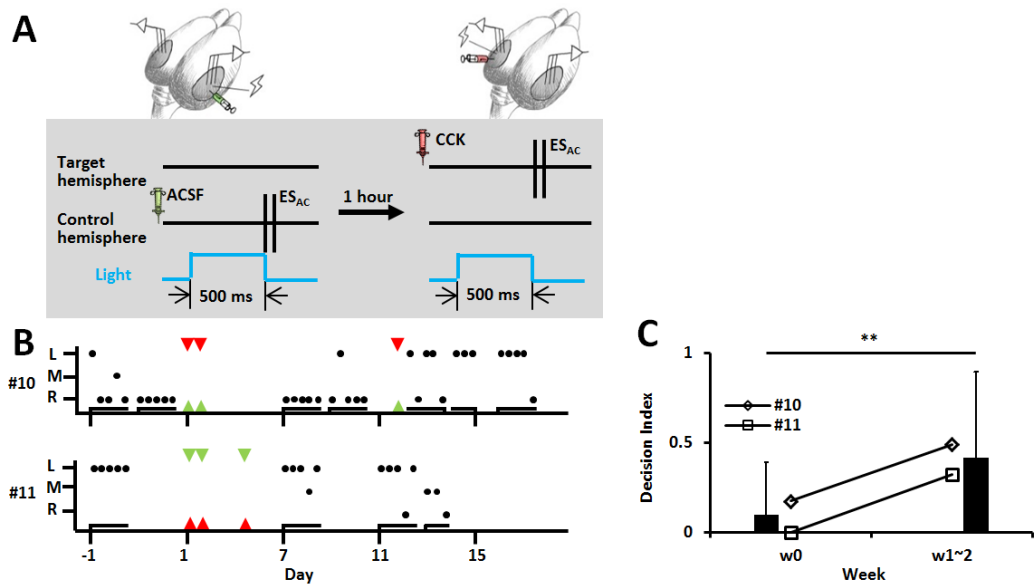
To avoid the disadvantage of the first control experiment, the second control was conducted. In this control experiment, ACSF or CCK was infused into one hemisphere, and the light stimulus was paired with electrical stimulation of the infused hemisphere under anesthesia. Two weeks later, the other substance (i.e., CCK or ACSF) was infused into the same hemisphere and the stimulus pairings were repeated (Fig. 25A). There was 1 rat (#10) that had initially approached to the right hole in most trials during baseline test and in all trials after ACSF infusion and stimulus pairings (Fig. 25B). However, after CCK was infused and the light stimulus was paired with electrical stimulation of the right auditory cortex, that rat started to approach the left hole on day 22 and 24 (i.e., 10 and 12 days after CCK infusion, respectively). The Decision Index maintained a low value in the first two weeks after pairings, but increased significantly after CCK infusion and pairings ( $p < 0.01$ , Chi-Square test; Fig. 25C).



**Figure 25.** (A) ACSF was infused into one hemisphere, and CCK was infused into the same hemisphere two weeks later. Stimulus pairings occurred after infusions. Shaded area, under anesthesia. (B) Behavioral responses to the light stimulus. Arrowheads represent stimulus pairings after infusion of ACSF (green) or CCK (red). (C) Decision Index for individual rat, and mean  $\pm$  SDs of Decision Index before and after stimulus pairings. N.S., not significant;  $**p < 0.01$ , Chi-Square test.

Since the second control experiment was very lengthy and data obtained from only one subject, a third control experiment was designed. ACSF was infused in the non-target hemisphere and the stimulus pairings was performed. One hour later but in the same period of anesthesia, CCK was infused in the target hemisphere and stimulus pairings was performed (Fig. 26A). After the intervention, the subjects in this group showed the tendency to approach the target direction (Fig. 26B). The Decision Index significantly increased corresponding to the stimulus pairings for the CCK-infused hemisphere ( $p < 0.01$ , Chi-Square test, Fig. 26C).

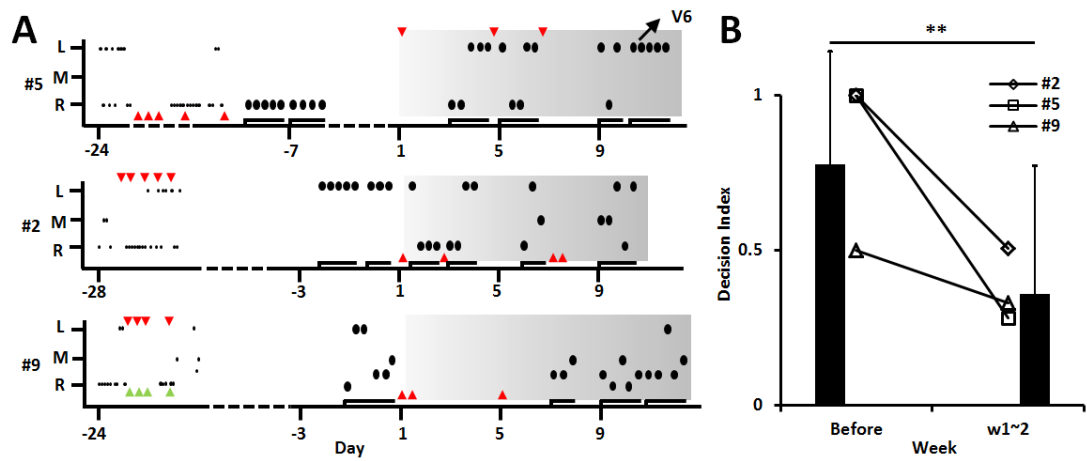
Together, these control experiments support the conclusion that the post-intervention behavioral changes are caused by CCK infusion and stimulus pairings, rather than by non-specific effect of anesthesia, infusions or stimulus pairings alone.



**Figure 26.** (A) CCK and ACSF were infused into different hemispheres within the same period of anesthesia. Stimulus pairings occurred after infusions. Shaded area, under anesthesia. (B) Behavioral responses to the light stimulus. Arrowheads indicate stimulus pairings after infusion of CCK (red) or ACSF (green). (C) Decision Index of individual rats, and mean  $\pm$  SDs of Decision Index before and after stimulus pairings.  $**p < 0.01$ , Chi-Square test.

### ***Reversal stimulus pairings***

For three rats that had already been infused CCK and paired in one hemisphere, CCK injections and reversal stimulus pairings were conducted in the other hemisphere three weeks after the previous intervention. Rats #5 and #2 showed behavioral switch toward the opposite hole after the reversal stimulus pairings (Fig. 27A, gradient shaded area). Rat #5 consistently approached the right hole before reversal pairings, but approached the left hole in most trials after the first reversal session and all trials on the last test day (see Video 6). Rat #2 also approached the opposite hole in most trials after reversal stimulus pairings. The Decision Indices of all three rats significantly decreased in the two weeks after reversal stimulus pairings ( $p < 0.01$ , Chi-Square test, Fig. 27B). These results again support the finding that the implanted memory induced by CCK was reflected from behavioral changes, and also demonstrate the flexibility of auditory neuronal plasticity in the presence of CCK.



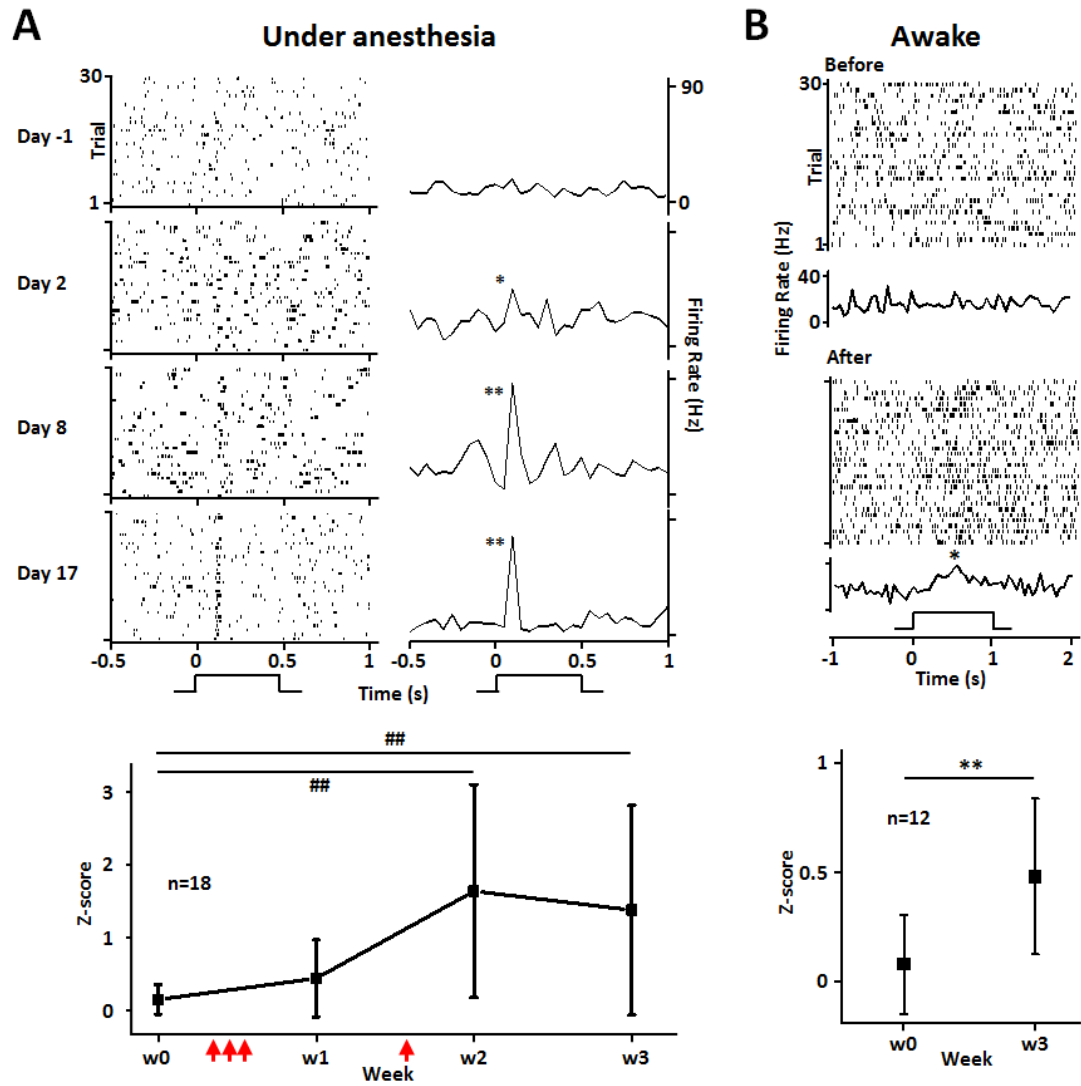
**Figure 27.** “Re-engineering” an artificial memory by reversal stimulus pairings. **(A)** Behavioral responses to the light stimulus after reversal stimulus pairings (gradient shaded area). **(B)** Decision Index of individual rats, and mean  $\pm$  SDs of Decision Index before and after reversal stimulus pairings.  $**p < 0.01$ , Chi-Square test.

## ***Responses of auditory cortex neurons to the visual stimulus after unconscious memory implantation***

We hypothesized that the unconscious implanted memory reflected from behavioral contexts was on the basis of neuronal plasticity induced by CCK application. Therefore, we examined the auditory neuronal activities in response to visual stimulus after CCK infusion and stimulus pairings under anesthesia and awake state. Neuronal activities before and after intervention were examined. A typical neuron in an anesthetized rat did not respond to the light stimulus before stimulus pairings (Fig. 28A, Day -1), but gradually started to respond to the light stimulus after CCK infusion and stimulus pairings (Days 2~17). The mean Z-score of neuronal response from group data significantly increased after stimulus pairings (Fig. 28A;  $n = 18$  neurons in 6 rats;  $F = 10.9$ ,  $p < 0.001$ ; w0 vs. w2: post-hoc  $p < 0.01$ ; w0 vs. w3: post-hoc  $p < 0.01$ ). We then examined whether the altered neuronal plasticity observed under anesthesia could also be observed during awake state. A typical neuron did not respond to the light stimulus before intervention, but showed responses to the light stimulus after the intervention (Fig. 28B). The mean Z-score from group data increased significantly after stimulus pairings (Fig. 28B,  $n = 12$  neurons in 2 rats; paired  $t$ -test,  $p < 0.001$ ). These findings suggest that the visual



responses of the auditory neurons induced by CCK infusion and stimulus pairings serve as the neuronal basis of the unconscious memory implantation.



**Figure 28.** Changes in response of auditory cortical neurons to the visual stimulus.

(A) Raster displays and peristimulus time histograms showing neuronal responses to the light stimulus under anesthesia, before (Day -1) and after (Days 2, 8, and 17) CCK infusion and stimulus pairings. Z-scores were calculated based on differences between average neuronal firing during a 150-ms period after the light stimulus and an equivalent period of spontaneous firing. Stimulation pairings in the present of CCK were marked as red arrowheads. (B) Neuronal responses to the light stimulus

in awake state before and after stimulus pairings. Z-scores were calculated based on differences between average neuronal firing during a 200-ms period after the light stimulus and an equivalent period of spontaneous firing. ## $p < 0.01$ , ANOVA; \* $p < 0.05$ , \*\* $p < 0.01$ , paired  $t$ -test.

## Chapter 4: Discussion

In the present study, infusion of CCK in the auditory cortex prevented the establishment of visuo-auditory associative memory induced by associative fear conditioning training. Infusion of CCK in the auditory cortex of anesthetized rats enabled neuroplasticity, which caused the auditory neurons to persistently respond to light stimulus after which was paired repeatedly with electrical activation of the auditory cortex. This artificial visuo-auditory association trace lasted for at least 20 days, and was transferred to the awake state. This artificial association was also translated into behavioral action. In the two-choice rewarding task, the animal's behavioral responses cued by the light stimulus reflected that the light stimulus was associated with auditory cortical stimulation. Several control experiments were performed and the results showed that the behavioral changes were not due to non-specific effects of drug infusion or other manipulations from the observer. The reversal pairings conducted on the animals that had already shown directional intention alternatively switched the direction of behavior, demonstrating that the neuroplasticity of the auditory cortex is highly flexible under the influence of CCK. As a whole, these findings provide a scientific foundation for “memory implantation”.

In a previous study, we have established an artificial association between a visual stimulus and electrical stimulation of the auditory cortex using fear conditioning training, such that the auditory neurons started to respond to a visual stimulus (Chen et al., 2013). Notably, the formation and retrieval of this association were blocked by inactivation of the entorhinal cortex, indicating that both processes were hippocampal dependent (Chen et al., 2013; Winters and Bussey, 2005). On the other hand, deep brain stimulation of the medial temporal cortex in humans boosts memory performance (Suthana et al., 2012). These findings suggest that the associative memory established in the present study is also hippocampal-dependent. It requires the interactions between the neocortex and the hippocampal system (Corkin, 1984; Scoville and Milner, 1957).

In the brain, CCK is the most abundant of all neuropeptides (Rehfeld, 1978), and heavy labeling of CCK-containing neurons is observed in perirhinal and entorhinal cortices (Innis et al., 1979). Recently, we found that after injection of True Blue tracer in the auditory cortex, the retrogradely labeled neurons in the entorhinal cortex are mostly CCK-containing neurons. Furthermore, activation of the entorhinal cortex enabled neuronal plasticity in the auditory cortex, but this effect was minimized by local administration of CCK antagonist in the auditory cortex (Li et al., 2014). Therefore, we hypothesize that the hippocampal system sends a

memory-encoding signal, the neuromodulator CCK, to the neocortex to enable the neuroplasticity and write new associative memory through its entorhinal/perirhinal pathway.

There are accumulated evidences in support of this hypothesis. In the behavioral relative studies, application of CCK-B receptor antagonist attenuates fear-potentiated startle (Josselyn et al., 1995), blockage of CCK receptors suppresser conditioned fear (Tsutsumi et al., 1999), and deletion of the CCK-B receptor gene reduces anxiety-like behavior (Horinouchi et al., 2004). CCK is considered to mediate anxiety-like behavior (Wang et al., 2005). Studies of CCK have also been extended to the neural system. Activation of CCK-B receptors in the amygdala potentiates the acoustic startle response (Fendt et al., 1995; Frankland et al., 1997).

The possible mechanism may be that CCK increases GABAergic inhibitory transmission in the amygdala (Chung and Moore, 2007). Related to the function of learning and memory, CCK is necessary for mice to maintain normal memory function in a passive avoidance task and spatial memory (Lo et al., 2008). Besides, CCK also facilitates neuronal plasticity in the vitro hippocampal neurons (Deng et al., 2010). These findings suggest CCK is involved in the memory process, especially for the declarative memory. The results in the present study add on new knowledge that CCK induced neuroplasticity in the neocortex which can be

translated into behavioral actions. This finding compensates our existing understanding that the declarative memory is hippocampus-dependent and hippocampus system highly participates at the early stage of memory encoding and storage.

Using transgenic and optogenetic techniques in mice, Ramirez et al. labeled memory-encoding neurons to create a false fear memory in a particular context in which foot-shock was never delivered (Ramirez et al., 2013). This finding provides the idea that artificial manipulation of memory and its relative behavior is technically possible. Along similar lines, we implanted an artificial association between a light stimulus and electrical stimulation of the auditory cortex while rats were under anesthesia and CCK infusion. Subsequently, the rats took advantage of the existing association between the auditory cortical stimulation and the water reward, and were able to consider the light stimulus as cue for retrieval of the reward. Notably, although the visuo-auditory associative memory trace implanted in the auditory cortex was quickly reflected in neuronal activities, it took several days before the association was reflected in animal's behavior. This delay may be due to the time necessary for the newly implanted memory in the neocortex to be correlated and registered in the hippocampus (Teyler and DiScenna, 1986), or to be linked with the "place cells" in the hippocampus for directional guidance (O'Keefe

and Dostrovsky, 1971). After all, the present study steps ahead from our current understanding and demonstrates the possibility to implant memory upon the existing ones.

However, it remains largely unknown how CCK induces plastic changes at the synaptic and molecular levels. CCK-B receptors are the predominant form in the brain (Van Dijk et al., 1984). It is G-protein coupled receptor, of which activation increases the activity of phospholipase C and results in intracellular calcium release and activation of protein kinase C (Wank et al., 1995). Recent studies elucidate that CCK promote glutamate release in the hippocampal pyramidal neurons (Deng et al., 2010) and facilitates neuronal excitability in the entorhinal cortex (Wang et al., 2011). Glutamate has been intensively studied to be essential in synaptic plasticity and memory formation. Retrograde labeling in previous study revealed that CCK expressed in direct cortical projection neurons in entorhinal cortex, which are long projection excitatory neurons (Li et al., 2014). Taking together, we postulate that CCK receptors coexist with glutamate receptors in excitatory neurons, and interact with glutamate receptors to facilitate synaptic plasticity.

It is notable that the subsequent reversal pairings in the rats that had already associated light with cortical stimulation of one side were still able to alter the animal's behavioral decision to the other side. Compared to the lately established



association by reversal pairings, the original association, or implanted memory ought to be consolidated and became more stable over time. However the behavioral results challenge this idea. Possible reason is that the previous implanted memory was not stable, since the lack of water reward during light testing trial may be a strong negative factor that drives animal's re-learning and weakens the established association over time. Thus a newly established association on the opposite side may facilitate the reversal of animal's behavior.

There are some limitations in the present study. First, after CCK infusion and stimulus pairings, visual responses in the auditory cortex were observed in both anesthetized and awake state of the animal. Such visual responses were clearly observed in raster plot when light stimuli were repetitively presented and the neuronal recordings were overlaid for more than 30 trials. However, the number of visual stimuli in a single testing day when the animal was doing behavioral task was no more than five, and recording data across days cannot be simply grouped for analysis. The neuronal recordings of single day during behavioral task did not reveal clear visual responses in the auditory cortex when light was presented instead of electrical stimulation. The possible reason may be that the neuronal signals were interfered by the robust behavioral activities of the animal, and the number of trials was not large enough to eliminate the interference and extract the visual responses.

Since the small number of testing trials is necessary in the experimental design in order to avoid additional learning effect, it is hard to overcome this problem in the present experiment. To solve this problem, a new behavioral task should be designed in which a larger number of testing trials is possible. Improvements can be made in extracellular recordings so that neuronal signals are less interfered by animal's movements. Besides, recordings of local field potentials will also give a hint on how cross-modal sensory information converges at the presynaptic level while partially overcome the problems in multi-unit recordings.

The second limitation is that the measurement on memory changes is not sensitive enough. In the present results, some behavioral changes of the animal can only be observed using statistical analysis. In some testing trials, animal did not show clear directional choice comparing with the pre-intervention direction. Animal's memory changes cannot be fully reflected in behavior under this situation. There are several factors that can interfere animal's behavior during the test, such as animal's habit, attention or arousal level, task strategy, etc. The visuo-auditory associative memory cannot be fully reflected in the behavioral measurement that we used. Fear conditioning training would be the alternative choice instead of the present rewarding training. Fear memory can be directly reflected by animal's freezing behavior, while less interfered by other factors if experimental condition is well

controlled. After all, a more sensitive parameter to connect memory and behavior would be better to measure the memory changes of the animal.

Finally, electrical stimulation in the neocortex can cause a lot of effects. Both excitatory and inhibitory neurons were activated. Neural changes may occur in the neural circuits related to the stimulation site. To dissect the auditory neural circuits that are involved in this process, it becomes necessary to selectively activate specific neurons. The combination of transgenic and optogenetic techniques is one of the ways to achieve this goal. Using gene recombination technique to selectively express light sensitive protein in specific neurons in transgenic mice, we are now able to stimulate the specific neurons with high resolution laser. For example, injection of adeno-associated virus carrying CaMKIIa-ChR2 sequence induces specific channorhodopsin-2 expression in excitatory neurons. Thus only excitatory neurons can be depolarized by laser stimulation, giving us possibility to investigate whether recruitment of excitatory neurons is sufficient to induce memory implantation.

Moreover, it is also possible to control the expression of CCK gene in the hippocampal system and neocortex, to further address the function of CCK in memory formation. One of the feasible ways is to use Cre recombinase system in mice (Yizhar et al., 2011). By expressing Cre recombinase specifically in CCK

neurons and catalyzing target gene expression, A CCK-Cre line will provide us numerous ways to manipulate different functions of CCK neurons in different brain regions, thus helping us to find out the functioning map of CCK. In the future, we are going to employ these novel techniques to reveal the details on how the CCK functions and how the memory is implanted in terms of neuronal changes at the synaptic and molecular levels.

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