THE INVOLVEMENT OF ENTORHINAL CORTEX IN CROSS-MODAL ASSOCIATIVE MEMORY

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The Involvement of Entorhinal Cortex in Cross-Modal Associative Memory

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Certification of originality

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Abstract

As the gateway of the hippocampal system to the neocortex, the entorhinal cortex (EC) is hypothesized as a hub in the transformation process of recent memory to remote memory. The purpose of this research is to explore the EC's role in the retrieval of recent and remote cross-modal associative memory and identify another behavioral indicator of this kind of association besides freezing percentage.

In the first part of the thesis, a within-subject approach was adopted to examine the EC's role in cross-modal associative memory. An association between a visual stimulus and an auditory stimulus was induced by classical fear conditioning. The rats in the EC-intact condition exhibited 96%±2% freezing to the light stimulus in Week 1, while the rats in the EC-inactivated condition showed 2%±1% freezing. This difference lasted for the first three weeks. The result indicated that the EC participated in the retrieval of cross-modal associative memory.

The freezing percentages in both conditions approached each other in the last two weeks. The mean freezing percentage in the EC-intact condition decreased

to 54%±17% in Week 4, while in the EC-inactivated condition it increased to 34%±5%. This trend indicated that the dependence of the retrieval on EC decreased over time.

In the second part of the thesis, a cross-modal association between light and sound was demonstrated by a delay fear conditioning and a cued-reward task. Firstly, the light and sound stimuli were combined as the conditioned stimulus and associated with a foot shock in the experiment group; only the light stimulus and the foot shock were presented to the control group. Secondly, the sound stimulus was used as the cue for water reward. Thirdly, the sound was replaced by the light to examine whether the cued-reward task was an indicator for the cross-modal associative memory. Six out of 7 rats in the experiment group, compared with 1 out of 8 rats in the control group, succeeded in getting the water reward in the first trials of the replacement. This difference between the two groups lasted for the next 20 trials. It indicated that visuoauditory association was formed in the conditioning phase and could be retrieved in the reward phase with the cued-reward task.

In summary, the retrieval of cross-modal associative memory depends on the EC and the dependence decreases over time. The cued-reward task can be used as an indicator to represent the cross-modal association between visual and auditory stimuli.

Relevant Publications

Working Paper

Chen X, Guo Y, **Liao Z**, Li X, Wang H, Li X, He J (2012) Encoding and Retrieval of Artificial Visuoauditory Memory Traces in Auditory Cortex Needs Entorhinal Cortex. The Hong Kong Polytechnic University.

Conference

Liao Z, He J. The Involvement of Entorhinal Cortex in Cross-Modal Associative Memory**.** *Guangzhou Neuroscience Symposium*. Guangzhou, People's Republic of China, *Jan 19-21,2013*

Liao Z, He J. The Establishment of Light-Sound Cross-Modal Association on Rat. *Beijing International Workshop on Auditory Neuroscience*, Beijing, People's Republic of China, *May 18-20, 2012.*

Liao Z, He J. The Establishment of a Stable Behavioral Model on Light-Sound Cross-Modal Association. *Zhuhai International Symposium on Auditory Neuroscience,* Zhuhai, People's Republic of China, *August 3-6,2011*.

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List of Abbreviations

AMPA	Amino-3-hydroxy-5-Methylisox- azole-4-Propionic Acid
CA	Cornu Ammonis
CAF	Central Animal Facility
CR	Conditioned Response
CS	Conditioned Stimulus
DNQX	6,7-dinitroquinoxaline-2,3-dione
EC	Entorhinal Cortex
PFA	Paraformaldehyde solution
SPL	Sound Pressure Level
UR	Unconditioned Response
US	Unconditioned Stimulus

Chapter 1 Introduction

1.1 Background of the study

This section presents a brief introduction of the interaction between hippocampus and entorhinal cortex (EC), followed by the cross-modal associative memory. It also describes the motivation of this research.

1.1.1 Hippocampus and entorhinal cortex

The role of hippocampus and parahippocampal region in forming new long-term memory has been recognized since the evidence of patient H.M (Scoville and Milner, 1957). The hippocampus and its adjacent cortical areas have thus received abundant attention in the memory formation and storage process (Squire, 1992, Wiltgen et al., 2004). These studies on amnesic patients found that the damage in hippocampus and its adjacent areas disabled memories of recent events, but left the long past memories intact.

The consistent findings of the hippocampus' role in learning and memory for all the commonly studied mammalian species, spurred the researchers

into investigation of the adjacent cortical areas (Squire, 1992). The hippocampus acquires information quickly and functions as a transient storage for recent memory. Processed by the hippocampus, the information is then sent back to neocortex for later retrieval. The remote memory is then formed in the neocortex (Graham and Hodges, 1997, Norman and O'Reilly, 2003). The anatomical adjacency of the EC to the hippocampus and neocortex makes the EC a key structure in the hippocampus-neocortex transfer model. The EC serves as the main gateway for cortical information sent from neocortex to the hippocampus and for the processed information sent from the hippocampus back to the neocortex.

Numerous studies have identified the indispensable role that the hippocampus plays in declarative memory (Tulving and Markowitsch, 1998, Eichenbaum, 1999, Chowdhury et al., 2005). The EC processes multimodal sensory information to the hippocampus (Lavenex and Amaral, 2000, Witter et al., 2000a). Fear conditioning provides a tractable behavioral paradigm for dissecting the role of the hippocampus (Sanders et al., 2003). Combining EC and the cross-modal associative memory is a

good starting point to understand the role of EC in memory formation, retrieval and EC-related memory diseases.

The research on EC is of great significance for understanding EC-related diseases. The impairment of EC function, such as novelty detection, associative learning and episodic memory processing, is responsible for its association with psychotic disorders and delusions (Prasad et al., 2004). Profound layer II EC neurons loss in Alzheimer's disease and the decreased volume of EC in Schizophrenia patients, suggest that the EC is closely related to human declarative memory deficits (Gomez-Isla et al., 1996, Baiano et al., 2008).

1.1.2 Cross-modal association

Cross-modal association involves stimuli from different modality. Through cross-modal associative learning, people could learn new knowledge, accept new concepts, and make complex decisions when environment changes based on their previous experiences.

The typical associative learning is the process of establishing relationship between conditioned stimulus (CS) and the unconditioned stimulus (US)

(Sanders et al., 2003). The research findings in CS-US have led to study about CS-CS association. The CS-CS pattern associates abstract stimuli. This association is analogy to the multisensory environment that people experience from day to day.

The visuoauditory association is a type of cross-modal association. Under normal conditions auditory stimuli evoke responses in visual cortex (McIntosh et al., 1998, McIntosh and Gonzalez-Lima, 1998); visual stimuli modulate responses in auditory cortex (Bizley et al., 2007, Kayser et al., 2008). Visual cortex in early blind people is activated by auditory stimuli in a Positron Emission Tomography study, and auditory cortex of early deaf people is excited by visual stimuli (Sadato et al., 1996, Weeks et al., 2000, Arno et al., 2001). These phenomena suggest that understanding and manipulating the cross-modal association can help improve our living ability.

1.2 Objectives of the study

The aim of this study is to contribute to the research on cross-modal associative memory by comparing the performance of the rat in the fear

memory retrieval with bilaterally inactivated EC and that of the same rat but with intact EC. The main objectives of this study are as follows.

1. To investigate how the EC participates in the retrieval of cross-modal associative memory.
2. To investigate the EC's role in the transformation of the cross-modal associative memory from recent memory to remote memory.
3. To explore the establishment of cross-modal light-sound association by fear conditioning.
4. To identify whether the cued-reward task can serve as an indicator for the cross-modal association.

1.3 Significance of the study

This research found that the retrieval of associative memory depended on the EC. The dependence of EC in memory retrieval decreased over time (see Section 3.2.1 – 3.2.4). This result echoes the earlier finding from human studies that recent memory depends on the hippocampal system, while remote memory is stored within neocortex and is less dependent on the hippocampal system.

In the behavioral experiment it was assumed that cross-modal association could be detected by combining fear conditioning and cued-reward task.

Ten trials of light-sound-foot shock conditioning were presented to subjects in the experimental group. Ten trials of light-foot shock pairings were presented to subjects in the control group. The significant difference between the two groups confirmed the validity of combining fear conditioning and the cued-reward task in testing the strength of cross-modal association (see Section 4.2.1 – 4.2.4).

1.4 Outline of the thesis

This thesis is organized as follows.

Chapter 1 introduces the general background and the research scope of the present study, including the objectives and the significance of the study.

Chapter 2 presents a review on recent memory, remote memory and the corresponding cortical areas. The status quo of research in cross-modal association is also covered.

Chapter 3 describes the methodology of the experiment on the EC's involvement in the cross-modal associative memory, in the manner of listing the procedure and data analysis methods, followed by presenting the results.

Chapter 4 delineates the experimental design and procedures of the visuoauditory association.

Chapter 5, the final chapter gives a summary of findings and attempts to arrive at general conclusions.

Chapter 2 Literature Review

This chapter begins with a review of the literature on the hippocampal–neocortex transfer model in recent and remote memory and goes on to provide current understanding on entorhinal cortex (EC) in that process. It then presents studies on cross-modal associative memory, followed by a discussion of the significance of combining EC and the cross-modal associative learning.

2.1 The hippocampal-neocortex transfer model

The hippocampus is widely recognized, with involvement of the encoding, consolidation and retrieval of the spatial, configural, multimodal representations of memory (Scoville and Milner, 1957, Anagnostaras et al., 2001). Research with diverse methods, including lesion and neuroimaging experiments in animals and humans, revealed the vital role of hippocampus in declarative memory (Kim and Fanselow, 1992, Barry and Doeller, 2010).

The participation of hippocampus in memory is time limited. Memory learned long ago was left intact after hippocampal damage (Teng and

Squire, 1999, Wang et al., 2009, Lesburgueres et al., 2011), while the acquisition of new memories was severely impaired (Buzsaki, 1989). Maviel et al. (2004) found that the expression of immediate early genes increased in neocortex of mice following the retrieval of remote spatial memory, but not recent memory. Wiltgen et al (2004) delineated the differential activation of recent and remote memory in the hippocampus and neocortex. Further research revealed the role of the neocortex in storing memories learned long ago, as neocortical damage impaired remote memory (Graham and Hodges, 1997, Squire et al., 2001).

The hippocampus and neocortex have a division based on their contrasted role following remote memory retrieval and their ability of affecting the memory performance after lesion (Wiltgen et al., 2004). It is believed that the newly formed memory is initially processed and encoded in various cortical areas and higher level processing of it takes place in the hippocampus. Connection between the different cortical areas is then formed gradually with the related cortical areas simultaneously activated by hippocampus. When the memory becomes sufficiently strong, it would be independent of the hippocampus and be transferred to related neocortex

(Alvarez and Squire, 1994, Bontempi et al., 1999, Takehara et al., 2003, Wiltgen et al., 2004).

According to the hippocampus-neocortex transfer model, recent memories primarily activate the hippocampus while remote memories primarily activate neocortex (Alvarez and Squire, 1994, Wiltgen et al., 2004). A possible explanation for the slow onset of cortical involvement is that new memories must be linked to existing memories within the cortex via a gradual interleaving process so to avoid damaging the existing memories (Shahaf and Marom, 2001).

2.2 The entorhinal cortex

With MRI studies, the extent of H.M.'s surgical lesion was elaborated, which involved almost the whole EC and part of the hippocampal region, including dentate gyrus, hippocampus and subicular complex (Corkin et al., 1997).

The EC is usually classified into the hippocampal region (Gluck and Myers, 2001, Andersen et al., 2006), due to its unidirectional connection feature in the information transforming process as the hippocampal circuit

does. Most connections between the hippocampus and neocortex consist of entorhinal-perirhinal-cortical connections and entorhinal-cortical connections (Swanson and Kohler, 1986, Brown and Aggleton, 2001).

The EC is not only the major cortical input area for the hippocampus with multimodal sensory information, but also the principal output station of the hippocampus (Lavenex and Amaral, 2000, Sewards and Sewards, 2003). As shown in Figure 2.1, the EC receives information in the superficial layers from the neocortex, including perirhinal cortex and parahippocampal region. The information is then projected to the dentate gyrus through the perforant pathway and also directly to Cornu Ammonis 1 (CA1) and CA3 fields of the hippocampus (Lavenex and Amaral, 2000, Witter et al., 2000b). After a largely unidirectional projection from dentate gyrus to CA3 and then to CA1, the information then travels through subiculum, and back to the deep layers of EC. From there, information projects back to the cortical areas where it originated. As EC transports multimodal sensory information, it is involved in cross-modal associative memory.

The hippocampal system is involved in several stages of memory. When a new event occurs, the acquisition of memory takes place. In this stage, the information of the event, e.g. the visual stimulus and the auditory stimuli as well as some contextual stimuli, is encoded based on their modalities. The information is thus encoded as a construct to be stored and recalled later. Following the encoding of memory, consolidation happens in several hours at synaptic level and after several weeks in a system level (Abel and Lattal, 2001).

In fear conditioning, retrieval tests reactivate the encoded memory and also establish new memories that occur during the retrieval tests themselves. In a typical fear conditioning paradigm, an associative relationship between a neutral stimulus (Conditioned Stimulus, CS) and an aversive stimulus (Unconditioned Stimuli, US) is established by several trials of pairing. After the pairing, the CS is able to evoke the conditioned response (CR) related to the US without the presence of it. In retrieval test, the CR is usually assessed by presenting the CS without the US. In that case, the response to the CS decreases as the animal learns that the CS no longer predicts the US; extinction of memory happens in this condition.

Chen et al. (2012) conducted an experiment with implanted electrodes in the auditory cortex and drug cannula in the EC on behaving rats. Pre-training unilateral EC inactivation impaired the establishment of an artificial association between a visual stimulus and bilateral auditory cortex stimulation. Post-training unilateral EC inactivation at early days affected retrieval of the memory. Following this line of research, the present study conducted a bilateral EC manipulation on cross-modal associative memory.

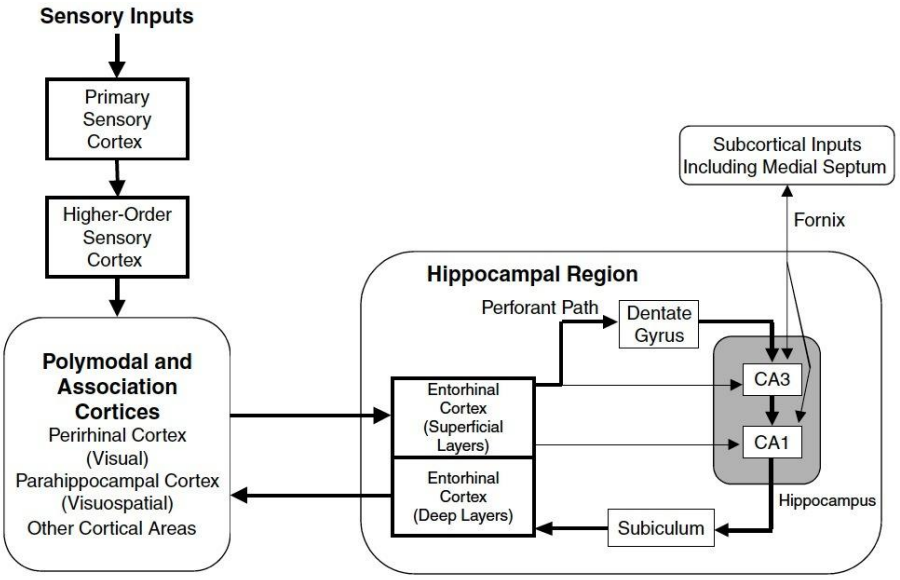


Figure 2.1 Sensory information flow within the hippocampal region

Adapted from Gluck and Myers (2001, Page 261).

2.3 Cross-modal associative learning

2.3.1 Associative learning

Associative learning is the process of associating an element with a separate, pre-occurring element. Associative learning is one elucidating mechanism involved in discrete learning sessions and an adaptive process that allows an organism to learn to anticipate events. It is the process of learning relationships between stimuli such as which stimulus predicts another or what the combination of stimuli that co-occurs is.

The cross-modal association refers to animals' ability to link information of different modality. It bears a much closer resemblance to the multisensory nature in human experience than a unisensory association.

There are findings that cross-modal visual-auditory stimuli could largely facilitate subjects' motion activity, and the extra-striate visual cortex of early blind subjects can be modulated by auditory stimulation (Arno et al., 2001, Franciotti et al., 2011, Alink et al., 2012).

Audiovisual interactions and representation enable the cross-modal processing of visual modulation of neurons in auditory cortex (Romanski,

2007, Kayser et al., 2008). Even non-auditory events, such as visual and somatosensory stimulation and movements, can activate auditory cortex (Brosch et al., 2005). Responses are generally faster and accurate when both auditory and visual modality are stimulated than when only one is (Patching and Quinlan, 2004).

Butler and James (2011) highlighted the importance of the role that multisensory information plays in memory. They discovered the behavioral and neural differences between cross-modal audiovisual associations and within-modal associations. The recall of information from cross-modal association activated the hippocampus to a greater degree than within-modal association.

2.3.2 Fear conditioning

Pavlovian fear conditioning has been widely used to investigate the neural mechanisms underlying learning and memory, since the Nobel Laureate Pavlov, a Russian physician, discovered this phenomenon (Sanders et al., 2003). It establishes an associative relationship between a neutral stimulus and an aversive stimulus. The neutral stimulus is termed conditioned

stimulus (CS) whereas the aversive stimulus is termed unconditioned stimulus (US). The US elicits physiological response inherently, which is called unconditioned response (UR). Presented together with the US in a certain spatial and temporal relationship, the CS is able to evoke the conditioned response (CR) without the presence of the US.

Delay fear conditioning and trace fear conditioning are the two types of fear conditioning (Han et al., 2003, Fanselow and Poulos, 2005). In delay conditioning, CS onset occurs briefly before US onset and its termination is either coincident with US onset or termination. In trace fear conditioning, CS termination briefly occurs before US onset and there is a time gap in between (Fanselow and Poulos, 2005). Researchers found that trace fear conditioning needs the involvement of the hippocampus while there is no affirmed conclusion about delay fear conditioning (Crestani et al., 2002, Fanselow and Poulos, 2005, Misane et al., 2005, Quinn et al., 2008).

Chapter 3 The Role of Entorhinal Cortex in Associative Memory

This chapter begins with the introduction to materials and methods, followed by the results. A within-subject design was conducted to answer the question of whether the entorhinal cortex (EC) is involved and to what extent the involvement is in the associative memory. Section 3.1 introduces the subjects, experiment procedures and data analysis. Section 3.2 presents the overview and statistical analysis of the results.

3.1 Materials and methods

The subjects and materials are presented along the experimental procedures in a chronological manner. The parameters used in the training and testing are illustrated in the training and the testing session respectively.

3.1.1 Subjects

The subjects were 5 male Sprague-Dawley rats. All of them were approximately 2 months of age at the start of the experiment, weighing between 280–350g. The rats were kept individually in plastic cages in the

Central Animal Facility (CAF), Hong Kong Polytechnic University. They all had free access to water and food. The temperature in the room was maintained at 21 ℃ and a regular 12-hour light-dark cycle was given to the subjects in the CAF center. The training and testing were all conducted during the light portion of the light-dark cycle. All experimental protocols were approved by the Animal Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University.

3.1.2 Surgery

Atropine sulfate (0.05mg/kg) was given subcutaneously 15 minutes before anesthesia and at regular intervals during the operation to inhibit tracheal secretion. Subjects were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (54.7mg/ml solution, Ceva Santé Animale Co., France) before the surgery. Supplemental doses (25mg/kg/h) were administrated regularly in order to maintain the anesthetized state throughout the surgery. The subject's body temperature was maintained at 37-38 ℃.

After the induction of anesthesia, the subject was mounted on a stereotaxic instrument (Narishige Scientific Instrument Lab, Japan). An incision was made along the middle line of the head. The stereotaxic instrument was adjusted to place bregma and lambda in a flat skull surface. Craniotomy and implantation of drug injection cannula (0.48 mm in diameter; RWD Life Science Co. Ltd, Shenzhen, China) were performed on the rats under sterile conditions. The drug cannula guides and the dummy inner guide cannula were fixed as a whole before implanting. Four small skull screws (1mm) were positioned to anchor dental cement (Mega Press NV, Germany) to the cannula.

Small holes were drilled bilaterally to the EC with the coordinates of 6.6mm posterior to bregma, 4.7mm lateral to the mid-sagittal suture, and 6.8mm ventral to dura. The drug injection site was identified in a pilot study conducted in anesthetized rats, in which the activation of the region modulated neuronal responses in the auditory cortex to the auditory stimulus.

The fixed cannulae would then be placed perpendicularly to the head and were driven bilaterally to the target location by a micromanipulator (Narishige, Japan). Five minutes were allowed for the cannulae to become steady in place and for the tissue to release the tension. Then a layer of silicon elastomer (Kwik-Cast, World Precision Instrument, Inc.) was used to cover the dura-removed space around the cannulae to protect the cortex from further damage in later operation. Another five minutes were allowed to let the silicon become hard and fixed at both sites. Dental cement mixture consisted of Mega Press liquid and powder was then applied to the skull to fix the cannulae.

After the surgery, the rat was kept on a cotton pad in a plastic basket till fully awake and freely moving. Later the animals were transported back to the CAF. Three to five days of recovery were allowed before the experiment.

3.1.3 Behavioral apparatus

The fear conditioning and the test of associative memory occurred in a homemade Plexi-glass and stainless steel chamber (27cm x 28cm x 51cm).

The chamber was placed in a double-walled soundproof room (NAP, Clayton, Australia). It had a grid with a grey plastic tray underneath. The grid consisted of 20 parallel steel rods (5mm diameter), with a 10mm spacing between each rod. The grid was connected to a Grass stimulator (GRASS s48 stimulator) to generate a scrambled foot shock, with the voltage set to a range of 32v to 42v. A computer running TDT auditory workstation software was used to control all stimulus presentations.

A white light lamp was always lighted with the direction pointing to the ceiling of the room to provide a dim environment so that the camera could capture the video. The chamber was flushed with water before and after each session during the experiment.

3.1.4 Auditory stimulus and visual stimulus

The auditory stimuli were delivered through an open-field speaker (Tucker-Davis Technologies, Alachua, FL). The sound pressure level (SPL) was calibrated with a condenser microphone (Center Technology, Taipei). A pure tone equal to 42 dB SPL was used in the experiment. The visual stimuli were generated by a homemade LED matrix. The

illumination of the light was 26 Lux measured at the bottom of the chamber.

3.1.5 Training

For fear conditioning, a classical trace fear conditioning paradigm was adopted. The parameter of the paradigm was set based on the previous research (Chen et al., 2012). After a 2-min baseline, each rat received a 60-trial session of trace fear conditioning.

In the conditioning, a combined 100ms light stimulus and a 20ms sound stimulus (200ms delay from the light's onset, 42dB) was used as the CS and a 0.5s' foot shock (500ms delay from the onset of the light) was used as the US, as illustrated in Figure 3.1. The inter-trial-interval was 30 seconds. Immediately following the last conditioning trial, the rat was taken out of the chamber and housed in a cage in another room in the laboratory. Usually, there were two subjects in a parallel experiment. After the second rat was presented with 60-trial conditioning, the two subjects were transported back to CAF center.

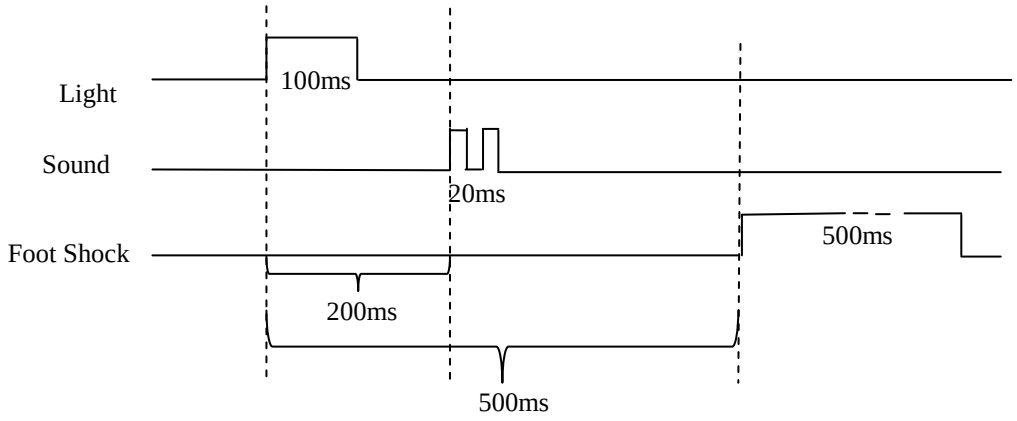


Figure 3.1 Schematic of the trace fear conditioning

The number of trails delivered in the experiment was a case-by-case issue. After 3 days conditioning, there were two trials with only light to test the freezing response. If the rat showed high freezing to the light, then the conditioning was ended. The rat was then promoted to the test phase. If the freezing percentage was low, there would be another 60 trials and another test to measure the conditioning result. Generally, the conditioning was delivered to the rat for 4-5 days with 240-300 trials in total.

3.1.6 DNQX

1. Rationale for infusing DNQX

In the mammalian nervous system, amino-3-hydroxy-5-methylisox-azole-4-propionic acid (AMPA) receptors (AMPARs) mediate fast, glutamatergic excitatory synaptic transmission and play important roles in

several forms of synaptic plasticity, such as short-term and long-term synaptic plasticity (Lee et al., 2010). As long-lasting change in synaptic function is widely acknowledged to be the cellular basis of learning and memory, a functional recruitment of AMPARs in a postsynaptic modification was strongly supported (Malinow and Malenka, 2002).

Quinoxaline derivatives have routinely been used over several decades as non-NMDA glutamate receptor antagonists. They can modulate synaptic transmission and alter neuronal excitability via transmembrane AMPARs regulatory proteins in hippocampal and cerebellar neurons (Lee et al., 2010). Quinoxaline derivatives were all good antagonists of glycine/NMDA currents and displayed a greater range of potencies against kainite and AMPA in a study on xenopus oocytes (Randle et al., 1992).

DNQX, a glutamatergic AMPA antagonist, is one of quinoxaline derivatives. It could be applied to selectively decrease or block excitatory responses (Finch et al., 1995). The DNQX-mediated depolarization is mediated via a postsynaptic mechanism (Lee et al., 2010). Reversal of

DNQX is usually slow and non-existent over several minutes, thus allow the examination of the behavioral response.

2. Infusion of DNQX

To explore the functional role of EC in the retrieval of memory, bilateral injection of 6,7-dinitroquinoxaline-2,3-dione (DNQX) was implemented to reversibly inactivate the area.

The freezing responses were recorded with or without DNQX drug injection at different time points from the establishment of the memory in 5 weeks. This post-training drug injection approach, alternatively administrating DNQX injection and no drug injection to the subjects only after the intensive training, was an efficient way to protect the animals' memory in the acquisition and the consolidation period.

At the infusion time point, animals were gently wrapped in a soft and dry towel. The dummy cannulae were removed and infusion was administered through an injection cannula (G = 0.5mm). The parameters of the cannula were as follows: Guide Cannula O.D. = 0.48mm, C = 10mm; Dummy Cannula G = 0.2mm; Injector for Guide Cannula G = 0.5mm), as illustrated in Figure 3.2. The injection cannula was attached to a 5μl

Hamilton syringe (Hamilton, Reno, NV) with polyethylene tubing filled with silicone oil (World Precision Instruments, USA). The syringe was then driven by a micro-infuser pump, a QSI-Quintesssential Stereotaxic Injector (Stoelting Co., USA).

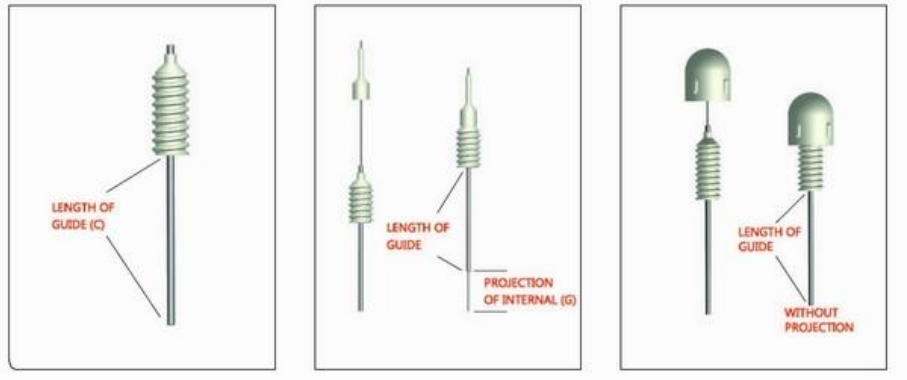


Figure 3.2 The cannulae system

Adapted from RWD Life Science (2006), retrieved on Nov 11, 2012.

DNQX disodium salt (Tocris Bioscience, UK), a glutamate antagonist, was dissolved in saline to a concentration of 10mM/L. Each hemisphere of the rats was treated with 1.5 µl DNQX.

To avoid the disadvantage of the chronically implanted cannulae, the infusion was set at a constant rate of 0.30 µl/min in 5 minutes for a total volume of 1.5 µl. The two hemispheres of the rat were infused sequentially: first left hemisphere, then the right one. Following the infusion of each hemisphere, the injector was left in place for five minutes to allow for

adequate diffusion of the compound into the EC. Once the infusions were complete, the injector was replaced by the dummy cannulae. Another 10 minutes was allowed for the drugs to take effect and for the subject to calm down before being placed into the behavioral apparatus. During the infusion, the animals were kept in a large basket and allowed to move freely. For the EC intact condition, no compound was infused to EC.

3.1.7 Testing

In the test, the animals were placed in the behavioral apparatus again as in the conditioning phase. Only the light stimulus was employed as the test stimulus. The defensive response that the rat displayed, which is termed freezing, was adopted as an indicator for measuring the strength of memory. Freezing was defined as the absence of movement except for respiration (Blanchard and Blanchard, 1969).

The time rats spent freezing within 30s of the presentation of light were collected for calculating the freezing percentage based on the previous experiments (Chen et al., 2012). The freezing was scored by the experimenter using the time frame provided by Windows Media Player.

The freezing time was reported as the percentage of time spent freezing in the 30s after the presentation of light stimulus.

The associative memory was assessed with two trials every week under each condition: the EC intact condition and the EC inactivated condition. The two trials were usually delivered within 5 minutes. However, in the EC inactivated condition, hyperactivity was always observed. Due to the hyperactivity, the time between the two trials was varied.

3.1.8 Histology

After 5 weeks of observation, the rats were anaesthetized with a lethal dose of sodium pentobarbital and were perfused intracardially with 250ml saline solution followed by a 300ml of 4% paraformaldehyde solution (PFA). The brains were then extracted and stored in 4% PFA solution for at least 24 hours, and then transferred to 30% sucrose solution. The fixed brains were sliced coronally into 40 μm sections with a cryostat (Shandon Cryotome E, Thermo electron cooperation). The sections were mounted onto gelatin coated slides and placed in a dry and clean environment for completely drying in the following two days.

The Cresyl Violet Staining was performed after the brain slices were completely dry on the gelatin-coated slides to verify cannulae position. Slices were immersed for 2 minutes in each of the following: xylene, xylene, 100% ethanol, 95% ethanol, 75% ethanol. Slices were then dipped in distilled water for 2 minutes and stained in 0.1% Cresyl Violet acetate for 15-20 minutes, in which the color of the staining slices was checked under a microscope frequently. Then the slices were rinsed in distilled water for 3 minutes and dehydrated and prepared for clearing with 1 minute in each of the following: 75% ethanol, 95% ethanol, 100%ethanol. The clearing was performed with a 5 minutes immersing in xylene and then the slices were kept immersed in a new solution of xylene till coverslipped with DPX mountant.

3.1.9 Data analysis

Statistical analyses were performed using Stata/SE (v 12.1, StataCorp LP, TX). Data were stored as a Windows Media Video (WMV) file on a computer for off-line analysis. Two-way analysis of variance (ANOVA) was used to evaluate the treatment effect across time. One-way repeated

measures ANOVA was employed to test the difference in the means of the freezing percentages under distinct conditions across time. Bonferroni's correction was used in post-hoc pairwise comparisons. For comparison between freezing responses of the condition that the EC was intact and the condition that the EC was inactivated within the same week, paired student's t-test was used. Statistical outliers, greater than 2 Standard Deviation (SD) away from the mean were excluded in the analysis. Results yielding a p-value of 0.05 are considered statistically significant. If the p-value is under 0.01, results are considered highly significant.

3.2 Results

In this experiment, there were two conditions, the EC intact condition (control) and the EC inactivated condition. In the EC intact condition the EC was not injected with DNQX. In the EC inactivated condition, the drug was injected bilaterally into the EC.

Freezing was defined as the absence of movement except for respiratory action (Blanchard and Blanchard, 1969). In this experiment, the freezing

percentage of the subjects was measured for 30s immediately after the presentation of each light stimulus in the test phase.

3.2.1 Overview of the raw data

Figure 3.3 presents a clear view of the comparison between the EC intact condition and the EC inactivated condition of SD0911. The observations of other subjects are presented in Appendix I.

To illustrate the raw data of the freezing responses across weeks, the behavioral responses of 5s before and 30s after the presentation of the light stimulus are depicted in a raster plot. For example, Figure 3.4 shows the responses of subject SD0911. The observations of other subjects are presented in Appendix II. The first trials in each week are supplemented with video records (see Appendix IV).

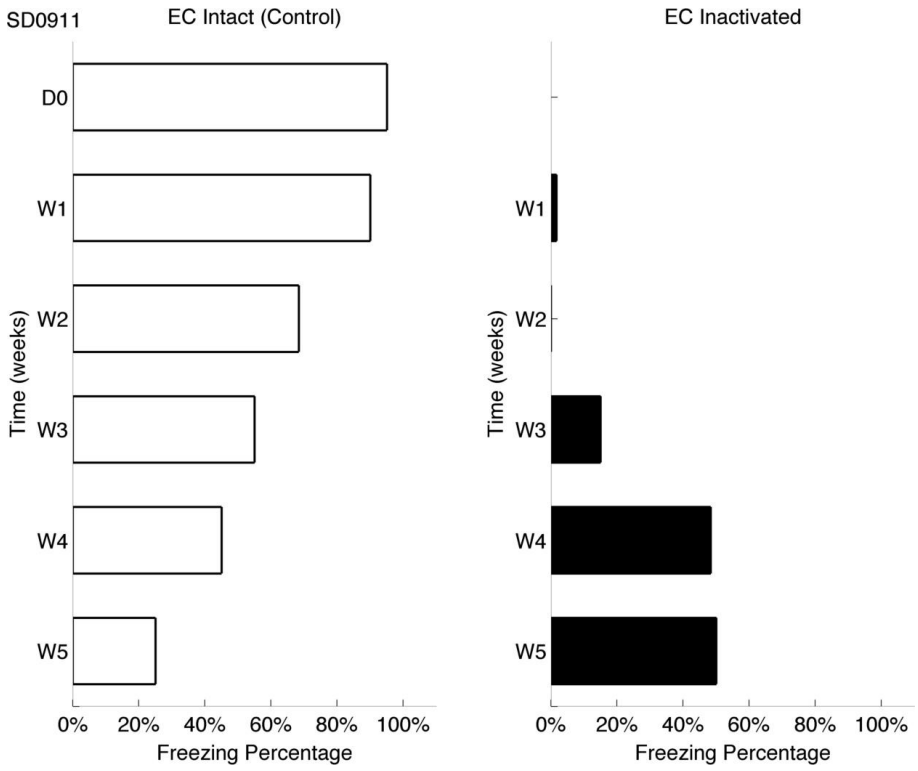


Figure 3.3 Freezing percentage across weeks

The left panel shows the EC intact condition, represented as control. The right panel shows the EC inactivated condition. *D0* is the last day of the conditioning. The freezing percentage obtained from *D0* is presented as the baseline. Under the control condition, the freezing percentage decreased across time, from around 85% freezing to around 35% freezing. Contrary to the control condition, the performance in the EC inactivated condition shows a growing trend in the percentage of freezing. The growing trend started with almost 0% freezing to around 50% percent of freezing.

As shown in Figure 3.4, the control showed almost complete freezing in the first two weeks after conditioning, while in the EC inactivated condition only 2s or 3s freezing was observed (see the dashed box).

The performances in both groups converged toward the same level of freezing in the last two weeks. Specifically, the freezing time approached

50% in the EC inactivated condition, while the performance in the control group decreased to 30%.

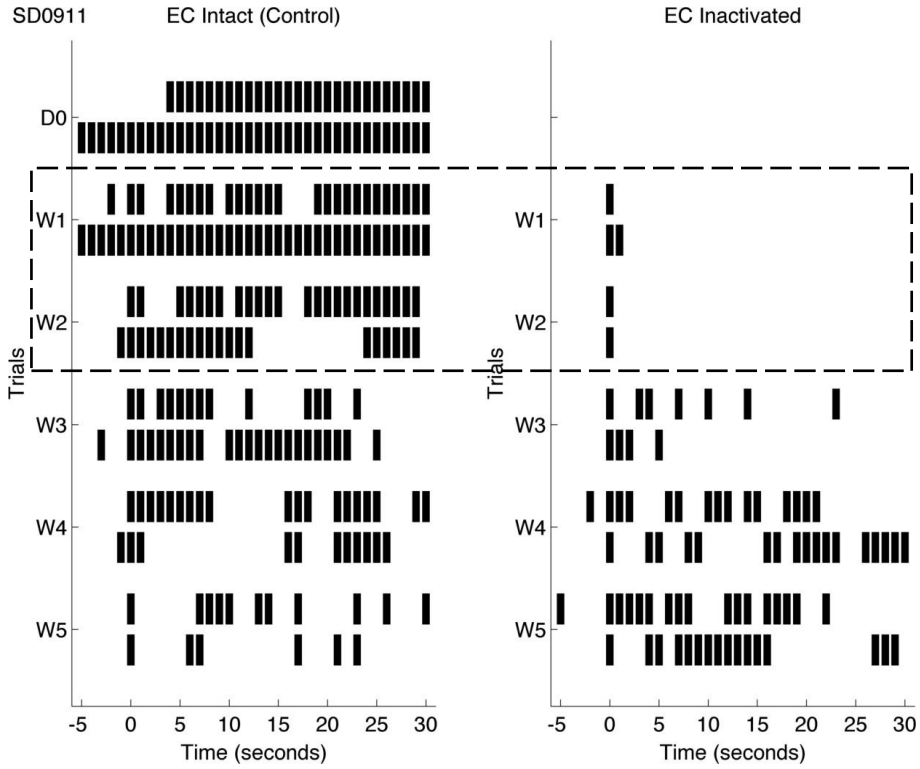


Figure 3.4 Raster plot of freezing responses

Each black bar indicates 1s of freezing and each blank bar indicates motion of the subject. Each subject was exposed to two trials under each condition every week for 5 weeks. On the last training day (labeled as *D0*), subjects were also presented with two trials.

3.2.2 Comparison of the EC-intact and EC-inactivated conditions

Table 3.1 presents the mean and standard deviation of freezing percentage over time under the two conditions for five weeks. The standard deviations of freezing percentages in both conditions increased with time. In Week 1,

the freezing percentage of each subject was clustered closely around the mean value. However, since Week 4, there was considerable variation in freezing percentages between subjects.

Table 3.1 Results of paired t-test of mean freezing percentage

Time (weeks)	P-value	Mean freezing percentage	
		EC intact (Control, %)	EC inactivated (%)
1	**0.000	96.3 (4.15)	2.3 (2.8)
2	**0.000	87.0 (16.3)	8.3 (7.7)
3	**0.003	76.0 (16.0)	8.3 (9.5)
4	0.408	52.0 (38.3)	34.3 (12.2)
5	0.473	34.0 (30.5)	23.3 (19.6)

Note: Standard deviations are embraced in the brackets.

For each week, paired t-test is performed to compare the mean freezing percentages of the two conditions. In Week 1, Week 2 and Week 3, there is highly significant difference, as shown in Table 3.1 (indicated by **). For example, in Week 1, the freezing percentage in the EC intact condition was 96%, while the freezing percentage in the EC inactivated condition was 2.3%, $t(4) = 50.25$, $p < 0.001$.

In Week 4 and Week 5, the freezing percentages in both conditions approached each other. In Week 5, the difference between the freezing percentage in both conditions was less than 10% (34.0% in the EC intact condition and 23.3% in the EC inactivated condition, $t(4) = 0.79$, $p = 0.473$).

3.2.3 Effect of time on associative memory

Each subject was exposed to two treatment conditions (EC intact and EC inactivated condition) over a period of five weeks. The two-way ANOVA was employed to analyze the effect of treatment condition and time (number of weeks since conditioning) on the freezing percentage.

The two-way ANOVA indicated a statistically significant effect of treatment condition, $F(1, 36) = 114.61$, $p < 0.001$, as well as a significant effect of treatment conditions by time interaction, $F(4, 36) = 11.10$, $p < 0.001$. However, the time effect was not significant, $F(4, 36) = 2.10$, $p = 0.101$. This result implied that the effect of time on the mean freezing percentage depended on the treatment condition.

To assess the effect of time, one-way repeated measures ANOVA is performed separately for each treatment condition. Post hoc analysis with Bonferroni's correction was conducted to compare the mean freezing percentage between weeks.

Figure 3.5 shows that the mean freezing percentage in the EC intact condition decreased with time. The highest mean freezing percentage was 96.3% in Week 1 and the lowest was 34.0% in Week 5. One-way ANOVA of freezing percentage in the EC intact condition indicated that the effect of time is statistically significant, $F(4, 16) = 9.60$, $p < 0.001$.

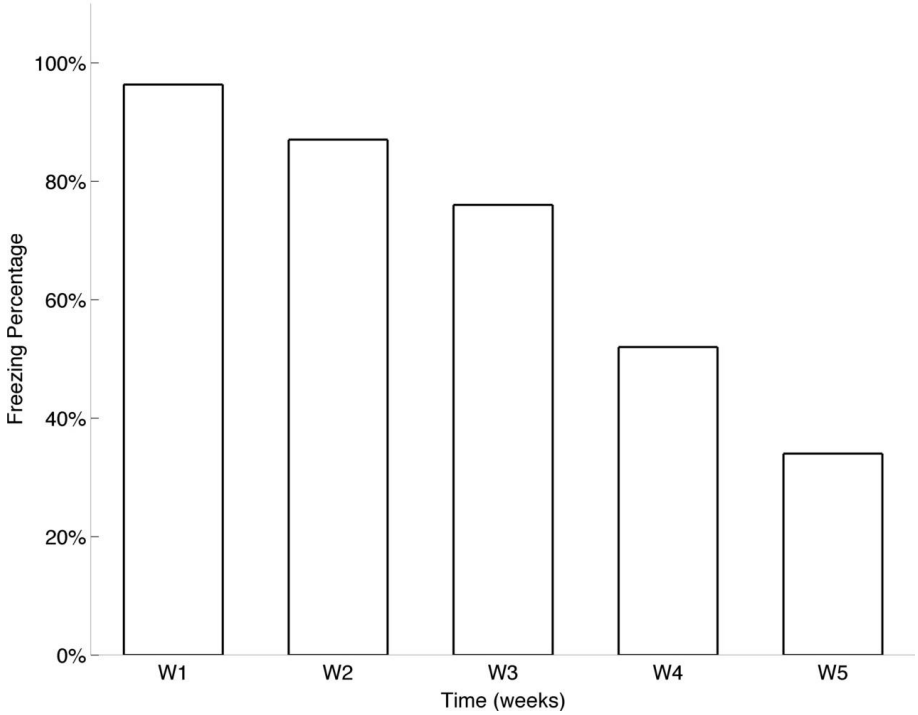


Figure 3.5 Mean freezing percentage in EC-intact (Control) condition

Figure 3.6 shows that the mean freezing percentage of Week 1, Week 2 and Week 3 in the EC intact condition were statistically different from that of Week 5. There was also significant difference between mean freezing percentages of Week 1 and Week 4 in this condition. Table 3.2 presents the p-values for the pairwise comparisons between weeks.

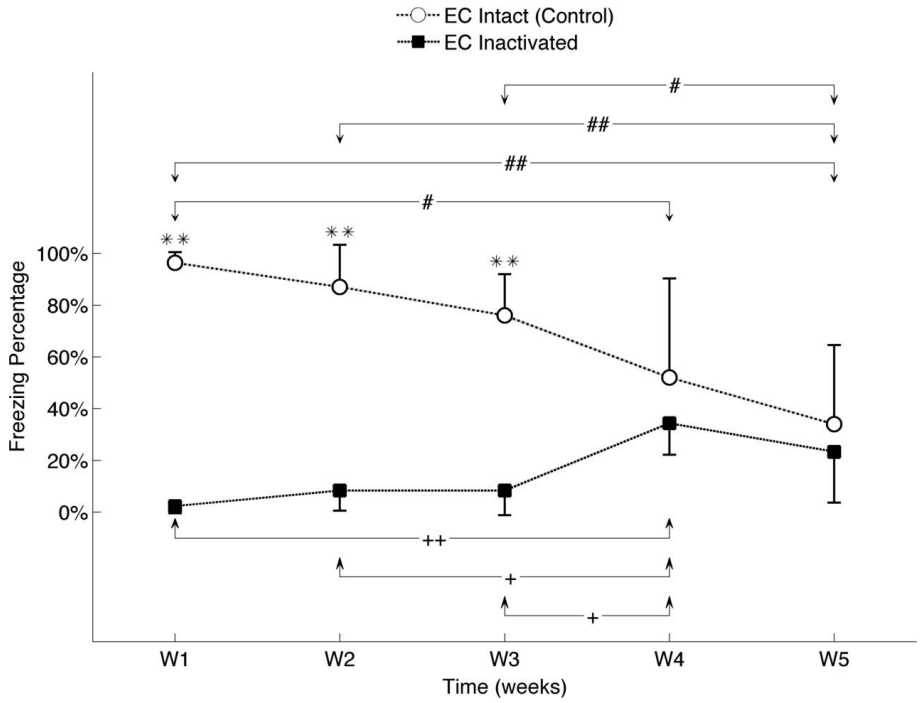


Figure 3.6 Entorhinal cortex in cross-modal associative memory

The statistical significance levels of one-way ANOVA are indicated by # ($p < 0.05$) and ## ($p < 0.01$) for control condition and by + ($p < 0.05$) and ++ ($p < 0.01$) for EC inactivated condition. In addition, statistical significance level of t-test is indicated by ** ($p < 0.01$).

Figure 3.7 shows that the mean freezing percentage in the EC-inactivated condition was maintained at a low level in the first three weeks (less than

10%). However, in Week 5 the mean freezing percentage in this condition increased to 23.3%. One-way ANOVA of freezing percentage in the EC inactivated condition indicated a significant effect of time, $F(4, 16) = 6.39$, $p = 0.003$.

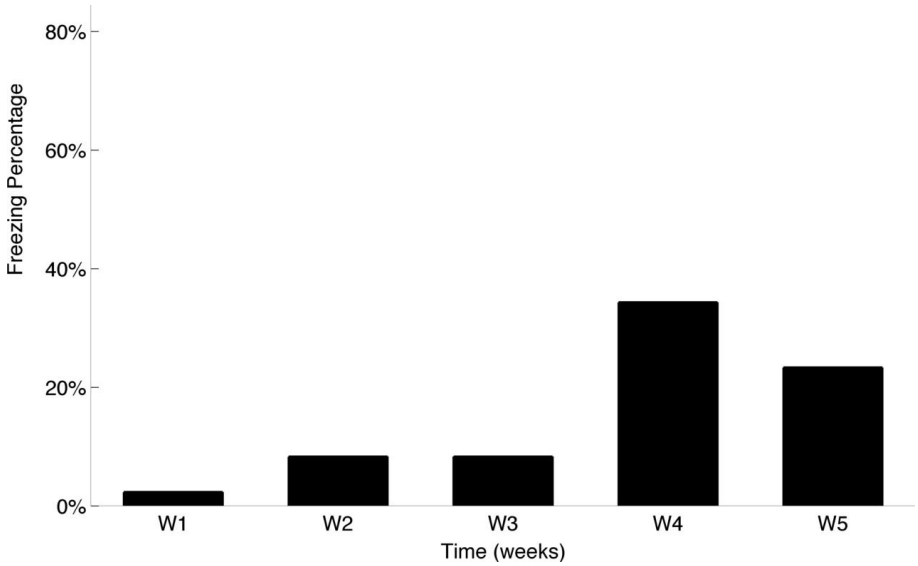


Figure 3.7 Mean freezing percentage in EC-inactivated condition

In the EC inactivated condition, the freezing percentage increased over time, as shown in Figure 3.7. Response in Week 1, Week 2, and Week 3 are all significantly different from that of Week 4, as shown in Figure 3.6. For detailed statistical results, see Table 3.2.

This result supports the hypothesis that recent cross-modal associative memory depends on the EC and this dependence decreases over time as the memory turns into a remote memory.

Table 3.2 Multiple pairwise comparisons between weeks

Week Comparison	P-Value	
	EC Intact (Control)	EC Inactivated
2 VS 1	1.000	1.000
3 VS 1	1.000	1.000
4 VS 1	*0.016	**0.005
5 VS 1	**0.001	0.115
3 VS 2	1.000	1.000
4 VS 2	0.087	*0.028
5 VS 2	**0.003	0.584
4 VS 3	0.572	*0.028
5 VS 3	*0.025	0.584
5 VS 4	1.000	1.000

Note: post-hoc analysis with Bonferroni's correction.

3.2.4 Verification of cannulae location

The location of the guide cannulae and the injector cannulae in this experiment should be verified to confirm the accuracy of drug infusion. The EC was targeted with the coordinates of 6.6mm posterior to bregma, 4.7mm lateral to the mid-sagittal suture, and 6.8mm ventral to dura bilaterally. The guide cannulae fixed with dummy cannula (G= 0.2mm) were combined as one to target the area. As the injector for guide cannula

(G=0.5mm) replaced the dummy cannulae in the infusion, it extended 0.3mm deeper to the ventral as expected. The exact position of the cannulae was 6.6mm posterior to bregma, 4.7mm lateral to the mid-sagittal suture, and 7.1mm ventral to dura bilaterally.

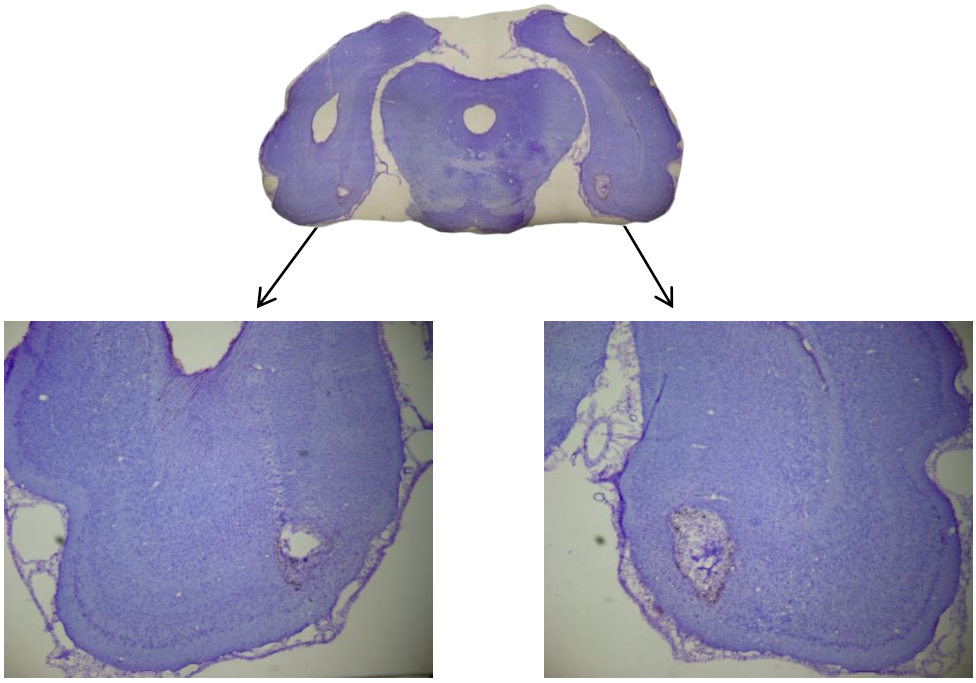


Figure 3.8 Photomicrograph of a Nissl–stained brain section

Following cresyl violet staining, the slices were examined with a microscope (Eclipse 80i, Nikon Japan). The coronal brain sections of subject SD0911 are shown in Figure 3.8. The tips of the injection cannulae extended to the medial EC bilaterally.

The histological results from other individual rats are presented in the Appendix III. Data on two rats (SD0815 and SD0828) was not available due to technical problem. The photomicrographs of 3 rats are shown with the sequence of SD0829, SD0911 and SD0912.

Chapter 4 Visuoauditory Association

This chapter presents the experiment designed to identify another behavioral indicator of cross-modal association besides freezing percentage. Unlike the conditioning paradigm described in Chapter 3 (see Section 3.1.5 and 3.1.7), this experiment used a delay fear conditioning paradigm and a cued-reward behavioral task to establish and demonstrate the association between a visual stimulus and an auditory stimulus. This establishment of this visuoauditory association is described in the training under Section 4.1. The demonstration of it is presented in the result section under Section 4.2.

4.1 Materials and Methods

This section emphasizes the experiment design and the cued reward procedures in the training. It also provides information about the general condition of the experiment, such as the subjects and the stimuli involved.

4.1.1 Subjects

The subjects were 16 male Sprague-Dawley rats: 8 in the experimental group, 8 in the control group. Each animal was deprived of water for two

days before the task and had 15 minutes of free water after training. The environment was the same as those described in Section 3.1.1. Before the experiment, the animal was transported from the CAF to the laboratory. It was kept outside the double-walled soundproof room for 30 minutes to achieve a normal state. Then it was placed in the behavioral apparatus for training.

4.1.2 Behavioral apparatus

The behavioral apparatus was a plastic storage box with the homemade foot shock grid inside (see Figure 4.1.). One side of the box was drilled with 3 holes. The left hole was sealed throughout the experiment. Only the right hole and the central hole were used. Those two holes were equipped with infrared sensors to detect the rats' nose poking activity. The base was a grey plastic tray. Foot shock grid consisted of 19 stainless bars with a distance of 1.5cm between each other. A sound speaker (Tucker-Davis Technologies, Alachua, FL), a homemade LED light and a video-recording camera (Logitech Webcam, CA, USA) were placed above the center. The training apparatus was placed in a double-walled

soundproof room (NAP, Clayton, Australia). The conditioning and the test were conducted in this chamber.

Figure 4.1 The training chamber in the visuoauditory association (seen from above)

4.1.3 Auditory stimulus and visual stimulus

Both stimuli were triggered by the TDT auditory physiology workstation, as described in Section 3.1.4. The auditory stimulus was a 60-70dB SPL white noise. The visual stimulus was a white light generated by light-emitting diodes placed at 40cm above the chamber. The illumination of the light was 26 Lux measured at the bottom of the chamber.

4.1.4 Fear conditioning

In the conditioning phase, the rat was placed in the behavioral apparatus individually. Five minutes were allowed to explore the conditioning chamber. The acclimation resulting from the exploration was assumed to avoid the contextual freezing.

As illustrated in Figure 4.2, a delay fear conditioning was used to establish the association. Rats in the experiment group were exposed to 2s light and 1.8s sound (combined CS) with a 0.6s foot shock (US). The paradigm for the control group was exactly the same except only the light was used as the CS. There were ten trials with a 60s inter-trial interval.

The control group was designed to examine the difference that the association would result in after the cued-reward task. If there were no such a control group, it would be impossible to tell whether the behavioral result in the test was due to the association or not. The control group would also exclude the possibility of sequential procedural knowledge, in which subjects managed the task by simply response to a stimulus then follow the steps in the task, despite of whether it was a sound stimulus or

light stimulus. With a further examination of the fear in the testing in the experiment group and the control group, it would be easy to tell whether the visuoauditory association was the reason for the difference.

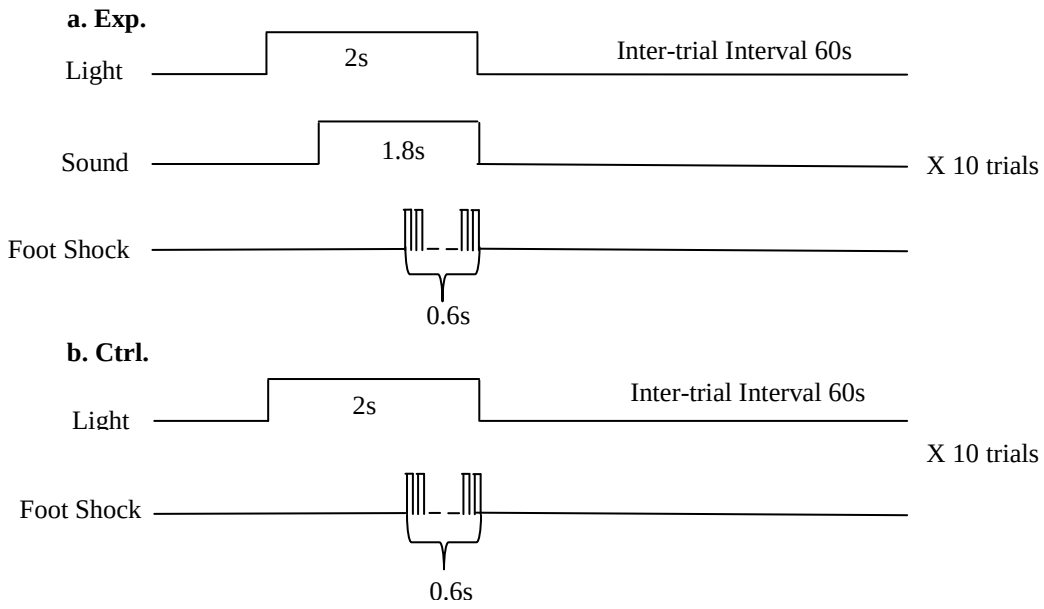


Figure 4.2 Fear conditioning paradigm in visuoauditory association

The conditioning was regarded as effective and successful if the rats showed freezing response to the CS on the next day of the conditioning. That is rats in the experiment group expressed fear to the combined visual-auditory stimuli and rats in the control group expressed fear to the visual stimulus.

4.1.5 Training

To establish an association with the auditory stimulus and the water reward, both groups were trained with the subject-initiated sound-reward protocol. There were 8 rats in the experiment group and 8 rats in the control group.

The key in this training process was to make sure that the rats utilize the auditory stimulus as a cue for the water reward. A five stages training scheme with varied holding time in the last three stages was adopted. The training procedures and criteria are illustrated in Figure 4.3.

Stage 1. The purpose of the first stage of the training was to let the rat know that the water was only delivered in the right hole. The rat could get water reward whenever it poked into the right hole. This step contained around 50 trials.

Stage 2. In this stage the rats were required to poke into the central hole to initiate the task and then move to the right hole to get water reward. The nose-poking signal was detected by the infrared sensor in the central hole and the right hole. After around 100 trials of the second step, most rats

could obtain a high percentage of correctness in this task. Around 300 trials were delivered in this stage.

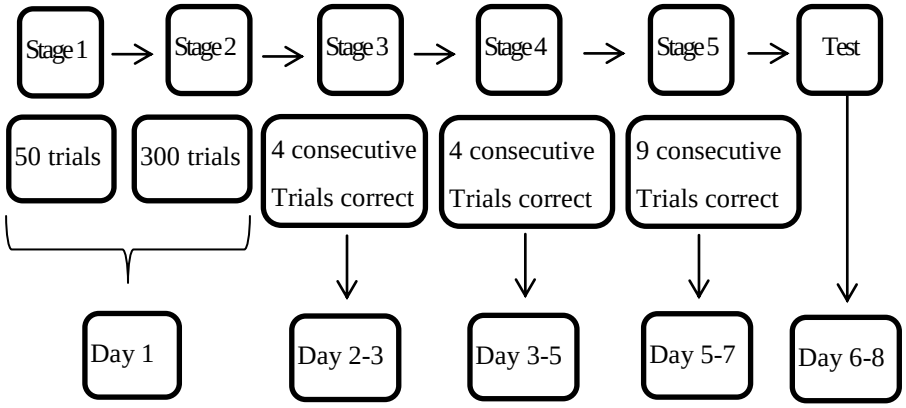


Figure 4.3 The five training stages

In stages 3-5, the sound was added as a cue of water reward. The rat had to poke into the central hole, wait for the onset of the sound cue and then poke the right hole to get water reward. Figure 4.4 gives an illustration of a cued reward trial in the task. The parameters involved in later analysis are indicated by the vertical lines.

Holding time. L1 represents the time that the signal in the central hole was detected. L2 illustrates the time of the onset of the sound. The holding time was defined to be the time after the poke in the central hole and before the onset of the sound stimulus. Here it is indicated by the time between L1 and L2.

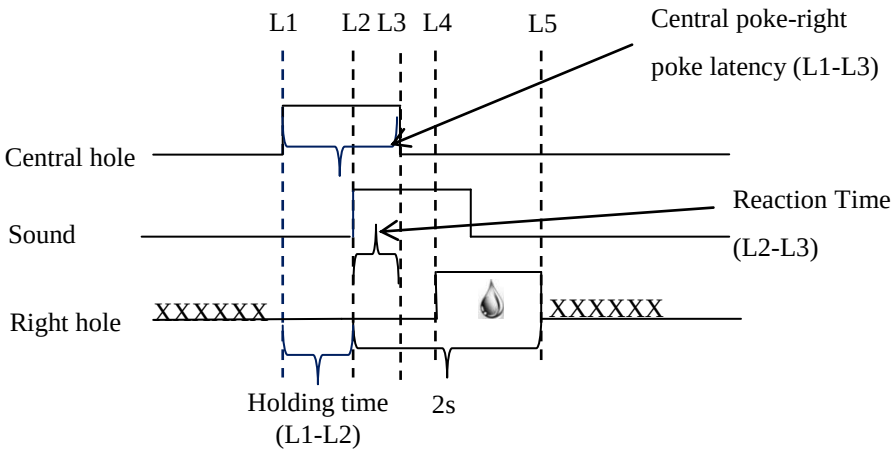


Figure 4.4 Illustration of the cued task

Reaction time. L3 indicates the offset of the signal in the central hole. It was the time that the animal left the central hole. The time between L2 and L3 is defined as the reaction time that the subjects needed to recall the association of the sound toward the water reward.

Central poke-right poke latency. The latency of the right poke to the central poke was shown between L1 and L4.

Movement time. L4 is the time that a signal was detected in the right hole. It is the time that the rat poked in the right hole for the water reward. The time between L3 and L4 indicates the time cost of the movement.

The 2s time window. L5 indicates the end of the trial. There was a 2s time limit from the onset of the sound and the delivery of water, implied

between L2 and L5. The subject had to take action within the 2s by quickly recognizing the sound as a cue and moving its head toward the right hole to get the water reward.

The water was delivered only in the limited time window. Under other conditions, there was no water delivered, indicated by *XXXXXX* in Figure 4.4. The no water delivered condition includes: (1) the rat did not poke in the central hole to initiate the task trial; (2) the rat poked in the central hole but left the hole before the onset of the sound; (3) the rat waited till the onset of sound but did not move to the right hole for water reward; (4) the rat waited till the onset of sound but failed to reach the right hole in the 2s time window for water delivery.

In stage 3-5, the subjects were required to successfully get water reward in one trial, so it could proceed to initiate another trial. If it failed in one trial, the computer would continue delivery of that trial till the subject fulfilled the requirement of the trial and get water reward. Only after that, another trial would be delivered to the rat. All the trials with different holding time were delivered pseudo-randomly by the computer.

Stage 3. It was the first stage that the sound was added to the task. The holding time in this stage was set to be very short, 200-800ms (see Video 1 in Appendix IV). The aim of this phase was to let the rat know that the sound was the cue and make it familiar with the protocol step by step. If the rat could get 4 consecutive trials right, then it was promoted to the next stage on the next day.

Stage 4. It was designed to make sure that the rat only utilizes the sound as a cue to get water reward. The random holding time was sampled from 500 and 1500ms. The criterion of completion was 4 consecutive correct responses.

Stage 5. The holding time overlapped with the former two stages. It enhanced the subject's ability in performing the task. The performance was considered stable when 9 consecutive correct responses were achieved.

4.1.6 Testing

In the test period, the light stimulus used previously in the conditioning replaced the sound stimulus to be the cue in the behavioral task in both

groups. At the beginning of the test session, each rat was given around 50 trials of the subject-initiated sound-reward protocol for the purpose of achieving a stable behavioral state and renewing the cued task. The sound was then replaced by the light. Similar to the subject-initiated sound-reward protocol, this task was named subject-initiated light-reward protocol because subjects had to poke into the central hole to initiate the task and use light as a cue for getting the water reward. The criterion for the completion of this stage was 9 consecutive correct trials.

4.1.7 Data analysis

There were 16 rats in the experiment, with 8 in the experiment group and 8 in the control group. All of the rats in this experiment were not used for other experiments. One rat from the experiment group did not complete stage 3 within one hour of training and was excluded from the data analysis. The training performance of 3 rats was not recorded, so in the analysis about the training process, only data from 12 rats were included.

One-way ANOVA was employed with the data of the central–right poke latency and the difference of reaction time with regard to different holding

time respectively. The central–right poke latency regarding to different holding time was analyzed to find out whether the sound stimulus was taken as a cue. The reaction time difference was plotted against holding time difference.

In the test, t-test was adopted for the analysis of the difference between the first trials in the test and the representative trials of the training period and the difference between the first trials and the performance in trials 72-80 in the test regarding to reaction time.

4.2 Results

This part presents the learning process in the training and demonstrates that the sound was used as the cue for water reward. It then describes the results of both groups in the test, followed by the analysis of the fear in both group.

4.2.1 Behavioral performance in the training

There were 16 rats in total, 8 in the experimental and 8 in the control group. With One rat excluded and three rats' data not recorded in the

training, there were data from 12 rats in the training included in the analysis of the training process.

Stages 1 and 2 were completed on the first day of training by all the subjects. The maximum trials required in each stage across the 12 rats were 118 trials in Stage 3, 141 trials in Stage 4, and 149 trials in Stage 5. The total number of trials in Stages 3-5 ranged from 176 to 338 trials. The curve in Figure 4.5 reflects the learning progress of the 12 rats in Stage 3 to Stage 5, as those are the stages that the sound stimulus was involved.

The percentage in visuoauditory association is the percent of correct subjects in each trial. % correct subjects = number of correct subjects ÷ total number of subjects. "% correct subjects" is the number of subjects that successfully used the sound as cue to get water reward divided by the total number of rats (n=12), i.e. 50% correct subjects represents that there are 6 subjects utilized the sound as the cue to get water reward on the corresponding trials on the x-axis.

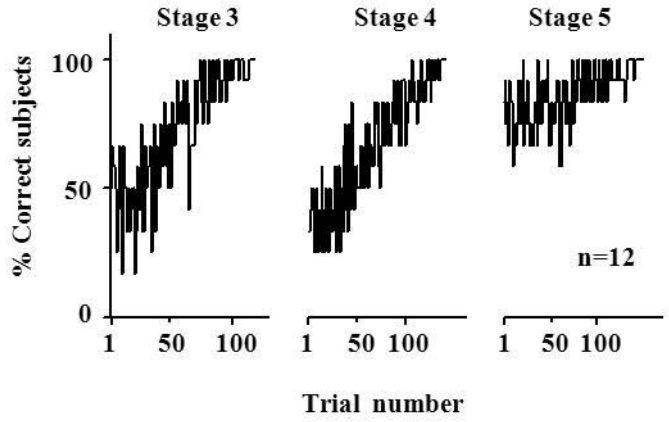


Figure 4.5 Behavioral Response in the training

In Stage 3, the performance of all rats improved gradually as training trials increased, indicating the process of learning. When promoted to Stage 4, all the rats started from a low percent of correctness state again. In Stage 5, as the holding time was within the range of Stage 3 and Stage 4, subjects showed a higher level of percentage of correct rate. A slightly more confined reaction time in Stage 5 is reflected in Figure 4.6. This result indicates that the performance of rats improved throughout the training and those rats were able to reach the expected stable state.

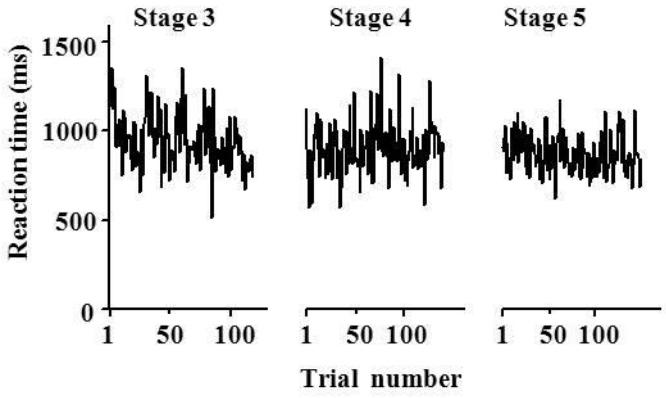


Figure 4.6 Reaction time in the training

4.2.2 The effect of sound

It is crucial to find out whether the rats managed to use the sound as a cue. They could have taken advantage of the overlap between the earliest and latest reward windows. For example, the shortest holding time 100ms allows a retrieval of reward at any time point between 100ms and 2100ms after initiating the trial. Likewise, a longer holding time, such as 1200ms, allows a retrieval of reward at any point between 1200ms and 3200ms. These reward windows overlapped from 1200ms to 2100ms. It is possible that a rat fulfilled the requirement of the task by simply withholding responding for about 1500ms. To address this issue, data from the 12 rats were reexamined.

Figure 4.7 shows the central poke–right poke latency for different holding times. The latency for the 100ms and 200ms holding times were pooled. Each data point represents a single subject in each stage, with 12 rats in total. The latency differed significant across holding times ($F = 51.74$, $P < 0.001$, one-way ANOVA). It indicates that when a longer holding time required, the subjects waited longer till the delivery of sound stimulus. This result supports the utilization of the sound cue.

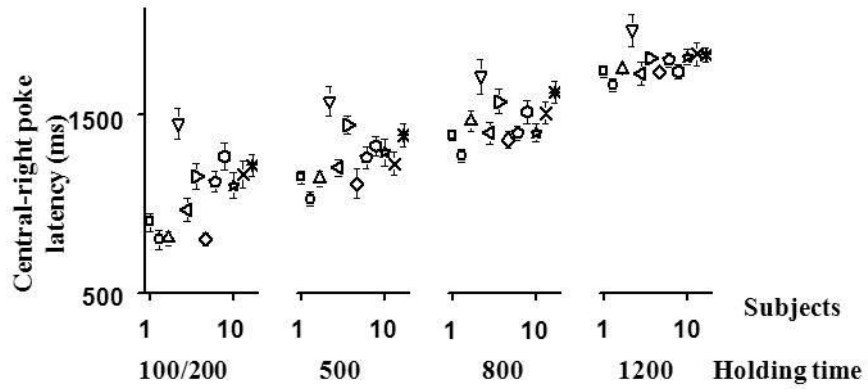


Figure 4.7 Latency of right poke after central poke as a function of holding time

As shown in Figure 4.8, the reaction time decreased while the holding time increased. It seems that the rats may adopt a middle holding time strategy in getting the reward.

It became clear that the decrease of the reaction time as the holding time increase was due to the increase of the subjects' preparation time for the

action. Subjects had enough time to get prepared both psychologically and physically, since a longer holding time allowed a longer waiting time for the trigger of the sound stimulus. Thus it was natural for reaction time to decrease.

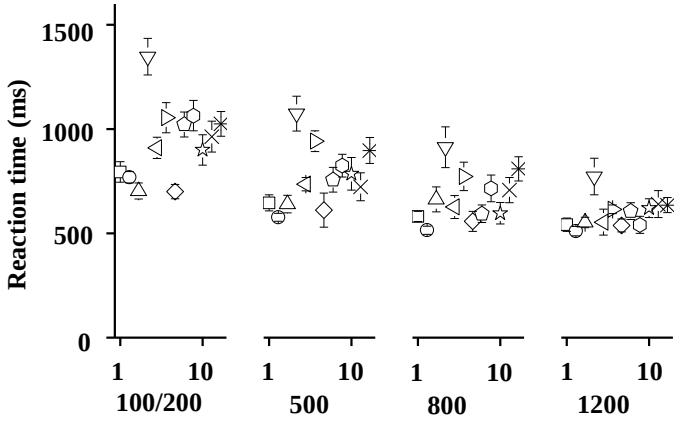


Figure 4.8 Reaction time to different holding time

The relationship between the reaction time difference and the holding time difference is illustrated in Figure 4.9. The difference between the reaction times was far below the difference in the holding time. The horizontal bars in the upper part indicate the difference between the two holding times.

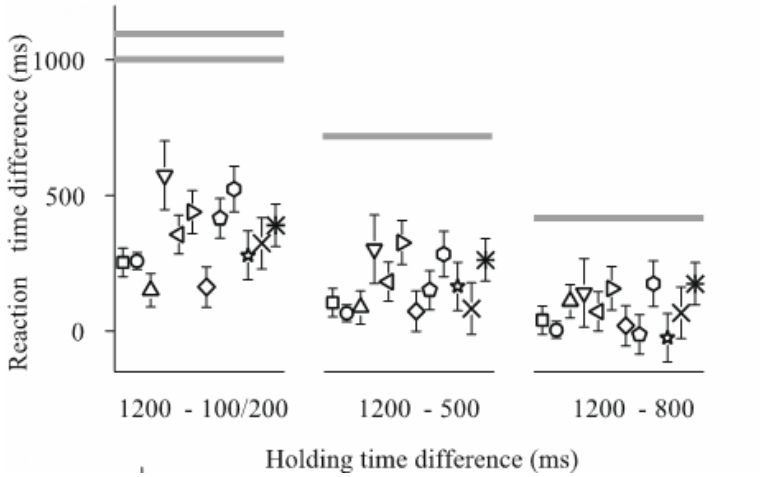


Figure 4.9 Difference in reaction time with different holding time

4.2.3 Behavioral performance in the test

In the test phase, the light stimulus replaced the sound stimulus. Correct trials were the ones that the rats successfully utilized the light stimulus as the cue to get water reward. Unsuccessful trials were the ones that the rats triggered the light stimulus but failed to get water reward. Premature trials were the ones on which the rats did not wait long enough for the light stimulus. To examine the association between the visual stimulus and the auditory stimulus, only trials on which rats triggered the light stimulus were included in the data analysis.

For the test phase, data from all 16 rats except the one excluded in stage 3 were obtained. As a result, 7 rats in the experiment group and 8 rats in the control group were included in the analysis of the testing phase.

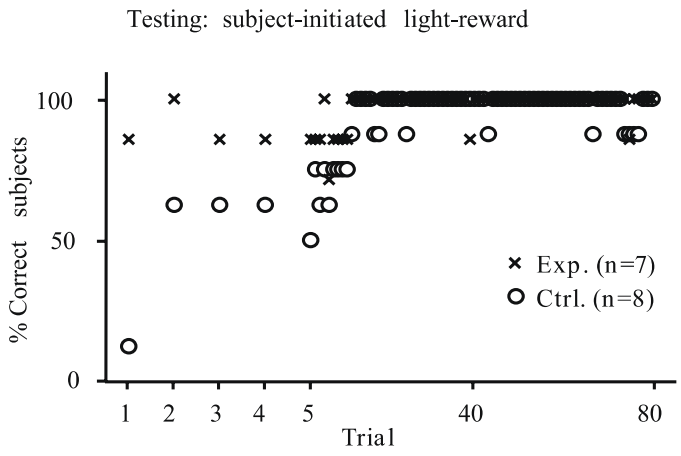


Figure 4.10 Performance in the test

As shown in Figure 4.10, the difference between the experiment group and the control group is significant in the first 20 trials. In the first trials, 6 out of 7 rats in the experiment group successfully got water reward, while only 1 out of 8 rats in the control group did. The percentage of correct subjects in the experiment group continually exceeded that of the control group in around 20 trials. The failure of the control group in the first few trials demonstrates that it was not due to procedural memory that rats in the experiment group succeeded. These results suggest that the experimental group successfully associated the visual stimuli and the auditory stimuli.

The performance of subjects in both groups improved quickly. Both groups got approximately 100% correct subjects. Such trend indicates the learning process of the subjects to the light-reward task in the test with the training. The slight difference is that subjects progressed faster in the test. The result is reasonable because rats had experienced the similar task in the training.

Figure 4.11 shows an averaged reaction time in each group. The trail labeled *1* is the beginning of the test. Trials labeled with negative numbers are the last 9 trials in the training. Trials labeled with positive numbers are in the test.

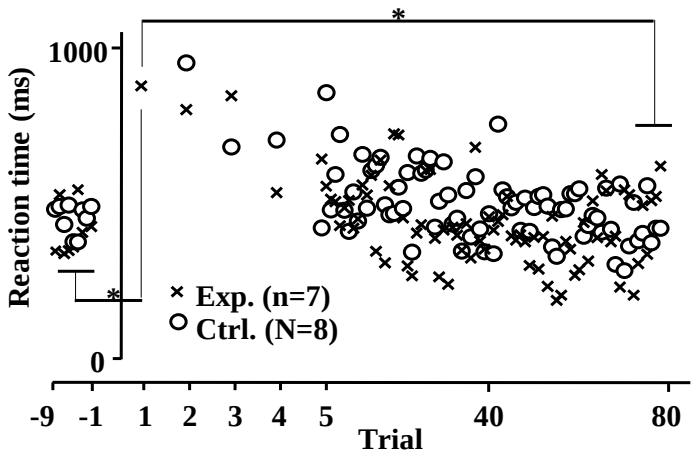


Figure 4.11 Reaction time in the test

There is significant difference between the reaction time of the first trials in the test and the last nine trials in the training ($P < 0.05$, t-test). The

reaction time in the final stage of the training period was 420±28.3ms. It changed immediately to 882±96.6ms on the first trial (n = 6) and 804±203.3ms on the second trial after replacement (n = 7) ($P < 0.05$, ANOVA) in the experiment group.

Difference was also found between the first trials and the performance in trials 72-80 trials regarding to reaction time in the test, as shown in Figure 4.11($P < 0.05$, t-test). The reaction time decreased to 452±36.8ms on trials 72-80 from the first trials (882±96.6ms on the first trial (n=6) and 804±203.3ms on the second trials, $P < 0.01$ vs. trial 1, t-test). The decrease of the reaction time was, perhaps, due to a learning process involving a switch from a light sound-reward association to a light-reward association. There was no difference in the reaction times of the experimental and control groups on trials 72-80 in the test (452±36.8ms vs. 412±34.4ms, $P = 0.42$, t-test).

It is of interest to note that the rats in the experimental group initially paused but then reacted quickly to retrieve the reward after replacement of the auditory stimulus with the visual stimulus. Their reaction time was

significantly longer on the first trial with the visual stimulus than on the first trial with the auditory stimulus (Video 2 and 3). The reaction time difference may indicate the time needed for recalling the auditory cue from the visual cue. Rats in the experimental group paused before moving to retrieve the reward when the auditory stimulus was replaced by the visual stimulus.

This result is consistent with the hypothesis that the subjects in the experiment group were able to associate the light stimuli and the sound stimuli and apply the learned association to facilitate later behaviors. Rats in the control group did not have the association of the light and sound stimuli. They might have taken the light as a new cue in the test.

4.2.4 The effect of fear conditioning

The subjects were exposed to an equal 10 trials of fear conditioning. The fear to the light in the experiment group could have extinguished due to the intensive training with the sound stimulus. Thus the fear to the light in the control group might induce the difference in the test and render the result unconvincing.

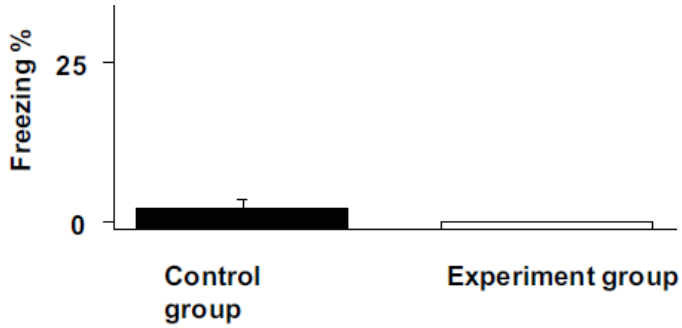


Figure 4.12 Freezing performance after the replacement

To address the above issue, the percent time spent in freezing during the 60s after the presentation of the light stimulus in the first trials was calculated. As shown in Figure 4.12, the percentages of freezing were near zero in both groups at the first trials in the test. This result indicates that it was not the fear in the control group but the cross-modal association that resulted in the difference in the test.

Chapter 5 Discussion and Conclusion

In summary, this research demonstrates that the retrieval of cross-modal associative memory depends on the entorhinal cortex (EC) and the dependence decreases over time. A cross-modal association between visual and auditory stimuli can be established with fear conditioning and be detected by a cued-reward task.

5.1 The role of entorhinal cortex in associative memory

The results of the experiment in Chapter 3 showed that the EC was involved in the early stage of the cross-modal associative memory. In the first three weeks, the freezing percentage was maintained at a high level in the EC intact condition, in contrast to the negligible level in the EC inactivated condition (see Table 3.1). The results implied that the EC was involved in the retrieval of the cross-modal associative memory.

The results from this study were consistent with the gateway function of the EC in the hippocampus-neocortex transfer modal. In the early stage of associative memory, the cross-modal associative memory was stored in the hippocampus as a recent memory. The retrieval of memory went

through a neocortex-EC-Hippocampus-EC-neocortex pathway, as indicated by Figure 5.1(a). If the EC was inactivated, the retrieval process could not access the hippocampus for the stored recent memory. Thus the associative memory could not be retrieved and there was little fear expressed in the test.

Despite the role of EC in the earlier three weeks, the freezing percentage in EC inactivated condition in the last two weeks increased and reached a comparable level as in the EC intact condition (see Figure 3.6). The results revealed that the participation of the EC in the retrieval of memory decreased over time.

This phenomenon was probably caused by the transition of cross-modal associative memory from recent memory to remote memory. If the remote memory had been formed, the cross-modal associative memory was stored in neocortex. The retrieval of the memory only needed to activate the neocortex and did not depend on the EC, as indicated by Figure 5.1(b).

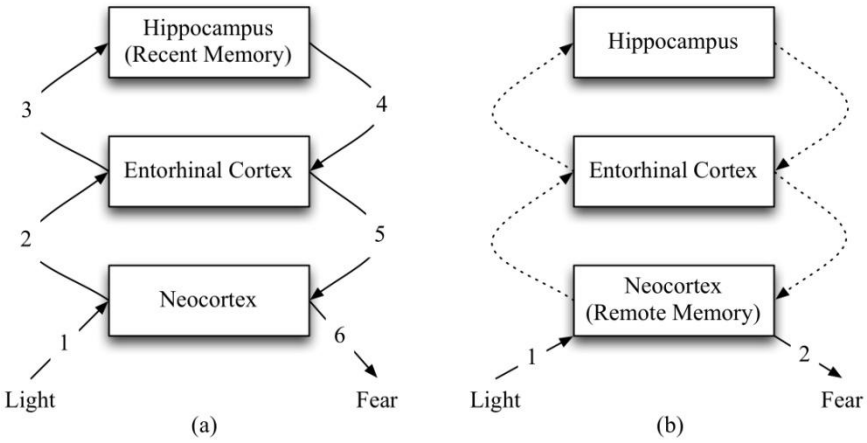


Figure 5.1 Retrieval of recent and remote associative memory

(a) Retrieval of recent memory. The recent memory is stored in Hippocampus. The light stimulus initiates the retrieval of the memory. The information travels through the neocortex to the EC and then arrives at the hippocampus to activate the recent memory. Then the hippocampus sends back the associative memory to the EC. Finally, the associative memory reaches the neocortex to express the fear memory. The path of the retrieval is represented by light-1-⋯-6-fear. (b) Retrieval of remote memory. The remote memory is stored in neocortex. If the remote memory has been formed, the retrieval of the associative memory needs only the participation of neocortex. The path of the retrieval is represented by light-1-2-fear.

This result is consistent with the blocked physiological change that post-training unilateral EC inactivation affects retrieval of the memory at early days (Chen et al., 2012). It also echoes the earlier finding on human study that recent memory depends on the hippocampal system, while the remote memory is stored within neocortex and is less dependent on the

hippocampal system (Graham and Hodges, 1997, Norman and O'Reilly, 2003).

In fear conditioning, the repeated given CS-US combination would likely induce habituation, in which the animals' conditioned response to the presented stimulus decreases as the conditioning trials increases (Storsve et al., 2010). However, in this experiment, the foot shock was delivered on a trial to trial basis, which depends on the state of the subject. With the aim to ensure the intensity of shock was strong enough to arouse attention to the combined CS-CS stimuli, the intensity of the shock varied in a range with obvious fear expression of the subjects during conditioning. After several trials, the animal could learn to predict the delivery of the foot shock with the CS-CS stimuli, as the temporary relationship between the CS and CS, the CSs and the US remained the same across trials.

Extinction depicts the situation, in which the conditioned response (CR) would be decreased in both amplitude and frequency due to repeated given the CS that have been conditioned with a US previously (Abel and Lattal, 2001, Phelps, 2004). In this experiment, the situation in the test is a typical

extinction condition. In the test, part of the CS, the light stimulus, was repeatedly presented alone across the whole experiment. However, only two trials of light stimulus were given in each test session. A small amount of memory extinction could be expected from the result and it does not impair the conclusion of the result.

The EC-intact condition can be treated as an observation of memory across time. The decreased freezing percentage across time corresponds to the possible feature of the extinction. However, the result under the EC-inactivated condition showed increased fear to the light stimulus. Possibly, extinction occurred as the observation time increased under the EC-contact condition in recent memory; in the EC-inactivated condition, there was little expression of fear memory: it was the EC that was needed for this process. As the observation time increases, the recent memory transformed into remote memory, the EC was not needed for the expression of the associative memory. Thus the subjects were able to express a fear memory after extinction under both conditions.

5.2 Alternative explanations of the memory retrieval path

There could be two possible explanations for the associative memory retrieval path. The first explanation is that in the early stage of associative memory, the cross-modal associative memory was stored in the hippocampus as a recent memory. If the EC was inactivated, the retrieval process could not access the hippocampus for the stored recent memory. However, when a remote associative memory has been formed, the neocortex becomes the place for memory storage. Thus even under the EC-inactivated condition, the memory could be retrieved as it does not need the EC for accessing the memory. This explanation is consistent with standard model of system-level consolidation (Alvarez and Squire, 1994, Takashima et al., 2009). In this model, the hippocampus is part of a retrieval network for recent memories. The memory trace is consolidated over weeks and gradually become independent of the hippocampal system and transferred to the neocortex. Remote memories are thus based on neocortical networks and can be retrieved independently of the hippocampus (Teng and Squire, 1999, Wang et al., 2009, Lesburgueres et al., 2011).

The other explanation states the hippocampal system in the medial temporal lobe as an integral part for retrieval of recent memory but the storage of recent memory is the neocortex not the hippocampus. The medial temporal lobe was only necessary for the retrieval of newly formed neocortical memory. The neocortical memory trace is consolidated over the weeks and gradually become independent of the media temporal lobe. That could explain why the animals started to show increasingly freezing time over the last a few weeks. With implanted electrodes in the auditory cortex and drug cannula in the EC on behaving rats, auditory activities were recorded in recent memory (Chen et al., 2012). The recorded activities suggest that the neocortex is participating in the recent memory. Through this point of view, it is possible the neocortex is the place for associative memory storage."

5.3 Visuoauditory association

The results from the experiment in Chapter 4 demonstrated that a behaviorally detectable cross-modal association between visual and

auditory stimuli could be measured by a cued-reward task besides freezing response.

A critical aspect of this study was the use of the reward retrieval paradigm to confirm that rats recalled the auditory memory on the first trial in which the auditory stimulus was replaced with the visual stimulus. Their ability to successfully retrieve the reward under these conditions indicated the strength of the association between the auditory and visual stimuli, as the visual stimulus up to that point had never been a signal for reward availability.

Reward retrieval in this experiment was linked to memory retrieval. There was an initial pause and subsequent rapid retrieval of the reward. This behavior was probably linked to the recall process. The visual stimulus triggered recall of the auditory stimulus, due to the association. The auditory memory led retrieval of the reward.

The different response times seen in the subject-initiated sound-reward paradigm vs. the first trial of the subject-initiated light-reward paradigm likely reflected the time needed for the auditory memory to be recalled by

the visual stimulus. The shortening of the reaction times after repeated trials may be indicative of a learning process involving a switch from an indirect association between the visual stimulus and reward (light → sound → reward) to a direct association (light → reward).

The demonstration of the association between the visual stimulus and the auditory stimulus suggested that animals were able to employ a cross-modal association previously learned to facilitate their later behaviors. The subject-initiated cued-reward behavioral task was effective in examining associative memories.

5.4 Applicability to human brain disorders

This research is closely related to one of the most important questions in brain research. It provides information on how short-term memory is transferred to long-term memory across sensory modalities. This work tested the role of the Entorhinal cortex and provides important information about the role of this brain structure in cross-modal memory transfer. Research on animals enriches the knowledge of the cortices that involved in

the process of learning and memory and gives direction in the later study on human with other technique, such as the PET and the functional MRI.

Several disorders of the human brain were closely related with EC. Layer II of EC was reported as the original location for the pathological changes of mild Alzheimer's disease (Gomez-Isla et al., 1996); layer III of EC is related to temporal lobe epilepsy with marked degeneration (Du et al., 1993). The volume reduction of EC is used as a reliable measurement of the risk for Alzheimer’s disease and proposed as a possible cause of schizophrenia (Baiano et al., 2008).

It is of great concern to use the animal model in studying human learning and memory, as it is hard to use the human as a subject to do invasive research. Research on animals enriches the knowledge of the cortices that involved in the process of learning and memory and gives direction in the later study on human with other techniques.

There is great consensus in finding on EC’s connectivity to the hippocampus across the human and nonhuman animals. The human EC is located in the ventro-medial portion of the temporal lobe. The reciprocal

connection between the EC and the hippocampus and other cortical and subcortical areas renders EC an integral part of the medial temporal lobe memory system.

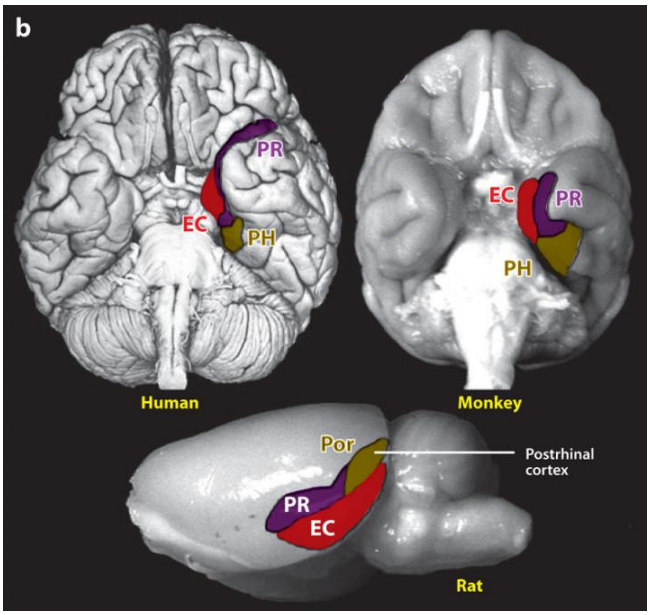


Figure 5.2 The view of human, monkey and rat brains

Adapted from Squire and Wixted (2011)

Work with rats, monkeys, and human is largely in agreement about the function of the hippocampus and related structures (Squire, 1992). The overall organization of the hippocampal-cortical connectivity in the rat is largely comparable to that in the monkey (Insausti et al., 1997). The hippocampal formation is needed for storage or retrieval of declarative memory in recent memory; long-term memory is fully dependent on the neocortex (Squire and Alvarez 1995).

The contribution of entorhinal cortex to learning and memory has been indicated by several studies. Stimulus specific activity of EC neurons were observed and the activity was found to be modified by the animals' experience with that stimulus (Zhu et al., 1995, Young et al., 1997).

Lesion studies of the hippocampus have identified its different roles in recent memory and remote memory, as shown in Figure 5.3 (Frankland and Bontempi, 2005). The lesion of hippocampus in recent memory resulted in great memory loss with the severity increased with the degree of lesion, while no such results were observed in remote memory.

The hippocampus is crucial for the encoding, formation and storage of recent memory. With an interleaving period between the hippocampus and the neocortex, the memory gradually leaves hippocampus and forms in the neocortex for later access. Lesion studies on animals have revealed that damage including EC leads to more severe impairment on memory than selective lesions of the hippocampus (Zola-Morgan et al., 1992, Jarrard, 1993).

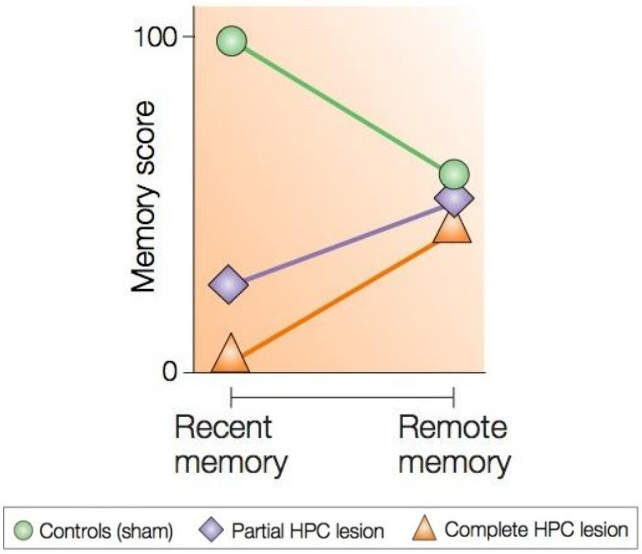


Figure 5.3 Lesion study on the hippocampus in memory

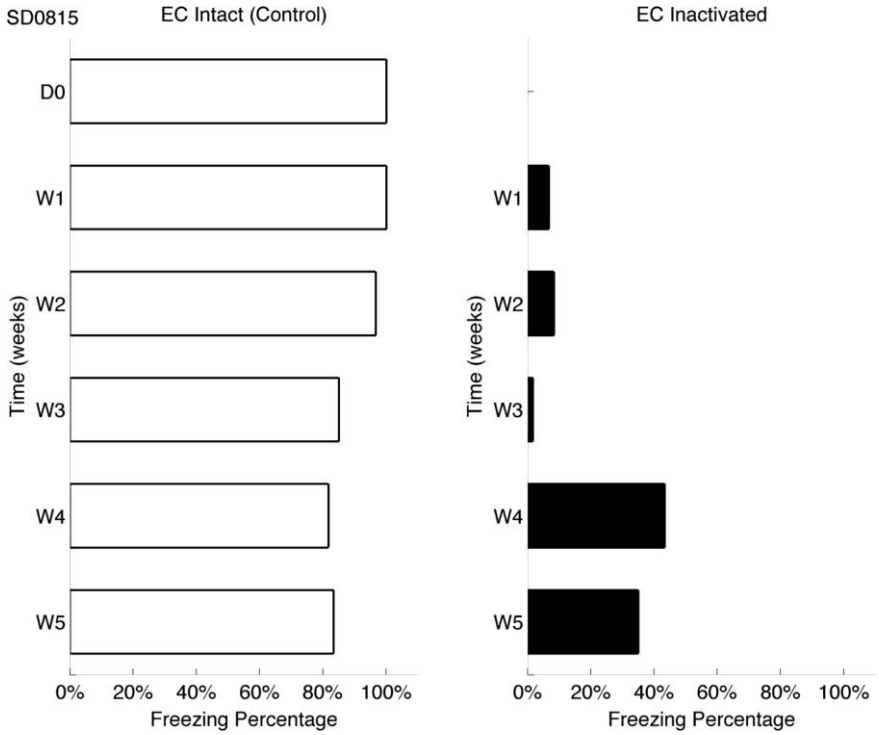
Adapted from Frankland and Bontempi (2005)

The result in this study on EC is consistent with the previous studies on human that reveal EC is embedded in the hippocampus circuit and the entorhinal functions in the process of information transformation.

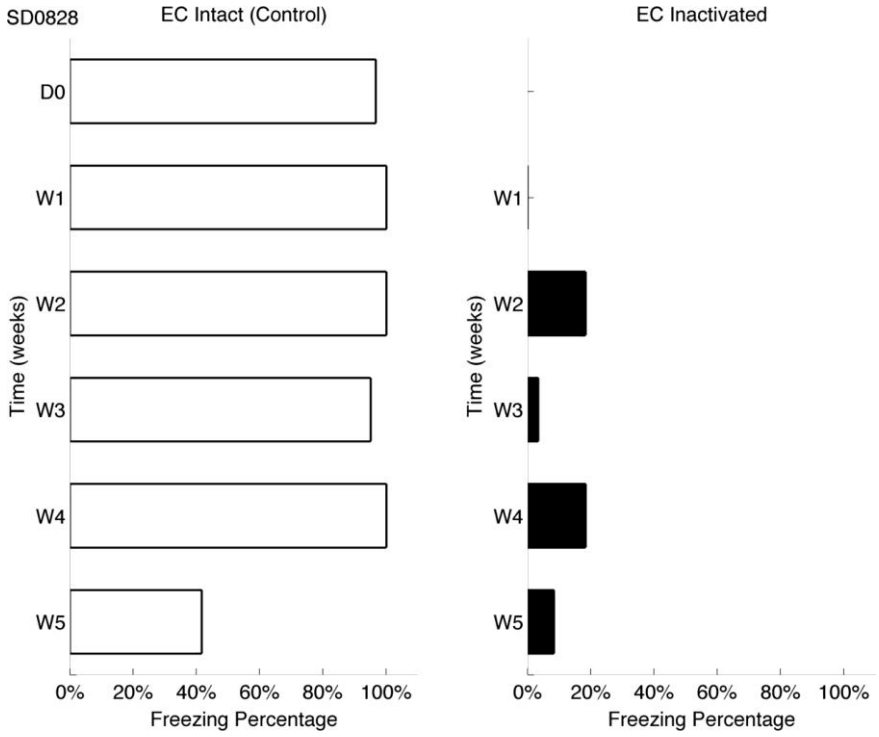
Appendix I Bar plot of freezing percentage

There are five subjects in the experiment on the entorhinal cortex (EC) and associative memory (see Chapter 3). They were named after the date of the surgery with prefix SD (Sprague Dawley Rat): SD0815, SD0828, SD 0829, SD0911 and SD0912. Figures below present a clear view of the comparison between the EC intact condition and the EC inactivated condition for each subject.

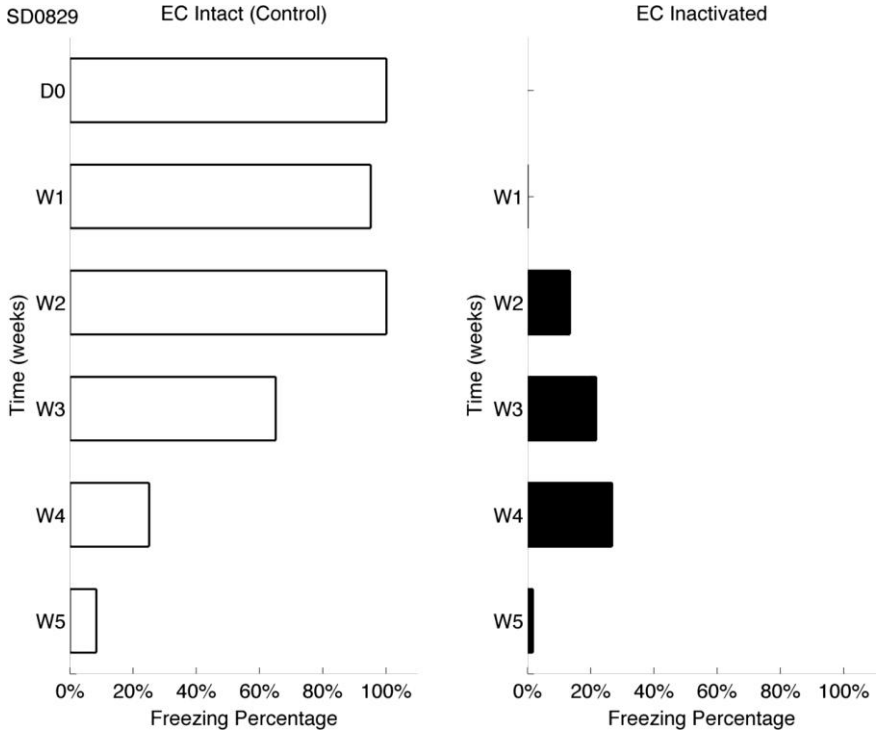
SD0815



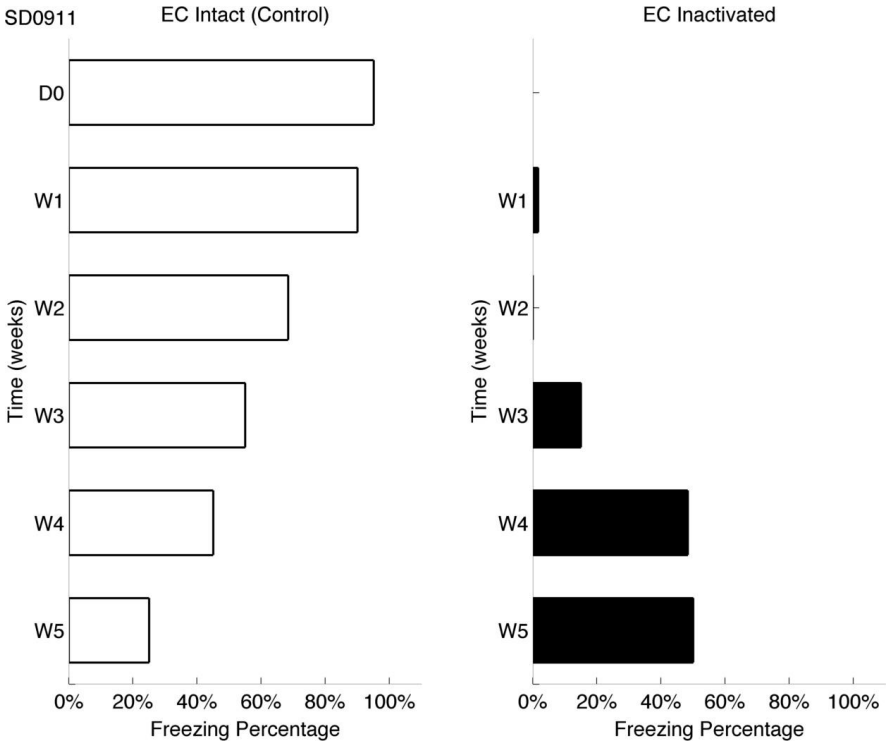
SD0828



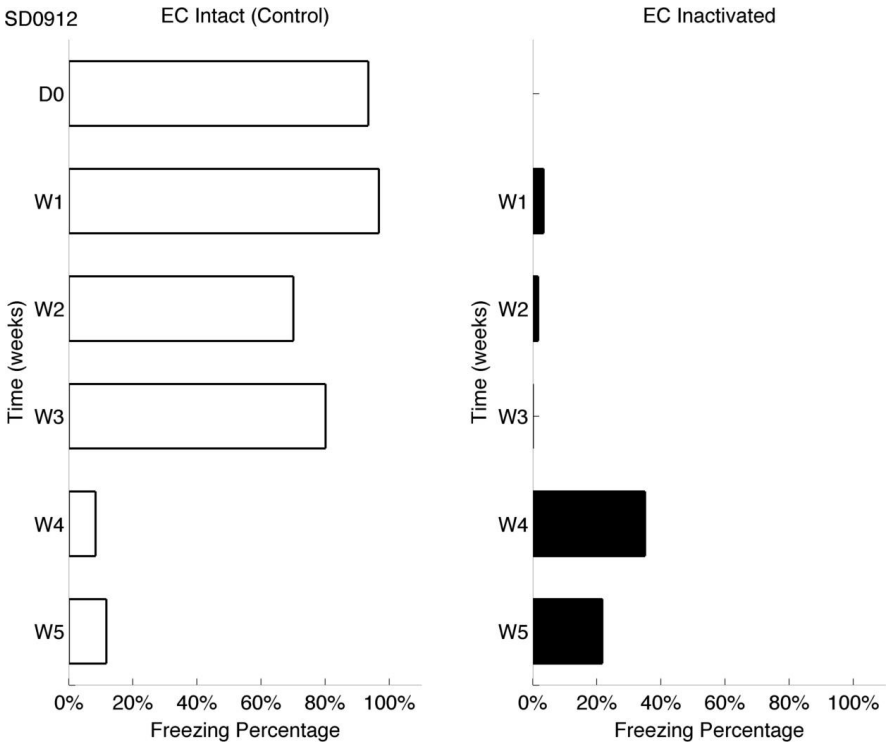
SD0829



SD0911



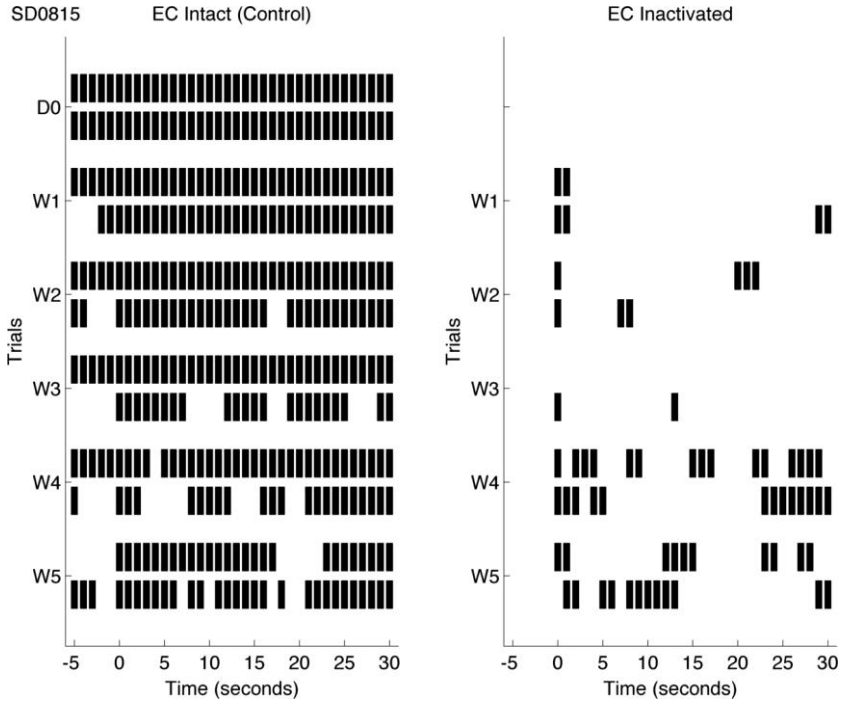
SD0912



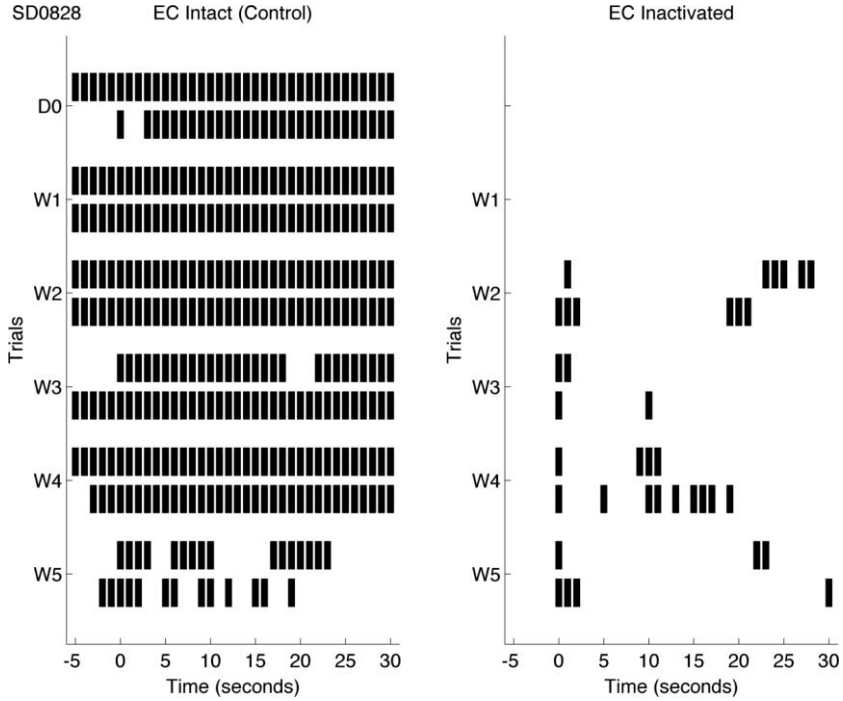
Appendix II Raster plot of freezing

To illustrate the freezing responses of the subjects, the behavioral responses of 5s before the presentation of the light stimulus and 30s after are depicted in the following raster plots. Each black bar indicates 1s of freezing and each blank bar indicates motion of the subject. Each subject was exposed to two trials under each condition for 5 weeks.

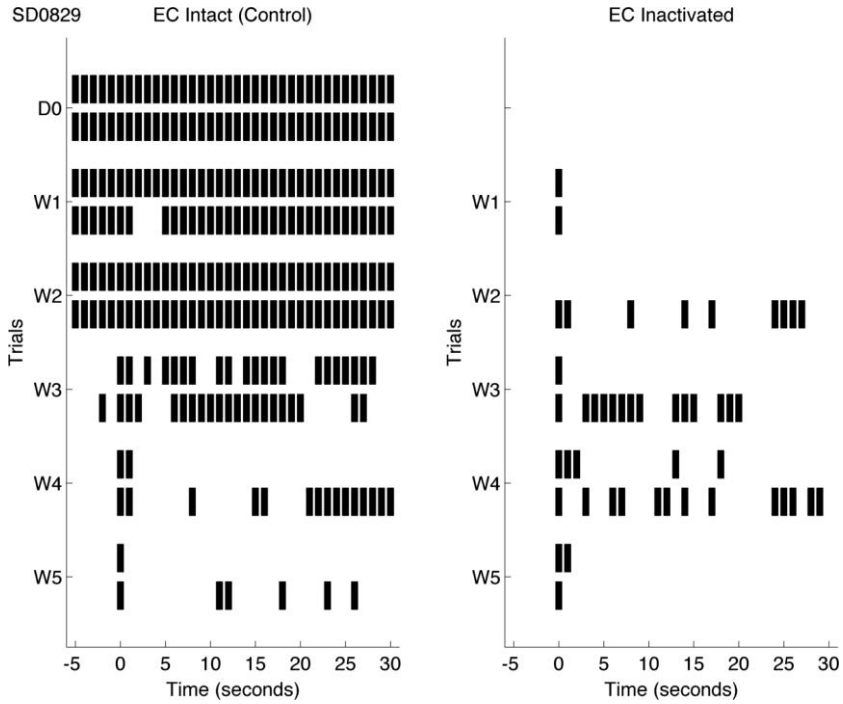
SD0815



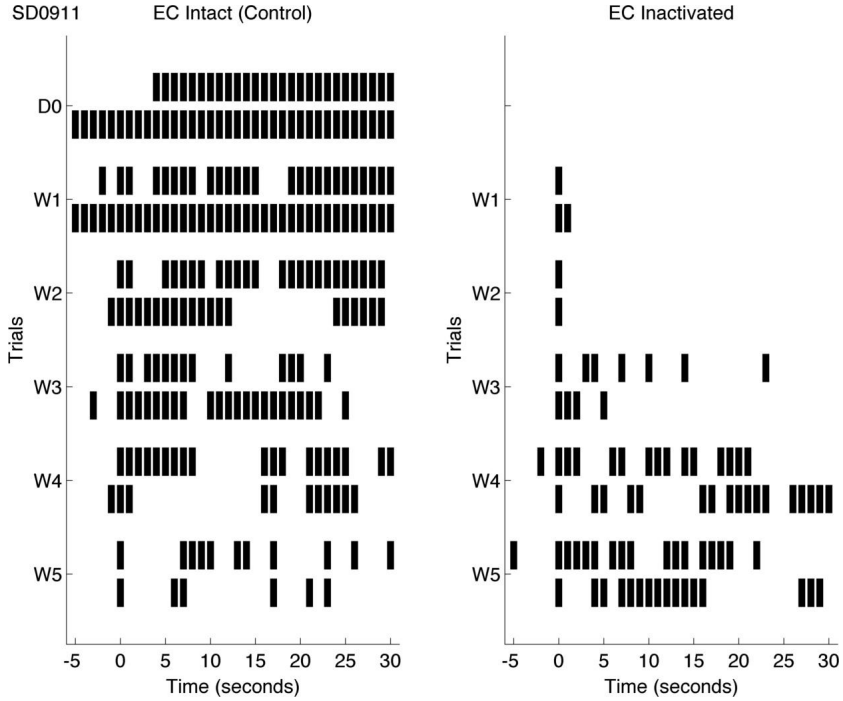
SD0828



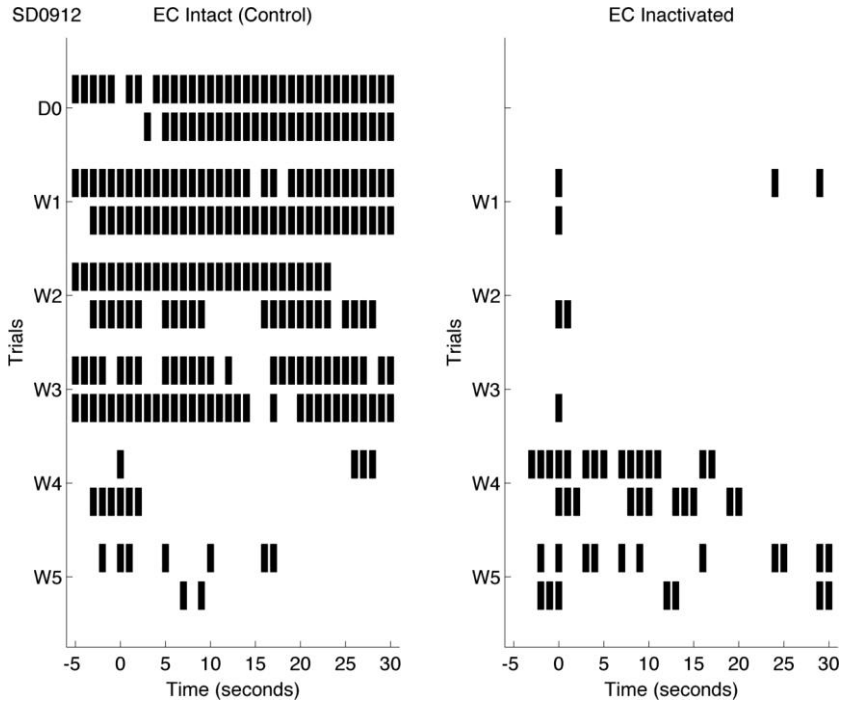
SD0829



SD0911



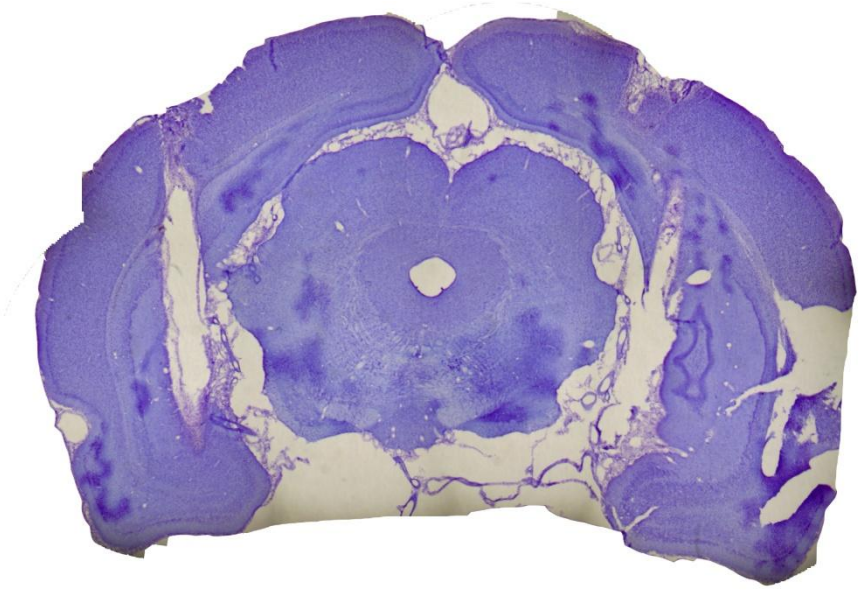
SD0912



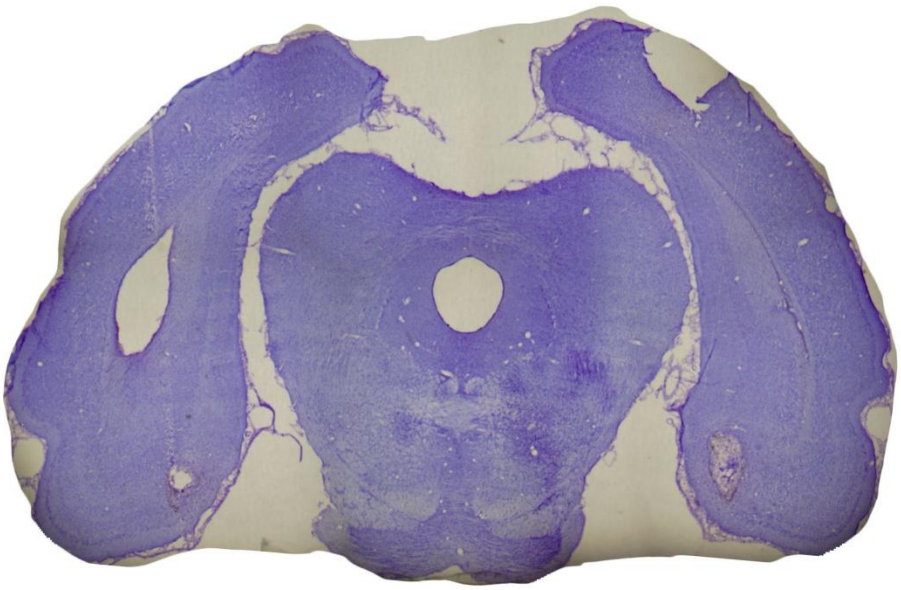
Appendix III Histological Results

Data on two rats (SD0815 and SD0828) was not available due to technical problem. The photomicrographs of 3 rats are shown with the sequence of SD0829, SD0911 and SD0912.

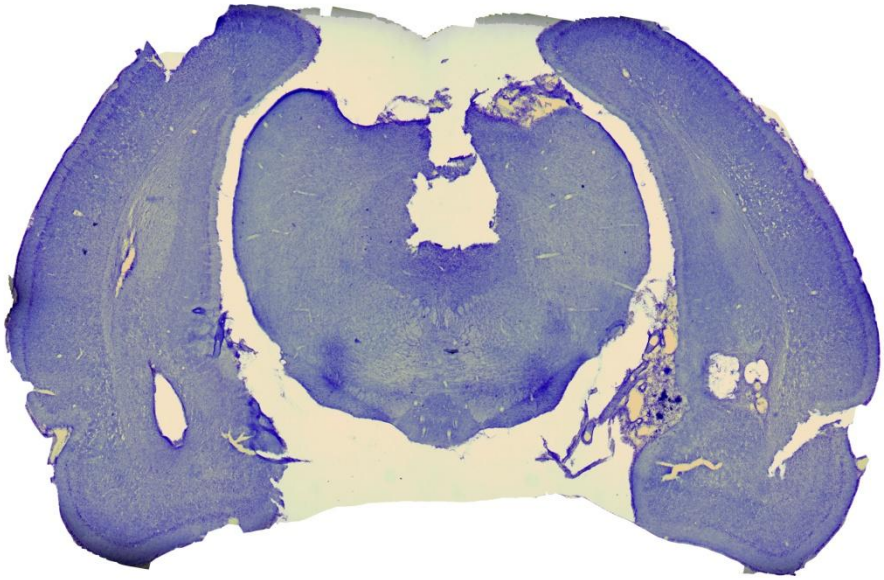
SD0829



SD0911



SD0912



Appendix IV Video records

The video records for Chapter 3 and Chapter 4 are stored on the attached DVD, in folders:

(1) `\Videos-for-Chapter-3` and,

(2) `\Videos-for-Chapter-4`, respectively.

Videos for Chapter 3 are all produced from subject SD0911 and named based on the test time: `Week 1, Week 2, Week 3, Week 4`, and `Week 5`. Each video file contains two sections. The first section presents the freezing response of the EC intact condition. The second section shows the freezing response of the EC inactivated condition. The length of each section is 35 seconds. The lights are given on the fifth second.

There are three videos for Chapter 4 and they are from the experiment on visuoauditory association. `Video 1` presents the subject-initiated sound-reward protocol. `Video 2` shows the behavioral response of two rats from the experiment group in the first trials of the tests. `Video 3` demonstrates the behavioral response of two other rats in the control group.

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