Ph.D. Dissertation of Natural Science

Dissociable roles of the medial and lateral divisions of the entorhinal cortex for scene memory-dependent behavior

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Abstract

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The medial temporal lobe, which includes the hippocampal system, has been known to play an important role in forming episodic memory. A number of studies have described visual scene as one of the most important sensory stimuli for the hippocampal system in humans as well in rodents and nonhuman primates. How scene-dependent memory, however, is processed by upstream structures of the hippocampus is largely unknown. In the present thesis, differential roles of the lateral and medial subdivisions of the entorhinal cortex (LEC and MEC, respectively) were examined in scene-dependent memory tasks. When the rat made spatial choices, namely turning left or right in a T-maze using scene stimuli, inactivation of the MEC, but not the LEC, by muscimol (MUS) severely impaired behavioral performance. However, when the task required the rat to make non-spatial choices between digging in the sand in the jar and pushing that same jar, based on scene stimuli, the LEC became more important than the MEC. This double dissociation demonstrates that (i) the subdivisions of the entorhinal cortex are involved in making distinct behavioral responses using scenes and (ii) motor responses seem to be important in determining the involvement of the LEC and MEC in scene-dependent memory tasks. While previous studies support a simple “what” versus “where” dichotomy for the roles of the LEC and MEC, respectively, the present thesis suggests the role of the LEC for “What should I do to this object in this context?” and the MEC for “Where should I go from here in this context?”.

Keyword: lateral entorhinal cortex, medial entorhinal cortex, medial temporal lobe, scene memory, scene information, spatial response, non-spatial response

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Introduction

1. Hippocampal episodic memory based on visuospatial information

The medial temporal lobe, including the hippocampal formation, has been considered critical in mediating episodic memory. In the case of human patient Henry Gustav Molaison, the surgical removal of the temporal lobe including the hippocampus induced partial retrograde and severe anterograde amnesia, while procedure memory, such as habits and motor skills, was spared (Scoville and Milner, 1957). Several experiments suggest that among other sensory input, visual scene-dependent stimuli especially are highly effective in engaging the hippocampal system in human (Epstein and Kanwisher, 1998; Hartley et al., 2007; Hassabis et al., 2007; Staresina et al., 2011; Zeidman et al., 2015) as well as nonhuman primates (Gaffan and Harrison, 1989; Wirth et al., 2003). Considering its role as a tool for storing various pieces of information, visual scenes can also be useful in organizing the information gleaned from these scenes. For most people, scene-dependent information appeals to process imagination, remembering the past, and planning for the future, for example, recalling where I parked my car, thinking about what I did on my birthday last year, et cetera. Through various cognitive tasks that required an episodic memory, such as recognizing visual environment or imagining future events, it has been found that the hippocampus is critical to the formation of scene-dependent memories [see (Maguire and Mullally, 2013) for review]. Prior studies also support the idea that even if damage was limited to the hippocampal formation, particularly to the bilateral Cornu Ammonis 1 (CA1), this damage is sufficient to cause amnesia (Zola-Morgan et al., 1986; Rempel-Clower et al., 1996). In experiments involving animals, it has also been demonstrated that the hippocampal neurons in rats exhibited significant modulation in firing rate when the animal was placed in a specific location containing external visual cues (O'Keefe and Conway, 1978; Muller and Kubie, 1987; Shapiro et al.,

1997; Knierim, 2002). In addition, those neurons formed memories of specific locations even in cue-absent environments (Muller and Kubie, 1987; Quirk et al., 1990), suggesting that two navigational systems, the internal one based on self-motion cues, and the external one, governed by environmental landmarks, may affect the spatial representation produced by the hippocampus in rodents, and these representations may constitute the basis of cognitive functions, including episodic memory. Evidence from behavioral experiments using animal subjects found that the hippocampus is involved in forming a mnemonic memory based on the visual contextual environment (Kim and Fanselow, 1992; Maren et al., 1997; Anagnostaras et al., 1999). More recent studies have reported similar results to the aforementioned paradigms in experiments when the entorhinal cortex (EC), which is one of the upstream structures of the hippocampus, was damaged (Esclassan et al., 2009). Some recent studies further argue that the hippocampus is critical to processing memory formed through scene-dependent stimuli. The studies confirmed this by inactivating the dorsal hippocampus via a GABA-A receptor agonist, muscimol (MUS), which severely impaired performance in scene-dependent behavior choice tasks (Kim et al., 2012; Delcasso et al., 2014; Lee et al., 2014). Furthermore, physiological evidence supports the result of these recent studies, in which the activity of the hippocampal neurons was highly dependent upon which scene was shown to subjects (Delcasso et al., 2014).

2. Anatomical properties and functional differences along the subdivisions of the entorhinal cortex

The EC in rats is located at the most caudal, ventral, and lateral part of the brain, and is regarded as a core structure connecting the neocortex with the hippocampal formation. According to its morphological description, the EC can be subdivided into the lateral and medial entorhinal cortex (LEC and MEC, respectively) (Krieg, 1946a, b; Blackstad, 1956; Insausti et al., 1997), and both of which can be further classified cytoarchitectonically into six sub-layers (Insausti et al., 1997). As one of the major upstream structures of the hippocampus, the EC sends strong input to the hippocampus (Lavenex and Amaral, 2000; Witter et al., 2000a; Knierim et al., 2006; Kerr et al., 2007) and as such, this upstream structure can help explain the functional roles of the hippocampus. As an example, the perforant pathway is known to be a major input from the EC to the hippocampus in which the layer II of the EC conveys cortical input to dentate gyrus (DG) and Cornu Ammonis 3 (CA3) as well as projecting from the layer III of the EC to Cornu Ammonis 1 (CA1) (Witter et al., 2000b). Moreover, the parallel input streams from the LEC and MEC for conveying non-spatial and spatial information, respectively, to the hippocampus have been described in prior studies as well (**Figure 1**) (Hargreaves et al., 2005; Knierim et al., 2006; Henriksen et al., 2010; Deshmukh and Knierim, 2011; Ito and Schuman, 2012; Tsao et al., 2013). Anatomical evidence for the dissociated connectivity of the LEC and MEC (Burwell and Amaral, 1998b, a; Burwell, 2000; Kerr et al., 2007) has been described in research stating that the LEC receives strong input from the perirhinal cortex (PER), which is considered a critical area in the recognizing or identifying of an object (Norman and Eacott, 2005; Winters and Bussey, 2005; Olarte-Sanchez et al., 2015), while the MEC receives a majority of input from the postrhinal cortex (POR), the rodent homolog of the parahippocampal cortex in humans and primates, which is known for its role in processing visuospatial information (Burwell and Amaral, 1998a; Furtak et al., 2007). Both the LEC and MEC not only convey inputs to the hippocampus, but these regions also reciprocally receive back-projections from the hippocampus to those deep layers (Tamamaki and

Nojyo, 1995; Dolorfo and Amaral, 1998; Naber et al., 2001; Kerr et al., 2007; Agster and Burwell, 2013). Taken together, it has been hypothesized that the two kinds of information, LEC-derived non-spatial memory and MEC-dependent spatial memory, are combined in an integrative memory representation at the level of the hippocampus.

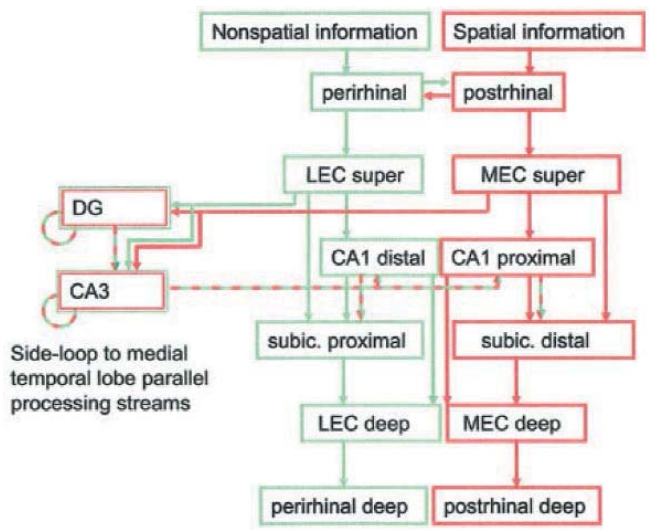


Figure 1. Parallel information streams of the medial temporal lobe (Knierim et al., 2006). Non-spatial and spatial information stream are independently processed through anatomical pathway including the LEC and MEC, and are combined in the DG and CA3. These combined information project back to the parallel processing streams. The superficial layer of the LEC receives non-spatial information from the PER, whereas those of the MEC receives spatial information from the POR. Cornu Ammonis 3, CA3; Dentate gyrus, DG; Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC; Perirhinal cortex, PER; Postrhinal cortex, POR.

3. The role of the medial entorhinal cortex in processing visuospatial information

The distinct roles of the LEC and MEC have been reported in physiological (Hargreaves et al., 2005; Henriksen et al., 2010; Deshmukh and Knierim, 2011; Yoganarasimha et al., 2011; Ito and Schuman, 2012; Tsao et al., 2013) and behavioral experiments (Hunsaker et al., 2013; Van Cauter et al., 2013). The neurons in the MEC were more spatially modulated than those in the LEC (**Figure 2**) (Fyhn et al., 2004; Knierim et al., 2006; Deshmukh and Knierim, 2011; Yoganarasimha et al., 2011).

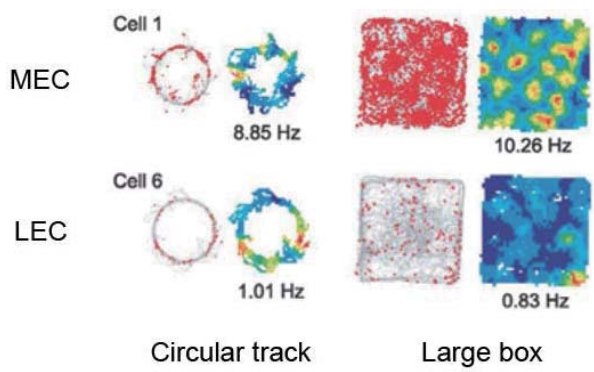


Figure 2. Properties of the activity of the MEC and LEC neurons on the circular track and in the large box (Yoganarasimha et al., 2011). Upper row represents the properties of the MEC neurons whereas lower row represents the properties of the LEC cells. The trajectory and the position of the rat (gray line and red dots, respectively) are shown on the left of each pair of diagrams, and on the right is shown the corresponding rate map. Red color indicates peak firing rate while dark blue color indicates no firing in the rate map. The MEC neurons show symmetric and spatially tuned firing patterns in both circular track and large box sessions, whereas the LEC neurons show dispersed and nonsymmetric firing patterns compared to the MEC neurons.

For instance, grid cells found in the superficial layers of dorsocaudal MEC may strongly support the concept of the MEC as playing a role in processing spatial information (Hafting et al., 2005) while cells in the LEC exhibited activity in a non-spatial manner (Hargreaves et al., 2005; Deshmukh and Knierim, 2011). Given the anatomical evidence that the POR receives and strongly conveys visuospatial sensory information to the CA1 and the MEC (Burwell and Amaral, 1998a; Furtak et al., 2007), it is highly implied that visual scene information is processed by the spatial information processing-stream involving the POR and MEC (Knierim et al., 2014) even if it has been described that both the PER and POR contribute to process fear memory based on the visually cued context (Bucci et al., 2000; Bucci et al., 2002; Burwell et al., 2004a; Burwell et al., 2004b). Physiological experiments showed that the neurons in the POR not only signaled object-location conjunctions in visual discrimination task, but also encoded egocentric motor responses (Furtak et al., 2012). In visual context-object association tasks, rats with lesions in the POR were significantly impaired in their performance, compared to the control and PER-lesion groups (Norman and Eacott, 2005). In this regard, several prior studies provided evidence for the functional role of the MEC as processing spatial information. Although bilateral lesions to the MEC induced performance deficits in a water maze task as significant as the deficits seen when lesions were made to the hippocampus (**Figure 3**), some types of hippocampal-dependent tasks were spared (Hales et al., 2014), suggesting the MEC is selectively associated with specific types of hippocampal-dependent memory, and is critical for egocentric spatial memory.

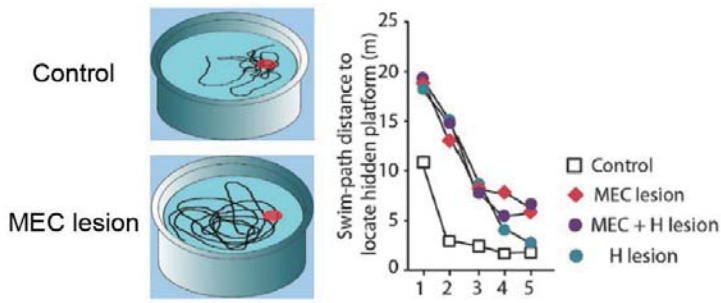


Figure 3. Impairment of place memory by lesion of the MEC (Hales et al., 2014). When the rat was tested in the water maze that required a rat to locate on the hidden platform, the MEC lesion group showed longer distance compared to control group. Note that behavioral performance of the MEC lesion group was impaired as much as hippocampus lesion group and the MEC+hippocampus lesion group as well.

Electrophysiological studies also reported that spatial coherence and the accuracy of spatial representation in the neurons along CA1's proximal and distal axes received different amounts of input from the MEC (Henriksen et al., 2010). When layer III of the MEC was damaged by a local infusion of neurotoxin, larger and more dispersed firing fields in CA1 were found, whereas CA3 cells still had stable firing fields, suggesting the significance of input from the MEC to the hippocampus in mediating spatial representations, as well as emphasizing layer-specific roles in processing spatial information between the MEC and hippocampus (Brun et al., 2008). In addition, grid, head direction, and conjunctive cells in layer III of the MEC are considered to coordinate egocentric spatial information, i.e. location, direction, and running speed, and as such, those cells might be the fundamental basis of a navigational system, especially in rodents (Sargolini et al., 2006). In contrast to the LEC's neurons, those of the MEC exhibited a robust spatial signal in cue-rich environments, demonstrating that the MEC uses a landmark-based spatial mapping system (Yoganarasimha et al., 2011).

4. The role of the lateral entorhinal cortex in identifying individual objects or characteristics of objects

Prior studies reported that in contrast to the MEC, the LEC is specialized for processing non-spatial information (Hargreaves et al., 2005; Deshmukh and Knierim, 2011; Deshmukh et al., 2012). Considering that non-spatial information is often distinguished based on individual sensory modalities, such as olfactory, tactile, taste, and auditory information, the unimodal and polymodal sensory areas might be the basis for forming non-spatial information. In addition, given anatomical evidence that the LEC receives direct input from these unimodal and polymodal sensory areas, i.e. insular, piriform, and perirhinal cortex, et cetera (Kerr et al., 2007), and indirectly through the PER, the functional role of the LEC is believed to be specialized for processing non-spatial information. In contrast, the POR and MEC are known to help process spatial information, which may play a significant role in forming scene-dependent memory. With regard to anatomical connections between the LEC and sensory modality areas, some studies provide evidence supporting the idea that the LEC contributes to the formation of episodic memory based on sensory information, i.e. the roles of the LEC in association with olfactory-tactile information (Boisselier et al., 2014) or with auditory-temporally discontiguous stimuli (Morrissey et al., 2012). Moreover, prior experiments have reported that the LEC plays a critical role in identifying individual items and the role of the LEC, especially, became more critical when the LEC-lesioned rats were required to discriminate the displaced object in association with the contextual environment (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a), suggesting that the LEC plays an intrinsic role in identifying characteristics of objects, as well as in associating the object with environmental cues (**Figure 4**).

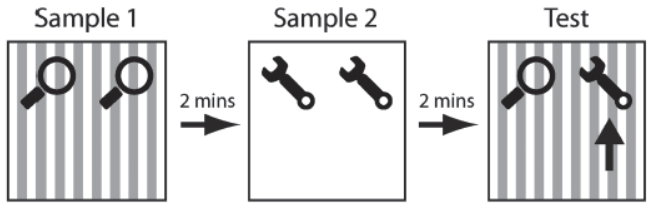


Figure 4. The role of the LEC in associating object and contextual information (Wilson et al., 2013a). In the object-context recognition test, rat was tested for object-context association. Two same objects and a specific context were paired for the first sampling session, and another pair of objects and context were paired for second sampling session. After two sampling sessions, rat was required to exploring the unfamiliar object that was not presented in the context of test session. The LEC lesion group showed a significant decrease in performance compared to control group.

In addition to the above background, no deficit in behavioral performance was found in LEC-lesioned rats when an object was provided as a cue by itself, rather than associating an object with other external cues (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). Physiological studies also showed that the neuronal activities of the LEC were modulated in a non-spatial manner (**Figure 5**) (Hargreaves et al., 2005; Deshmukh and Knierim, 2011; Deshmukh et al., 2012; Keene et al., 2016), while the neurons of the MEC demonstrated spatially tuned activities (Yoganarasimha et al., 2011).

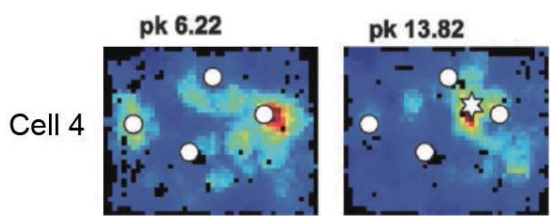


Figure 5. Object-related activity in the LEC neurons (Deshmukh et al., 2012). The LEC neuron of rat was recorded during freely exploring in a platform (left square platform) where contains four objects (white circles in each square platform). When a new object was introduced in the same

environment (right square platform), same neuron showed firing remapping to a new object. Red color indicates peak firing rate while dark blue color indicates no firing in the rate map. The numbers at the top of each rate map show peak firing for the recorded neuron.

5. Limitations of the previous behavioral paradigms in examining the functional roles of the lateral and medial entorhinal cortex

Although the existing theories have studied the roles of the LEC (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a) and the MEC (Steffenach et al., 2005; Hunsaker et al., 2013; Van Cauter et al., 2013; Hales et al., 2014) by testing animals in various memory tasks, it is unclear exactly which variables induced behavioral errors. For example, if a subject with a lesion to the MEC committed an error in a spatial navigation task, it could be either an error in its processing of visuospatial sensory information, an error in its motor responses, or a combination of these factors that affected behavioral performance (Steffenach et al., 2005). In object recognition tasks using subjects with lesions to the LEC, the animal's behaviors such as sniffing or rearing at an object were commonly recorded as a responsive behavior (Hunsaker et al., 2013). These unsystematically controlled stimuli and motor responses become problematic as individual experimenters could interpret subjects' behaviors in a subjective way, therefore confusing the observed outcome of a study. To address these issues, it is necessary to test subjects in various memory tasks that provide explicitly measurable sensory stimuli (i.e. projecting a visual pattern in a dark space with no external cues) as well as requiring clearly measurable motor responses (i.e. turning left or right with no other behaviors allowed). In addition, considering that the way spatial information is processed depends upon the specific contextual environment, it is reasonable to claim that sensory information and motor responses are integrated to form a specific behavioral outcome. So, in the execution of behavioral experiments, it is necessary to clearly separate the sensory information provided to and the motor responses asked of the animal subject in order to discriminate which components are critical in determining behavioral outcomes. The dominant theory, however, simply divided the spatial and non-spatial information processing streams based on the characteristics of the sensory information provided to subjects (Burwell and Amaral, 1998a; Witter et al., 2000a; Eichenbaum et al., 2012) and this classification seems to oversimplify the way the brain functions. Although the results

of some previous experiments might indirectly support the understood functional roles of the LEC and MEC in relation to spatial or contextual memory tasks (Hunsaker et al., 2013; Van Cauter et al., 2013), scene-based stimuli are rarely systematically controlled in these experiments. This issue could be resolved by testing animal subjects in various memory tasks which share the same sensory stimuli, but require distinct motor responses (i.e. requiring a spatial or non-spatial response based on the same scene-based stimuli). Another shortcoming of prior behavioral studies is a result of permanent damage caused by using lesion techniques, such as injecting an excitotoxic drug (Brun et al., 2008; Hunsaker et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a; Boisselier et al., 2014), or passing a radio-frequency current (Kesner and Giles, 1998; Parron et al., 2004; Van Cauter et al., 2013) into the LEC or MEC. The results of these studies can become confused, as permanent damage to specific brain regions may result in plastic changes to these functionally related neural circuits (Schenk and Morris, 1985). Alternatively, temporary inactivation of a specific brain region through the use of MUS or lidocaine, for example, can preserve normal circuits during testing. Temporary inactivation also allows the normal function of a targeted circuit to be compared to its inactivated mode within the same animal subject, whereas the lesion paradigm only allows for between-group comparisons. Surprisingly, no previous models have examined the distinct roles of the LEC and MEC by using the reversible inactivation paradigm, though the functional role of the LEC was examined using this method in some eye-blink conditioning experiments (Morrissey et al., 2012; Tanninen et al., 2013).

Hypothesis

In this thesis, I present various memory tasks in examining whether the LEC and MEC were differentially involved in processing spatial versus non-spatial responses based on the scene-based stimuli. At first, I tested rats in scene-based spatial choice (SSC) task, in which the rats were required to make a spatial choice (turning either right or left) based on scene-based stimuli that comprised of four different pictures of patterns (types of patterns), which were displayed on LCD screens during the trials. Given that findings of prior studies emphasized the role of the MEC as processing spatial information and creating visual, context-based memory, I hypothesized that the MEC, but not the LEC, would play an important role in the SSC task, which required associating scene-based stimuli with spatial responses. Secondly, I tested rats in scene-based non-spatial choice (SNSC) task. In contrast to the SSC task, the non-spatial choice between digging in the sand in the jar and pushing that same jar, based on scene stimuli, was required to complete the SNSC task. Given that findings of previous studies emphasized the role of the LEC in associating object with contextual information, I hypothesized that the LEC would play a critical role in the SNSC task, which required associating scene-based stimuli with non-spatial responses.

In the next part of the study, I examined the reliability of the main tasks by conducting different versions of the tasks. In the masked scene-based spatial choice (MSC) and the masked scene-based non-spatial choice (MNSC) tasks, I tested whether the pattern projections provided were a proper stimulus for triggering the rats' behavioral responses by concealing some parts of scene pattern. If rats chose behavioral responses based on the overall scene stimulus rather than a specific spot within the pattern, no performance deficit would be observed in the MSC and MNSC tasks.

In experiments which I describe in following sessions, the sensory and motor components involved in the SSC and SNSC tasks were separated. In the tactile cue-based

spatial choice (TSC) task, rats were required to make a spatial choice, whereas non-spatial choices were associated with object cues in the object-based non-spatial choice (ONSC) task. Given findings of the previous behavioral study that the behavioral performance was unaffected when an object by itself was cued instead of associating an object with other external cues in the LEC-lesioned rat (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a) and MEC-lesioned rats (Hales et al., 2014), I hypothesized that neither the LEC nor the MEC would be involved in associating object cue with behavioral responses. In addition, if only scene-dependent stimuli were critical factors in triggering specific behaviors in the rats, other types of sensory stimuli such as tactile cue would not affect behavioral performance.

EXPERIMENT 1. Scene-based spatial choice task

Introduction

Previous studies showed that the rodents' hippocampus is critical to processing scene-based memory (Kim et al., 2012; Delcasso et al., 2014; Lee et al., 2014). These studies confirmed this by inactivating the dorsal hippocampus of rat via MUS, which induced a significant performance deficit in scene-dependent behavioral choice tasks. Physiological evidence, in addition, supports these results in which the hippocampal neurons showed increased activity when scene-based stimuli was presented to subjects (Delcasso et al., 2014). How scene-based memory, however, is processed by upstream structures of the hippocampus is rarely identified. As one of the major upstream structures of the hippocampus, the EC sends strong anatomical inputs to the hippocampus (Kerr et al., 2007) and the functional roles of its subdivisions, namely the LEC and MEC, have been investigated in which the LEC and MEC are specialized to process object-related information and visuospatial information, respectively (Deshmukh and Knierim, 2011; Yoganarasimha et al., 2011; Deshmukh et al., 2012; Hunsaker et al., 2013; Van Cauter et al., 2013; Hales et al., 2014). Although previous studies might support the functional role of these regions indirectly by behavioral studies testing animals with spatial and contextual memory tasks, visual scene has hardly been systematically controlled (Morrissey et al., 2012; Hunsaker et al., 2013; Van Cauter et al., 2013). Furthermore, behavioral responses were not clearly measured in a subjective way in examining the roles of the LEC and MEC (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). In the scene-based spatial choice task, I strictly controlled both sensory stimuli and behavioral responses to clearly examine whether the LEC and MEC would be differently involved to make spatial responses, turning left or right, based on scene-based stimuli.

Methods

Subjects

Eight male Long Evans rats weighing 340–400 g (9-11 weeks after birth) were used. All animals were housed individually and underwent a two-week acclimation period in Plexiglas cages in a temperature- (26℃) and humidity-controlled environment (40-60%). Rats were kept on a 12-h light-dark cycle. For behavioral experiments, all animals were food-restricted to 80% of their free-feeding weights with free access to water. All protocols were in compliance with the Institutional Animal Care and Use Committee of the Seoul National University.

Behavioral apparatus

A T-shaped linear track (86 x 40 cm) elevated to 88 cm above the floor was used in a scene-based spatial choice (SSC) task (**Figure 6**). A guillotine door-operated start box (22.5 cm x 16 cm x 31.5 cm) was attached to the end of the stem of the T-track. A food well (2.5 cm in diameter and 0.8 cm in depth) was placed at the end of two side arms of the T-track. Three 17-inch LCD monitors were installed as an array to surround the upper portion of the T-track to provide background visual scenes. Four optic fiber sensors (Autonics, Korea) were installed on both side walls of the linear track at different points (1 cm, 27 cm, 47 cm and 67 cm from the start box) to record the animal's position on the track. The activation of the first optic fiber sensor triggered the onset of scene stimuli on the LCD monitors. The apparatus was surrounded by black curtains, and no external visual cues were provided on the curtains. The experiment room was dimly lit by an overhead array of LED lights (4 lx) and two loud speakers played white noise (80 dB) during behavioral sessions to mask environmental noise. A digital camera installed on the ceiling recorded the behavioral testing session and transferred the data to a PC outside the testing room.

Figure 6. Behavioral apparatus used in the SSC task. When the start box door (omitted for visualization) was opened, a trial was begun. The scene stimulus was displayed on the three adjacent LCD monitors right after opening the start box door by triggering an optic sensor. Note the zebra stripes on the LCD monitors provided as a scene stimulus. A rat was required to choose either the left or right arm based on the specific scene stimulus to get a food reward in the food well (the white circles at the end of each arm). Scene-based spatial choice task, SSC.

Handling and Familiarization

Naïve rats were handled by an experimenter for 30 minutes a day for 3 days. Following the handling phase, rats were placed on a lab cart (99 cm x 45 cm x 84 cm) to forage for multiple pieces of cereal scattered on the surface of the cart. After acclimation to the environment during the foraging session on the cart for 3 days, rats were familiarized to the experimental room and the behavioral apparatus. During this period, rats were allowed to freely explore the apparatus while consuming cereal pieces scattered over the track and in the food wells. Once rats consumed over 80 pieces of cereal within 30 minutes for two consecutive days, a shaping phase began.

Shaping

A rat was first placed in the start box. When the start box door was opened by an experimenter, the rat exited the start box. Once the rat reached the choice point of the T-track, the rat was trained to enter the arm in which a silver metal washer (4 cm in diameter, 0.5 cm in height) had been placed over the food well. Once the rat displaced the washer, a quarter of a piece of cereal was found. The rat was allowed to eat it and was then gently guided back to the start box by the experimenter. The food well in the opposite, unrewarded arm was covered with a black metal washer. Rats were trained until the following criteria were satisfied. First, the median choice latency (from the opening of the start-box door to the displacement of the washer) was less than 5 s. Second, rats could correctly remove the silver washer covering the baited food well in more than 20 out of 40 trials. A daily session was forcibly finished when the rat conducted 40 trials or when 30 m had passed, whichever came first.

Pre-surgical training

A trial began when the rat exited the start box and triggered a sensor placed 1 cm from the door. The activity of the sensor led to a visual scene display (e.g. pebbles or zebra stripes) on the LCD monitors (**Figure 7**). Rats were trained to choose the right arm for the pebbles scene and the left arm for the zebra stripes scene. A correct choice resulted in a piece of cereal available in the food well when a black metal washer covering the food well was displaced by the rat. The rat was trained to consume the reward in the start box after being guided back into the start box. A wrong choice resulted in the rat being returned to the start box without a reward. Each scene appeared, in a pseudo-randomly intermixed fashion for 20 trials in a daily session and the identical scene was not presented over three consecutive trials. The rat was trained until its performance level reached greater than 80% correct responses per scene or above 75% correct responses on average for all scenes, with a response bias of less than 0.15. The response bias was calculated by first subtracting the number of right response-associated trials from left response-associated trials and then dividing the difference by the sum of the trials. When the rat reached the performance

criterion for the first pair of scene stimuli for two consecutive days, it was trained with the second pair of scene stimuli, e.g. mountain and bamboo patterns, until it reached the same criterion level with the first pair of scene stimuli. Then, when the rat reached the performance criteria for both the first and the second scene pairs, all four scenes were pseudo-randomly presented across trials to train the rat until it reached greater than 80% correct responses for each scene.

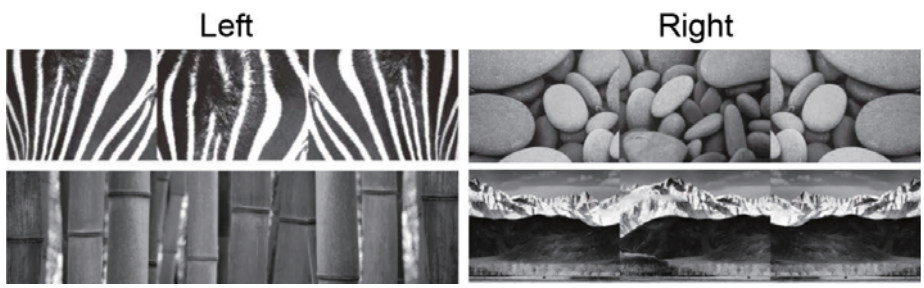


Figure 7. The background visual scenes used in the SSC tasks. Zebra stripes and pebbles patterns were provided for the first pair of scenes in the SSC task. When a rat reached to performance criterion for the first pair of scenes, next pair, bamboo and mountain, were provided. Each scene was associated with either left or right choice behavior. Scene-based spatial choice task, SSC.

Surgery

Once the rats reached the performance criteria for four scene stimuli for two consecutive days, a 3D-printed cannula complex (20 mm x 9 mm x 26 mm) was surgically implanted. The 3D-printed cannula complex was composed of four stainless steel guide cannulae targeting both the MEC and LEC bilaterally. The dummy cannula was inserted into each guide cannula and the tip of it protruded 1mm from the tip of guide cannula to prevent the leakage of drug along with the guide cannula track. Once the 3D-printed cannula complex was prepared, it was accurately calibrated along the horizontal and longitudinal axis on

the aluminum calibrator before the surgical procedure began (**Figure 8A**). When the surgery was conducted, the rat was deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 65 mg/kg), and its head was fixed in a stereotaxic frame (Kopf Instruments, USA). Anesthesia was then maintained by isoflurane inhalation (0.5–2% isoflurane mixed with 100% O_2) throughout the surgery. An incision was made along the midline of the scalp and the skull was exposed. The skull surface was leveled after adjusting the levels of bregma and lambda on the same horizontal plane. Six small burr holes were made on the skull to place anchoring skull screws to hold the 3D-printed cannula complex with dental cement. Four burr holes were also drilled to insert the guide cannulae of the 3D cannula complex into the LEC and the MEC at the following coordinates: 5.9 mm posterior to bregma, 6.8 mm lateral to midline, 6.4 mm ventral from dura for the LEC, and 7.8 mm posterior to bregma, 4.8 mm lateral to midline, 5.9 mm ventral from dura for the MEC. Following insertion of the guide cannulae into the target areas, medical grade silicone (Kwik-Sil, World Precision Instruments, USA) was applied to fill the burr holes to block the influx of dental cement into the exposed area of the brain during the cementing procedures. Dental cement was then applied to the skull surface to firmly hold the 3D-printed cannula complex (**Figure 8B**). The rat was recovered for 7 days before behavioral testing began.

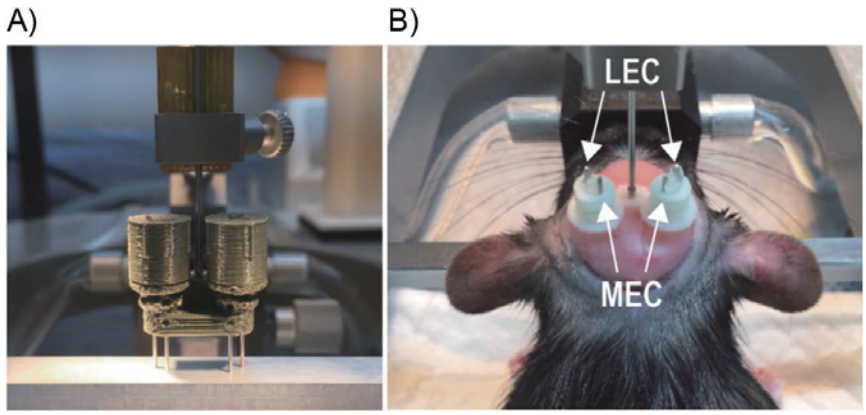


Figure 8. A 3D-printed cannula complex implantation surgery. A) When the rat reached to performance criterion in the pre-surgical training for two consecutive days, a 3D-printed cannula complex was implanted into the brain. Before inserting it into the brain, it was accurately calibrated along the horizontal and longitudinal axis on the aluminum calibrator to precisely target the expected brain regions. **B)** Four stainless steel guide cannulae bilaterally targeted the LEC and MEC (indicated by upper and lower white arrows). Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC.

Post-surgical testing and reversible inactivation of the EC

Rats were retrained to the pre-surgical criteria with the four scene stimuli for two consecutive days after recovery from surgery. Then, on the next day, artificial cerebrospinal fluid (aCSF) was simultaneously injected into the EC (i.e. both the LEC and the MEC, 0.3 μL per site) 20 min before behavioral testing. The vehicle solution was injected into both the LEC and MEC in the current study because the prior pilot studies showed no difference in performance between the two conditions. More importantly, prolonging the testing period with aCSF might carry the risk of overtraining the rats, which I wanted to avoid to properly test the functions of the LEC and MEC while those areas were engaged in the normal performance of the task. Over the next two days, the same animal received an injection of a GABA-A receptor agonist, muscimol (MUS) into

the LEC and then into the MEC (0.3 μL per site), or vice versa (the injection sequence was counterbalanced among the rats).

Histology

When all experimental testing was completed, fluorophore-conjugated muscimol (f-MUS; Sigma, USA) was injected bilaterally into both the LEC and MEC (0.3 μL per site) to verify the diffusion range of the inactivating agent. Twenty minutes after the injection of f-MUS, rats were killed by CO_2 overdose and were perfused transcardially with phosphate-buffered saline, followed by a 4% v/v formaldehyde solution. The brain was extracted from the skull and was soaked in 4% v/v formaldehyde-30% sucrose solution at 4°C until it completely sank. The brain was then gelatin-coated and soaked again in a 4% v/v formaldehyde solution-30% sucrose solution at 4°C until it completely sank. Then, the brain was cut into 40-mm coronal section on a freezing microtome (HM 430, Thermo Fisher Scientific, USA). Every second section was collected for thionin staining to verify the position of the 3D complex cannula, and every third section was used to examine the diffusion of f-MUS with a fluorescent microscope (Eclipse 80i, Nikon, Japan).

Data analysis

Performance was quantified as the proportion of correct trials in each session. Two-way repeated measures ANOVA was used to examine the effects of the drugs. A t-test corrected for multiple comparisons was used to examine the presence of any significant differences according to injection sequences. An unpaired t-test was used to examine the presence of any significant differences according to locations of cannula tips along the deep versus superficial layer in the SNSC task group. The response latency was defined as the duration from the time that the animal exited the start box to the time when the animal began to exhibit behavioral responses to obtain food rewards. The proportions of correct trials over the overall trials were regarded as behavioral performance assessments for each session.

Results

Critical role of the MEC, but not the LEC, for the scene-based spatial choice behavior

To examine whether the MEC was required for making spatial responses based on visual scene stimuli, I tested the rats from the SSC task group. When the rats from the SSC task group (n = 8) reached the performance criterion for post-surgical training for two consecutive days after surgery, drug injection procedures were performed 20 m before testing as described in the methods section. In the vehicle group, in which aCSF was simultaneously injected into both the MEC and LEC (EC-aCSF), the rats performed at above 90% correctness (**Figure 9**). However, when the rats were tested with MUS injection into the MEC (MEC-MUS) on the next day, behavioral performance was significantly impaired. On the other hand, when MUS was injected into the LEC on the following day (LEC-MUS), behavioral performance showed no impairment. A one-way repeated measures ANOVA showed the significant effect of the drug on performance ($F_{(2,14)} = 19.3$, $p<0.0001$). A Bonferroni-Dunn post-hoc test revealed significant differences in performance between animals in the vehicle group and the MEC-MUS group ($p<0.0001$), as well as between the LEC-MUS and MEC-MUS groups ($p<0.001$), suggesting that the MEC is the critical brain region for the scene-based spatial choice behavior, whereas the LEC is not. There were no significant differences in choice latency among the drug conditions ($F_{(2,14)} = 0.21$, $p=0.8$; One-way repeated measures ANOVA), suggesting that the impairment in performance in the LEC-MUS group was not attributable to generic sensory-motor deficits.

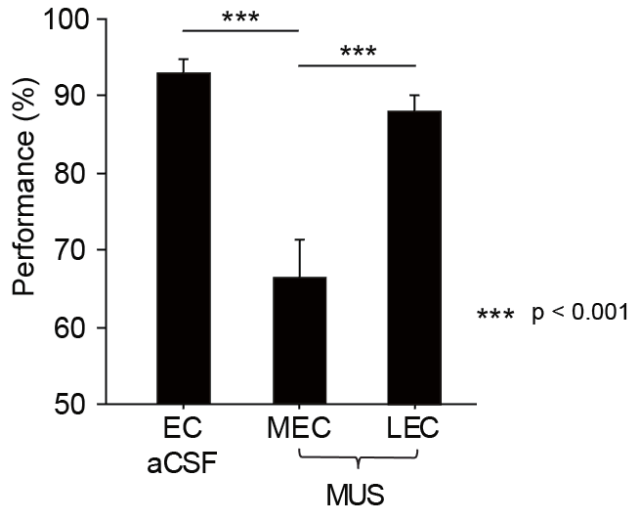


Figure 9. Post-surgical performance in the SSC task. The MEC-MUS group demonstrated a significant decrease in performance compared to the LEC-MUS group, as well as compared to the EC-aCSF group. ***p<0.001. Artificial cerebrospinal fluid, aCSF; Entorhinal cortex, EC; Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC; Muscimol, MUS; Scene-based spatial choice task, SSC.

Discussion

The functional role of the MEC in associating scene-based information with egocentric spatial response

Given that the rodent POR, like the parahippocampal cortex in humans, is uniquely involved in scene information processing (Epstein and Kanwisher, 1998), it is reasonable to assess that the MEC, as a downstream structure of the POR, is also heavily involved in processing scene-based information. Moreover, considering the anatomical connections between the MEC and a number of brain regions such as the parasubiculum and retrosplenial cortex that process idiothetic spatial information (McNaughton et al., 2006; Knierim et al., 2014), and prior behavioral experiments which described the functional role of the MEC in spatial navigation (Steffenach et al., 2005; Van Cauter et al., 2013; Hales et al., 2014), the results of the MEC-MUS group in the SSC task may be caused by the functional role of the MEC in associating egocentric spatial information with visual scene-based stimuli. In addition to the above description, when rats with MEC lesions were tested in a water maze task, which is typically considered a hippocampal-dependent task and requires subjects to process egocentric spatial information, performance was profoundly impaired. On the other hand, the MEC did not play an important role in other tasks, such as contextual fear conditioning, novel object recognition, and displaced object recognition, all of which are sensitive to hippocampal damage but do not require the processing of spatial information (Hales et al., 2014), suggesting that the MEC does not process all types of hippocampal-dependent memory. On the other hand, compared to the control group in the SSC task, the LEC-MUS group's performance was not impaired when MUS was injected. Considering that the LEC is only weakly connected to the areas such as the pre- and parasubiculum that process egocentric spatial information (Kerr et al., 2007), the result of the LEC-MUS group in the SSC task suggests that the LEC plays no critical role in producing egocentric spatial responses based on visual scene-based

information.

No degradation of experiment integrity due to unintentional damages

Although almost cannulae targeted the expected brain regions, cannulae that targeted the LEC inevitably damaged several areas including auditory cortices, the temporal association cortex (TE), and the PER, and cannulae targeting the MEC partially damaged visual cortices. Despite this cortical damage, no significant difference in performance of tasks was found when comparing aCSF condition across the tasks. These results clearly argue against the possibility that the effects of MUS may be due to unexpected damage in the overlying cortices. Considering the normal performance of the control group, it is also clear that attention and visual capabilities in said group were normal.

EXPERIMENT 2. Scene-based non-spatial choice task

Introduction

Previous studies have reported an important role of the LEC in identifying individual items (Deshmukh and Knierim, 2011; Wilson et al., 2013a). When rats with lesion of the LEC were required to discriminate the changed object in association with the contextual environment, more severe deficits were found (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a), suggesting that the LEC is also critical to recognizing objects in association with environmental cues. However, the external cue and contextual environment were not systematically controlled in prior studies. Furthermore, behavioral outcomes of the subjects seem not to have been well controlled. Although behavioral experiments using animal subjects are required to rigorously measure those behaviors with objective indicators, some behavior measurement tools used in previous studies, such as time spent sniffing or rearing at a novel object, inevitably required subjective interpretation. In the scene-based non-spatial choice task, I systematically controlled scene-based stimuli in the same way as the SSC task, and behavioral responses were also strictly controlled in which non-spatial responses, digging in the sand in the jar or pushing that same jar according to scene-based stimuli.

Methods

Subjects

Nine male Long Evans rats weighing 340–400 g (9-11 weeks after birth) were used. Animal care protocol was identical with the SSC task.

Behavioral apparatus

The T-track was also used in a scene-based non-spatial choice (SNSC) task. In the SNSC task, two side arms at the end of the track were made unavailable to the rat by the insertion of a linear track (82.5 cm x 7.5 cm). Two side walls (each 10 cm x 7.5 cm x 0.7 cm) and a food well were found at the end of the track. A wide-mouth amber glass jar (5.8 cm in diameter and 7 cm in height) was placed above the food well (**Figure 10**). The amber jar contained play sand mixed with cereal powder (Froot Loops, Kellogg's, USA) at a 7:1 ratio.

Figure 10. Behavioral apparatus used in the SNSC task. In the SNSC task, two side arms at the end of the track were blocked and a rat was required to respond either digging in the sand in the

jar or pushing that same jar based on the specific scene stimulus. The start box door was omitted for visualization. Scene-based non-spatial choice task, ONSC.

Shaping

A trial began when the experimenter opened the start box. Once exiting the start box, the rat was required to respond by either digging in the sand in the jar or pushing that same jar to obtain a food reward. To shape the digging behavior, rats were allowed to find a piece of cereal placed on the top of the play sand in the jar. The cereal reward was then gradually hidden in the sand over many trials and was eventually completely hidden in the sand (0.5 cm from the surface of the sand). When the rat found the cereal reward, it was allowed to consume it and was then gently guided back into the start box. For pushing behavior, in the earlier shaping stage, the food well was half-closed by a glass jar so that the rat could see the food reward. The food well was gradually closed by the glass jar over the course of many trials and was eventually completely closed. To shape both digging and pushing responses, rats were trained until the following two criteria were satisfied: first, the median latency from the opening of the start box door to the moment the rat received food reward was less than 5 s. Second, the rat could completely displace the glass jar covering the baited food well (for the pushing response) or could find a food reward completely hidden in the sand in more than 20 out of 40 trials. A daily session was forcibly finished when the rat completed 40 trials or when 30 m passed, whichever came first. Once the criteria were met, pre-surgical training for the main task began.

Pre-surgical training

Upon opening the start box door and the rat exiting the start box, one of the first pair of scene stimuli, pebbles or zebra stripes, was displayed on the LCD monitors in a pseudo-random sequence (**Figure 11**). Rats were trained to respond by either digging in the sand or pushing the jar in association with the scene stimulus (e.g. pushing for pebbles and digging for zebra stripes) for 40 trials a day. If the rat stood up and touched the sand with

both paws or if the rat moved the jar with its snout (even if the food well was not exposed), it was recorded as a digging or a pushing response, respectively. When the rat performed a correct response, a piece of cereal was available in the sand or in the food well. The rat was trained to consume the reward in the start box after being guided back into the start box. If the rat performed a wrong response, however, the rat was gently returned to the start box by the experimenter without a reward. Each scene appeared, in a pseudo-randomly intermixed fashion for 20 trials in a daily session and the identical scene was not presented over three consecutive trials. The rat was trained until its performance level reached greater than 80% correct responses per scene or above 75% correct responses on average for all scenes, with a response bias of less than 0.15. When the rat reached the performance criterion for the first pair of scene stimuli for two consecutive days, it was trained with the second pair of scene stimuli, e.g. peacock feathers and palmtrees, until it reached the same criterion level with the first pair of scene stimuli. Then, when the rat reached the performance criteria for both the first and the second scene pairs, all four scenes were pseudo-randomly presented across trials to train the rat until it reached greater than 80% correct responses for each scene.

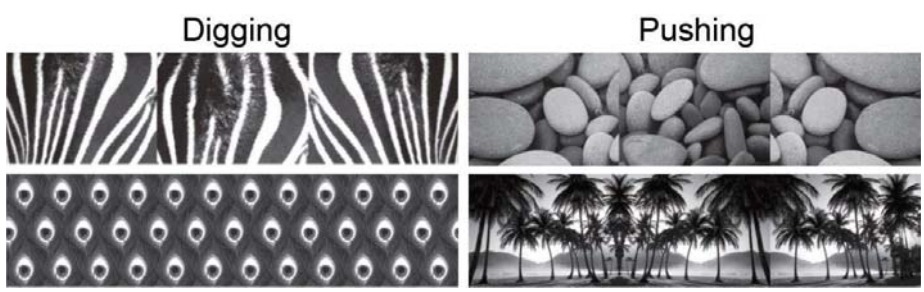


Figure 11. The background visual scenes used in the SNSC tasks. Zebra stripes and pebbles patterns were provided for the first pair of scenes in the SSC task. When a rat reached to performance criterion for the first pair of scenes, next pair, peacock feathers and palmtrees, were provided. Each scene was associated with either left or right choice behavior. Scene-based non-spatial choice task, SNSC.

Surgery

Surgical procedure was carried out in the same way as the SSC task.

Post-surgical testing and reversible inactivation of the EC

Post-surgical testing and reversible inactivation procedure were carried out in the same way as the SSC task.

Histology

Histological procedure was carried out in the same way as the SSC task.

Data analysis

Data analysis was carried out in the same way as the SSC task.

Results

Critical role of the LEC, but not the MEC, for the scene-based non-spatial choice behavior

Following the SSC task, I also conducted the SNSC task to examine if the performance deficits found in the SSC task also could be observed when a non-spatial choice behavior, digging or pushing, was required based on the scene stimuli (Lee and Shin, 2012; Lee et al., 2014). When the rats from the SNSC task group (n = 9) were tested after surgery, their performance was, surprisingly, reversed in comparison with the SSC task group. In the vehicle group (EC-aCSF), the rats performed at above 90% correctness (**Figure 12**). However, MUS injections into the MEC (MEC-MUS) did not affect behavioral performance whereas MUS injections into the LEC (LEC-MUS) significantly disrupted performance in the same rats, suggesting that the LEC plays the critical role for making non-spatial responses based on the visual scene stimuli whereas the MEC does not. A one-way repeated measures ANOVA showed a significant effect of drug on performance ($F_{(2,16)}$ = 10.1, p=0.01). A post-hoc test (Bonferroni-Dunn) showed significant differences between the EC-aCSF and LEC groups (p<0.001) and between the LEC-MUS and MEC-MUS groups (p=0.01). There were no significant differences in choice latency among the drug conditions ($F_{(2,16)}$ = 0.29, p=0.74), suggesting that the impairment in performance in the LEC-MUS group was not attributable to generic sensory-motor deficits.

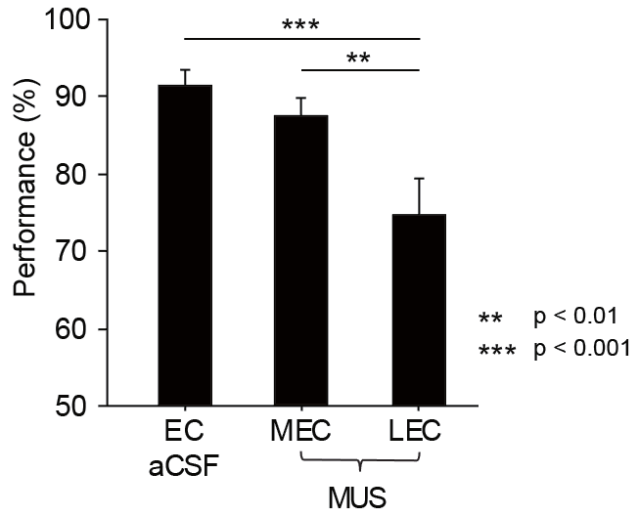


Figure 12. Post-surgical performance in the SNSC task. The LEC-MUS group exhibited a significant decrease in performance compared to the MEC-MUS group, as well as the EC-aCSF group. **p<0.01, ***p<0.001. Artificial cerebrospinal fluid, aCSF; Entorhinal cortex, EC; Lateral entorhinal cortex, LEC; Materal entorhinal cortex, MEC; Muscimol, MUS; Scene-based non-spatial choice task, SNSC.

Layer-specific functions along the superficial versus deep layers of the LEC in the SNSC task

Despite the significant impairment in performance with MUS injections to the LEC, some rats seemed less affected by LEC-MUS, which was noticeable in the large variance in the performance data for the LEC-MUS condition compared to the other drug conditions. I examined whether there was a relationship between the locations of the cannula tips in the LEC and performance levels in individual rats. If one of the cannula was only placed laterally, the animal's cannula position was still labeled lateral (e.g. rat 417 in **Figure 28**). Interestingly, it turns out that the rats with their LEC cannulae implanted more laterally along the superficial layers (marked in blue circles in **Figure 28**) were not as severely impaired as the animals with their LEC cannulae implanted more medially along the deeper layers (marked in red circles in **Figure 28**) when performance of the two subgroups under MUS were directly compared ($t_{(7)} = -4.35$, $p<0.01$) (**Figure 13**). The two subgroups were not significantly different from each other in the aCSF condition ($t_{(7)} = 0.3$, $p=0.77$), suggesting that the performances differed according to locations of the cannula tips along deep and superficial layers in the SNSC task group. The rats with the LEC cannulae implanted in a deeper layer exhibited a larger deficit in performance than those implanted in a more superficial layer. On the other hand, there was no significant difference in performance in the EC-aCSF group according to location of the cannula tip. Additionally, almost cannulae implanted in the MEC of the SSC task group were located in the superficial layer of the MEC, except only one rat that its cannula implanted in the MEC were located in the deep layer.

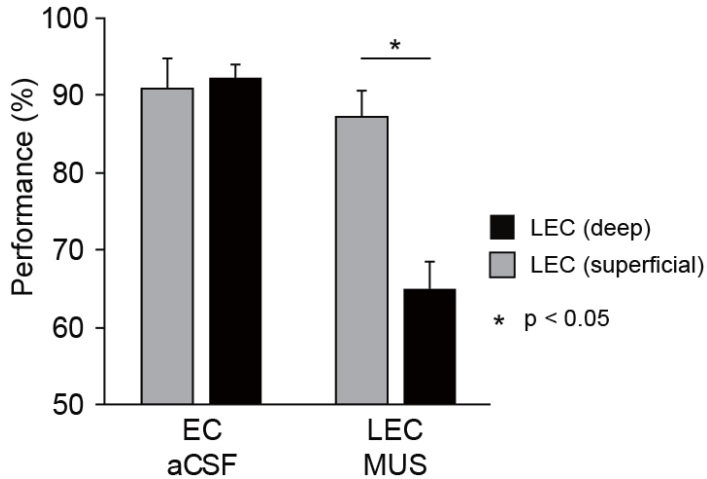


Figure 13. Differences in performance according to location of the cannula tips along superficial or deep layer in the SNSC task. Rats with the LEC cannulae implanted in a deeper layer exhibited larger deficit in performance than those implanted in a more superficial layer, whereas no differences in performance were found in the EC-aCSF group. *p<0.05. Artificial cerebrospinal fluid, aCSF; Entorhinal cortex, EC; Lateral entorhinal cortex, LEC; Scene-based non-spatial choice task, SNSC.

Functional double dissociation within the EC for the scene-based choice behavior

The functional double dissociation between the LEC and MEC can be more clearly seen when the performance deficit (measured by subtracting performance under MUS from performance under aCSF) was plotted for all MUS conditions for the LEC and MEC (**Figure 14**). An ANOVA was performed with the task as a between-group factor and the drug condition as a within-group factor, revealing a highly significant interaction between the task and drug conditions ($F_{(1,15)} = 34.76$, $p<0.0001$). Post-hoc tests (Bonferroni-corrected t-tests) showed that the LEC-MUS group showed significantly larger deficits compared to the control condition in the SNSC task ($p<0.05$), but not in the SSC task ($p>0.5$), whereas the reverse was true of the SSC task ($p=0.001$ with MEC-MUS). These results collectively show that the LEC and MEC were both involved in visual scene-based memory tasks, although different task demands recruited the two regions differentially. That is, the LEC was important when rats made non-spatial choices (digging versus pushing) about the same object (sand-filled jar) using scenes in the background, but not for spatial choices (left turn versus right turn). Conversely, the MEC was more involved in the SSC task, but not so much in the SNSC task.

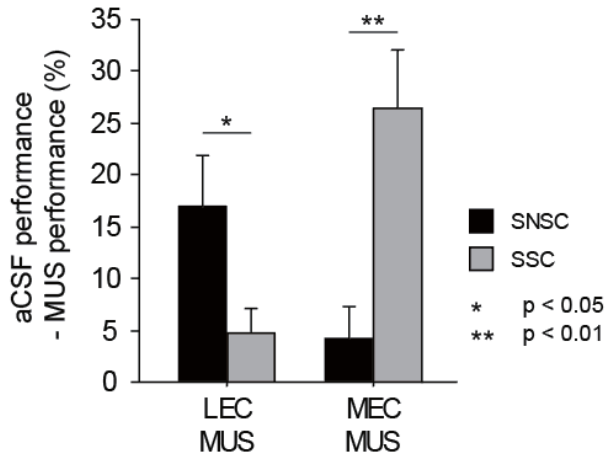


Figure 14. Double dissociation between the LEC and MEC in the SSC and SNSC tasks. Ordinate represents the performance difference between aCSF and MUS conditions. Note the significant differences between deficits for both MUS groups according to the SSC and SNSC tasks. *p<0.05, **p<0.01. Artificial cerebrospinal fluid, aCSF; Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC; Muscimol, MUS; Scene-based non-spatial choice task, SNSC; Scene-based spatial choice task, SSC.

Discussion

The functional role of the LEC in the scene-based disambiguation of objects-associated responses

The functional role of the LEC was examined in previous studies that lesions to the LEC caused a severe impairment in recognizing a newly replaced object in association with an array of adjacent objects (Van Cauter et al., 2013), and in identifying novel or newly presented objects in association with the contextual environment (Hunsaker et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). In the SNSC task, the temporary inactivation of the neurons of the LEC induced a significant deficit in performance compared to the control and MEC-MUS groups. Because the rats in the SNSC task were required to make appropriate behavioral responses when facing a common object, the background visual scene were the only cue provided to disambiguate the correct response. By contrast, the presentation of an object by itself may not produce responses sufficient to explain the intrinsic functions of the LEC. When a rat was required to choose a behavioral response in the SNSC task based on an object cue alone, compared to both the control and MEC-MUS groups, the LEC-MUS group exhibited no performance deficit. Previous studies yielded similar results in which a permanent lesion to the LEC did not affect a subject's ability to identify an object by itself (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). In agreement with the results of previous studies, the current study demonstrate that the functional role of the LEC can be extended to associating behavioral responses to the specific object with scene-dependent stimuli. So, the functional role of the LEC is critical when object-associated ambiguity must be reduced using scene-based or contextual information. Regarding spontaneous object exploration paradigms from previous studies, presented objects may become more ambiguous as animals experience more objects in the same environment (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). Contrary to previous studies, the current study asserts that the cause of object-associated ambiguity in the SNSC task may derive from the task demand that

two distinct behavioral responses should be conditionally performed to the same object based on the scene-dependent stimuli. Interestingly, the MEC-MUS group exhibited no performance deficit compared to the control group in the SNSC task, suggesting that a particular brain area may be specifically required for a task's specific demands.

The layer-specific functional roles of the LEC in the SNSC task

Although individual differences between rats such as weight or age were well controlled in the current study, uncontrollable variables, such as brain shape or its size, might cause the relatively different locations of cannulae implanted in the LEC across rats, though identical 3D complex cannulae were used for all subjects. Furthermore, behavioral performance in the SNSC task varied according to the locations of cannulae implanted in the LEC. Although it has been known that the LEC mainly receives anatomical input from the PER, it also receives a modest input from the POR that process visual scene information, but not as much as MEC (Kerr et al., 2007). Thus, it can be assumed that the visual scene information is also processed in the LEC, which can be explained by the results of the SNSC task that behavioral performance from the rats with LEC cannulae implanted deeper was more severely impaired than those whose cannulae were implanted more superficially. These results suggest a distinct functional difference along the superficial and deep layer of the LEC. The LEC receives the external sensory input from the upstream structures via its superficial layer and then conveys the received input to the DG, CA3, distal CA1, and proximal subiculum (Witter et al., 2000a; Naber et al., 2001). Based on the information received from the LEC, and also from the MEC, the hippocampus forms a conjunctive representation, which is, presumably, considered to be an important step in forming a coherent episodic memory, and this information is then transmitted to the deep layers of the LEC and MEC via the subiculum and CA1 (Witter et al., 2000a; Witter et al., 2014). Although the superficial layer of the LEC is inactivated by injecting MUS during the SNSC task, the superficial layer of the MEC still sends external sensory input to the hippocampus on behalf of the superficial layer of the LEC, and the

scene information formed in the hippocampus is transmitted to the deep layer of the LEC and MEC. This presumable process contributes to forming a distinct behavioral output, in this case, non-spatial responses to a specific object, and this assumption is proven as a behavioral results of the SNSC task that no performance deficit was found in the rats with cannulae implanted in the superficial layer of the LEC when MUS was injected. In addition, considering that no performance deficit was found in the ONSC task when the deep and superficial layers of the LEC were inactivated, respectively, it can be assumed that the LEC is specialized in the role of associating only visual scene stimuli with specific behavioral responses. Unlike the LEC, since the cannula tips implanted in the MEC are mostly located in the superficial layer, except one rat (no. 280), it is limited to distinguishing functional differences according to the superficial and deep layer of the MEC.

EXPERIMENT 3. Object-based non-spatial choice task

Introduction

The roles of the LEC in object-related memory have been described in previous studies (Deshmukh and Knierim, 2011; Hunsaker et al., 2013; Keene et al., 2016). Although the role of the LEC became more critical when the LEC-lesioned rats was required to discriminate the displace object in association with the contextual environment (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a), no performance deficit was found in the LEC-lesioned rat when an object was provided as a cue by itself, rather than associating object cues with contextual environment (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). In contrast with the SSC task, the animals were required to interact with an object in the SNSC task, (a wide-mouth amber glass jar). Accordingly, the performance deficits of the animals in the LEC-MUS group during the SNSC task might be caused by the involvement of the object, but not by the involvement of visual scene-based memory itself. To examine whether the performance deficit was caused by the object factor alone without scene stimuli, I tested a subset of rats from both the SSC (n = 6) and SNSC tasks (n = 3) in the ONSC task. The experimental procedures for the ONSC task were identical to those from the SNSC task, except that the background visual scene stimuli were replaced by object cues, ice cream and burger attached to the jar. In the ONSC task, rats were required to make the non-spatial response between digging in the sand in the jar and pushing that same jar after sampling an object cue attached to the jar.

Methods

Subjects

Nine rats (n = 6 from the SSC task group and n = 3 from the SNSC task group) were used in the ONSC task. Animal care protocol was identical with the SSC task.

Behavioral apparatus

An object-based non-spatial choice (ONSC) task was conducted in a rectangular acrylic box (55 cm x 23 cm x 23 cm) made of transparent red acrylic panels (**Figure 15**). A guillotine door-operated start box (22.5 cm x 16 cm x 31.5 cm) was attached to the end of the acrylic box. A linear track (52 cm x 20 cm) was placed inside the box by installing side walls (each 52 cm x 8 cm x 0.5 cm). A food well was placed 10 cm from the end of the linear track. A wide-mouth amber glass jar (filled with play sand and cereal powder) was also used in the task, and a magnet was attached to the glass jar so that an object could be attached to the jar during the task.

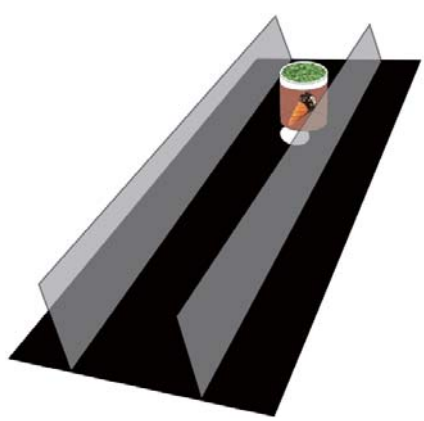


Figure 15. Behavioral apparatus used in the ONSC task. In the ONSC task, an object cue was

provided instead of the background visual scene. A rat was required to respond either digging in the sand in the jar or pushing that same jar after sampling the object cue. A rectangular acrylic box and start box were omitted for visualization. Object-based non-spatial choice task, ONSC.

Shaping

The animals were trained and then tested in the ONSC task. The experimental procedure of the ONSC task was identical with the SNSC task except for that the background visual scenes were replaced to the two object cues (ice cream and burger). No additional cues were provided during the training and testing except for the object cues. When the start box was opened by an experimenter, a trial began and the rat was trained to respond by either digging in the sand or pushing the amber jar after sampling an object cue attached to the amber jar (i.e. pushing for the ice cream and digging for the burger or vice versa) attached to the jar (**Figure 16**).

Figure 16. The non-spatial choice behavior based on an object cue in the ONSC task. In the ONSC task, the rats were trained to respond either pushing or digging behavior based on one of the two objects. The photo on the left shows the ice cream object attached to the amber jar, whereas the photo on the right shows the burger object attached to the amber jar. Object cue was attached

to the jar by a magnet and the inside of amber jar was filled with play sand mixed with cereal powder. When the ice cream object was presented, for example, the rat had to push the jar. Object-based non-spatial choice task, ONSC. If the rat stood up and touched the sand with both paws or if the rat moved the jar with its snout (even if the food well was not exposed), it was recorded as a digging or a pushing response, respectively. When the rat performed a correct response, a piece of cereal was available in the sand or in the food well. The rat was trained to consume the reward in the start box after being guided back into the start box. If the rat performed a wrong response, however, the rat was gently returned to the start box by the experimenter without a reward. Each object cue was applied in a pseudo-randomly intermixed fashion for 20 trials in a daily session and the identical object cue was not presented over three consecutive trials. To avoid latent olfactory cue, each object cue and the amber jar was cleaned with 70% isopropyl alcohol before a trial began. The rat was trained until its performance level reached greater than 80% correct responses per object cue or above 75% correct responses on average for both object cues, with a response bias of less than 0.15. No correction was allowed when the rat made an error. When the rats reached the performance criterion for two consecutive days, on the next day, aCSF was simultaneously injected into both the LEC and the MEC (0.3 μL per site) 20 min before behavioral testing. Over the next two days, the same animal received an injection of MUS into the LEC and then into the MEC (0.3 μL per site), or vice versa (the injection sequence was counterbalanced among the rats).

Reversible inactivation of the EC and testing

When the rats reached performance criterion for two consecutive days, drug injection procedure was begun, and injection protocol was carried out in the same way as the SSC task.

Results

No involvement of the LEC and MEC in the ONSC task

When the rat was tested with the injection of aCSF into the EC (EC-aCSF) after reaching the performance criterion, performance correctness was at above 90% (Figure 17). Interestingly, the injection of MUS into neither the MEC nor LEC induced a performance deficit compared to the aCSF condition. A one-way repeated measures ANOVA showed no significant effect of the drugs on performance ($F_{(2,16)} = 1.03$, $p<0.38$). No significant differences in choice latency among the drug conditions ($F_{(2,16)} = 0.28$, $p=0.75$; One-way repeated measures ANOVA) were found. These results clearly demonstrate that neither of the subdivisions of the EC were necessary when rats associated a single object to make a digging versus pushing response to the jar, and furthermore, emphasizes the importance of visual scene stimuli to elicit specific behaviors for rats.

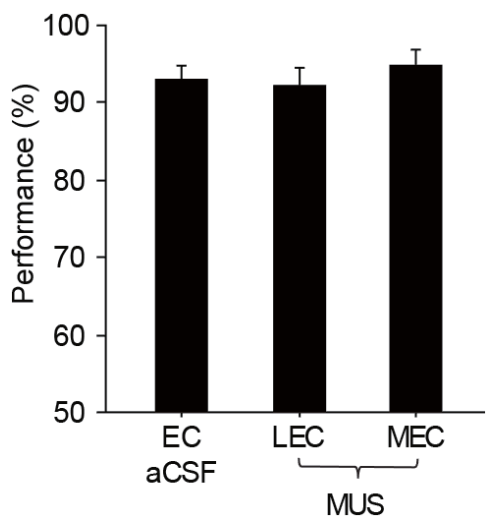


Figure 17. Performance comparison for the ONSC task. No performance deficits were found

among the different conditions for ONSC task. Note that the MUS injection into the MEC or LEC, respectively, did not influence performance. Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC; Muscimol, MUS; Object-based non-spatial choice task, ONSC.

No differences in the aCSF condition among tasks

The performance level under aCSF (mean = 92.9%) in the ONSC task was equivalent to the performance levels of the control conditions in the SSC (mean = 92.9%) and SNSC (mean = 91.7%) tasks, and one-way repeated measures ANOVA showed no significant difference on performance among three groups ($F_{(2,14)}$ = 0.36, p=0.7) (**Figure 18**). This suggests that the null effect of MUS in the object cue-based non-spatial choice task was not attributable to the ceiling effect for performance in the control condition. The results also demonstrate that the digging and pushing behaviors themselves were not necessarily affected by MUS injections into the LEC or MEC. These findings thus suggest that the performance impairment observed in animals that received the MUS injection into the LEC in the SNSC task may not be solely based on the presence of the object variable when compared to the SSC task.

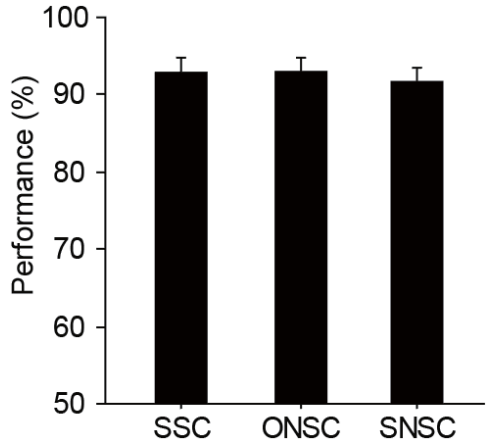


Figure 18. Performance comparison among the different tasks under the aCSF condition. The aCSF injection into the EC did not induce any significant differences in performance among the SSC, ONSC and SNSC tasks. Note that all performances from each group were above 80% correctness, suggesting a null effect of MUS in the ONSC task, which was not attributable to the ceiling effect for performance in the aCSF condition. Artificial cerebrospinal fluid, aCSF; Entorhinal cortex, EC; Object-based non-spatial choice task, ONSC; Scene-based non-spatial choice task, SNSC; Scene-based spatial choice task, SSC.

Discussion

In previous studies, the LEC-lesioned rat showed no performance deficit in identifying an object cue by itself (Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a), whereas a significant impairment in performance was found when the same rat was required to associate an object with contextual environment (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). Considering task components involved in the SNSC task, it is hard to know that which components, scene stimuli or non-spatial responses, affected behavioral performance. As an extension of the SNSC task, the ONSC task was conducted, which required rats to make the non-spatial response between digging in the sand in the jar and pushing that same jar after sampling an object cue attached to the jar. The main reason to conduct the ONSC task was to discriminate sensory stimuli from response component used in the SNSC task. Because behavioral performance in the SNSC task might be attributable to the involvement of the object, not necessarily to the involvement of visual scene-based memory, the task that contains different type of sensory stimuli, such as an object cue, with the same behavioral responses is required to overcome this issue. When the rat was tested in the ONSC task with MUS injection bilaterally into either the LEC of MEC, no groups showed performance deficit, whereas a significant deficit in performance was found in the LEC-MUS group in the SNSC task. These results demonstrate the significance of scene-based stimuli as a critical sensory stimuli in engaging the functions of the subdivisions of the LEC and MEC.

EXPERIMENT 4. Tactile cue-based spatial choice task

Introduction

In the SSC task, two major components, spatial response and the background visual scene, were included, and the animals were required to respond by either turning left or right based on the background visual scene. When the MEC-MUS group was tested in the SSC task, a significant performance deficit was found, and thus, this result emphasizes the involvement of the role of the MEC in performing the SSC task. However, it is unclear that which components were the critical factor to induce the performance deficits in the MEC-MUS group during the SSC task. If the rat's capability of associating spatial responses with scene stimuli during the SSC task were the critical factor, the same rat might show behavioral impairment when any type of sensory stimulus was presented instead of background visual scene. To examine this issue, as an extension of the SSC task, I tested a subset of rats from both the SSC (n = 4) and SNSC tasks (n = 6) in the TSC task, in which the rat was required to make either a left or right turn based on tactile cues (wire mesh and sandpaper).

Methods

Subjects

Ten rats (n = 4 from the SSC task group and n = 6 from the SNSC task group) were used in the TSC task. Animal care protocol was identical with the SSC task.

Behavioral apparatus

A apparatus used in the tactile cue-based spatial choice (TSC) task was identical with the SSC task except for that the tactile cue (30 cm x 7.5 cm x 0.2 cm), instead of the background visual scene, was applied on the bottom of the linear track at 8cm from the end of the intersection (**Figure 19**).

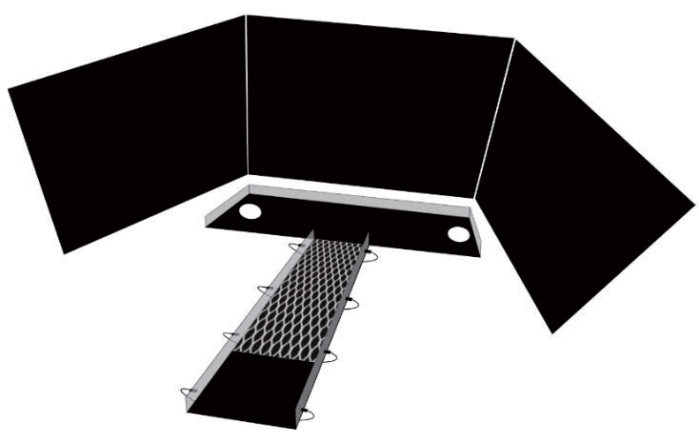


Figure 19. Behavioral apparatus used in the TSC task. In the TSC task, the background visual scene was replaced to the tactile cue. Tactile cue-based spatial choice task, TSC.

Shaping

The experimental procedure of the TSC task was identical with the SSC task except for that the background visual scenes were replaced to two tactile cues, wire mesh and sandpaper. Except for the tactile cues, no additional cues were provided during the training and testing. Once the start box was opened by an experimenter, a trial began and the rat was trained to choose either the right arm for the wire mesh or the left arm for the sand paper or vice versa (**Figure 20**).

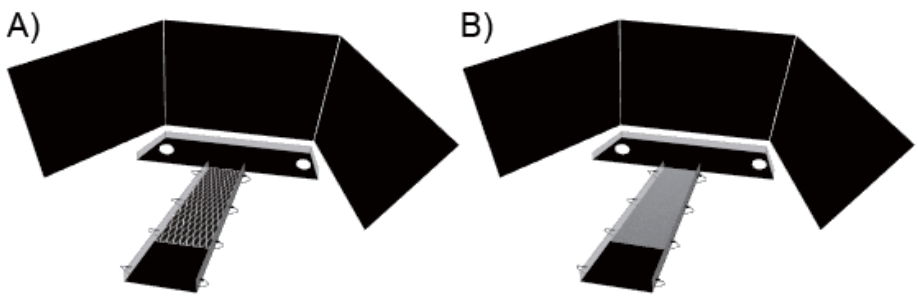


Figure 20. The spatial choice behavior based on the tactile cue in the TSC task. Two different types of tactile cues, **A)** wire mesh and **B)** sandpaper, guided rats to respond either right or left turn, respectively. For example, when the wire mesh cue was provided on bottom of the linear track, the rat had to turn to the right arm. Tactile cue-based spatial choice task, TSC.

If the rat chose a correct direction (when a black metal washer covering the food well was displaced by the rat), a piece of cereal was available in the food well. The rat was trained to consume the reward in the start box after being guided back into the start box. If the rat chose a wrong direction, however, the rat was gently returned to the start box by the experimenter without a reward. Each tactile cue was applied in a pseudo-randomly intermixed fashion for 20 trials in a daily session and the identical tactile cue was not presented over three consecutive trials. To avoid latent olfactory cues, each tactile cue was

cleaned with 70% isopropyl alcohol before a trial began. The rat was trained until its performance level reached greater than 80% correct responses per tactile cue or above 75% correct responses on average for both tactile cues, with a response bias of less than 0.15. No correction was allowed when the rat made an error. When the rats reached the performance criterion for two consecutive days, on the next day, the same method and procedure of the drug injection progressed in the ONSC task was conducted.

Reversible inactivation of the EC and testing

When the rats reached performance criterion for two consecutive days, drug injection procedure was begun, and injection protocol was carried out in the same way as the SSC task.

Results

No involvement of the LEC and MEC in the TCS task

When the rat was tested with the injection of aCSF into the EC (EC-aCSF), performance correctness was at above 85% (mean = 88.8%) (**Figure 21**). Interestingly, the injection of MUS into the MEC induced no significant difference in performance compared to the EC-aCSF group and the LEC-MUS group ($F_{(2,18)} = 1.39$, p=0.27; One-way repeated measures ANOVA). No significant differences in choice latency among the drug conditions ($F_{(2,18)} = 0.03$, p=0.96; One-way repeated measures ANOVA) were found. These results demonstrate that the performance deficits found in the MEC-MUS group during the SSC task were not caused by rats' capability of associating spatial responses with sensory stimuli, and the MEC is critically involved in using background visual scenes for making spatial responses, but not the other types of sensory stimuli, such as tactile cues.

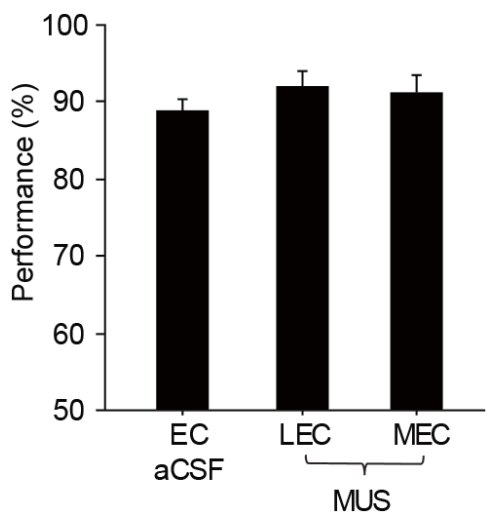


Figure 21. Performance in the TCS task. No performance deficits were found among the

different conditions in the TSC task. Note that the MUS injection into the MEC or LEC did not influence performance. Tactile cue-based spatial choice task, TCS; Muscimol, MUS; Medial entorhinal cortex, MEC; Lateral entorhinal cortex, LEC.

Discussion

As a downstream structure of the POR, the MEC is also heavily involved in processing scene-based information, and in the SSC task, it has been found that the functional role of the MEC to make spatial responses based on scene stimuli. Regarding that the rodent POR is uniquely involved in scene information processing (Epstein and Kanwisher, 1998), it is reasonable to assess that the MEC would be a critical brain region where process scene-based memory. Although findings of the previous behavioral experiments have been described the functional role of the MEC in spatial navigation (Steffenach et al., 2005; Van Cauter et al., 2013; Hales et al., 2014), the results of the MEC-MUS group in the SSC task may be caused by the functional role of the MEC in associating egocentric spatial information with external sensory stimuli. If performance deficit found in the SSC task was attributable to rats' ability of associating sensory stimuli with spatial responses, any types of sensory stimuli would affect behavioral performance. In this regard, I conducted the TSC task that provides tactile cues instead of scene-based stimuli. Interestingly, neither the MEC-MUS nor LEC-MUS group showed performance deficits during the TSC task, demonstrating the performance deficits found in the MEC-MUS group during SSC task were not caused by rats' capability of associating sensory stimuli with spatial responses.

EXPERIMENT 5. Masked scene-based choice task

Introduction

Following the post-surgical testing for the SSC and SNSC task, the MSC and MNSC were conducted to test the reliability of scene-based stimuli used in the SSC and SNSC task. Systematic control of the scene-based stimuli is one of the advantage of the present thesis, and through a series of experiments, it has been proved that visual scenes can be used as a sensory stimulus to recruit the functional role of specific brain region. However, it is still unclear whether or not rats made their behavioral choices based on overall scene pattern. In some cases, it might be possible that rats made their responses based on a specific spot of the provided scene. To address this issue, I conducted additional tests involving MSC and MNSC tasks, and used manipulated versions of the original pattern I had used as the visual scene stimuli. Two types of scene pattern, one in which the original pattern was projected through a grid of medium-sized circles and another one in which the original pattern was projected through a grid of small circles.

Methods

Subjects

Ten rats (n = 7 from the SSC task group and n = 3 from the SNSC task group) were used in the MSC and MNSC tasks. Animal care protocol was identical with the SSC task.

Behavioral apparatus

The identical apparatus from the SSC task was used in the MSC and MNSC tasks.

Testing

(a) MSC task: The animals from the SSC task group (n = 7) were tested in the MSC task. In the MSC task, four original visual scenes (pebbles, zebra stripes, mountain and bamboo pattern) used in the SSC task were modified in which each scene stimulus was masked by a gray-solid color, but restrictively provided through either medium- or small-sized circles. Two sizes of circles were not simultaneously presented in a same trial. Those shaped a regular diagonal patterns and led to form four different types of patterns in which a location of each circle was slightly shifted along the horizontal axis (**Figure 22**). Following the first 10 trials out of total 80 trials with the original visual scenes, two out of four types of the modified patterns applied to the medium- and small-sized circles, respectively, were presented twice during the test session in a pseudo-randomly intermixed fashion. Upon the modified scene trial, no food reward was given to prevent the response bias in which the rat, for example, might choose only the right arm whenever a modified scene was provided, no matter what the original background visual scene was. When the rat opened a washer in a modified scene trial, the rat was gently guided back to the start box without a reward. The MSC task was conducted only for a single day without any drug injection.

(b) MNSC task: The animals from the SNSC task group (n = 3) were also tested in the

MNSC task. In the MSNC task, the four visual scenes (pebbles, zebra stripes, palmtrees and peacock feathers) used in the SNSC task were modified in the same manner with the MSC task. In brief, the experimental procedure of the MSNC task was identical with the MSC task except for that the behavioral responses were replaced from the spatial choice (left and right turn) to the non-spatial choice (digging and pushing behavior). The MNSC task was also conducted only for a single day without any drug injection.

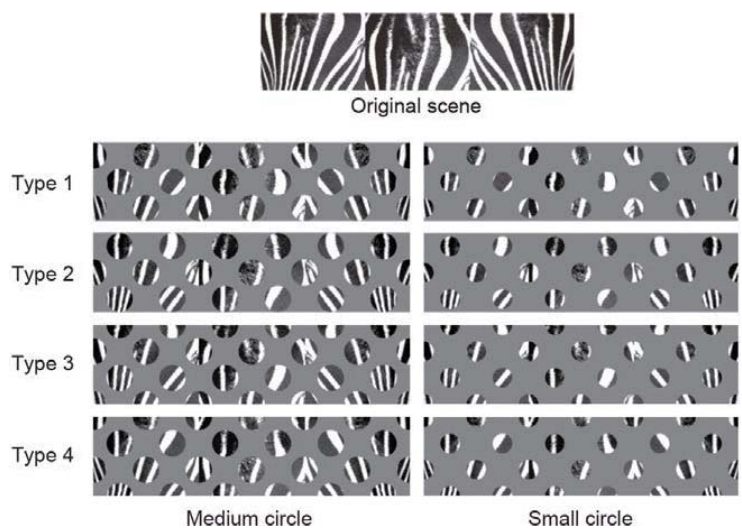


Figure 22. The modified-scene stimuli used in the MSC and MNSC tasks. To confine the amount of visual scene information, the modified-scene stimuli were provided in a pseudo-randomly intermixed fashion in the MSC and MNSC tasks. Note that the original scene stimulus, zebra stripes, was masked by a gray-solid color, but restrictively provided through either small or medium-sized circles. Masked scene-based spatial choice, MSC; Masked scene-based non-spatial choice task, MNSC.

Results

Effects of modified scene information on choice behavior

Although the background visual scenes were predominantly used to elicit specific behaviors in rat, it was uncertain whether they chose their behaviors based on the scene stimuli per se. Because rats may have made their choices based on a specific spot in the scene instead of the overall scene, it was important to verify whether the performance deficits found in both the SSC and SNSC tasks were derived from a selective sampling bias. To examine this issue, I tested the rats that were selected from the SSC task group (n = 7) and SNSC task group (n = 3) in the masked scene-based spatial choice (MSC) and non-spatial choice tasks (MNSC). In the original scene condition, the behavioral performance was at above 85% correctness (**Figure 23**). There was no significant difference in performance between the original scene condition and the medium-sized circle condition. However, the small-sized circle condition showed significant differences in performance compared with the original scene condition as well as with the medium-sized circle condition. A one-way repeated measures ANOVA showed a significant difference in performance ($F_{(2,18)} = 7.67$, $p<0.01$). A Bonferroni-Dunn post-hoc test not only revealed significant differences in performance between the original scene condition and the small-sized circle condition ($p<0.01$), but also between the medium-sized circle condition and the small-sized circle condition ($p<0.05$).

Although the original scenes were systematically masked with symmetric patterns in a masked scene trial, it might be possible that rat could make their behavioral choices based on a specific spot of the remained scene stimuli. If rat's behavior was dependent on a specific spot in a given masked scene trial, another trial that may conceal the same specific spot from the previous trial could affect behavioral performance. Individual rat experienced eight masked scene trials for each medium- or small-sized circle condition and two out of four different types of masked pattern were applied to each medium- and

small-sized circle condition. To examine if there were a significant difference in performance among each type of the medium- or small-sized circle condition, respectively, I conducted a paired t-test to compare the performance between type 1 and type 2 for each medium- and small-sized circle conditions. The results showed no significant difference in performance between types of each the medium circle-sized condition ($t_{(5)}$ = 2, p=0.1019) and medium circle-sized condition ($t_{(5)}$ = -0.307, p=0.7711), suggesting that rat chose their behavioral performance based on overall background scene, but not based on a specific spot. The results of the masked scene-based tasks suggest that the amount of scene information provided could be critical to making a behavioral response. In addition, this test verified that rats did not make their behavioral choices based on a specific spot in the scene stimulus.

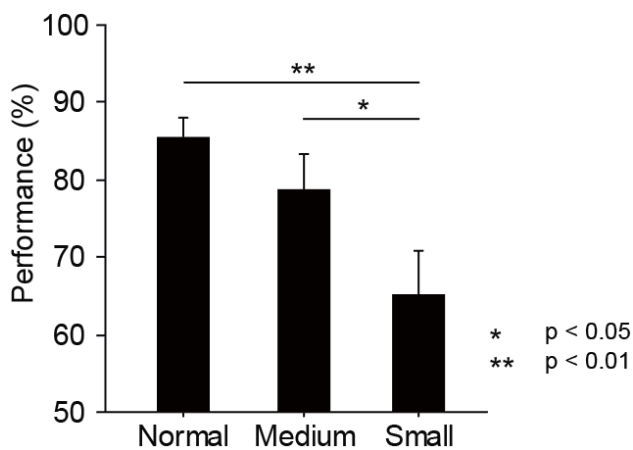


Figure 23. Variable performances depending on the amount of scene information. In the small-sized circle condition, the behavioral performance was significantly decreased compared to the normal scene condition as well as to the medium-sized circle condition. *p<0.05, **p<0.01.

Discussion

Reliability of scene-based stimuli used in behavioral tasks

An advantage of the present thesis as compared to previous studies is the systematic control of the visual stimuli, which directly can induce specific behavioral responses. Although the visual stimuli were used as the sole cue for both the SSN and SNSC tasks, it was unclear whether or not a specific part of the scene pattern was serving as the real cue eliciting specific behaviors from the rat. To address this issue, I conducted additional tests involving MSC and MNSC tasks, and used manipulated versions of the original pattern I had used as the visual scene stimuli. There were two manipulated versions of the pattern, one in which the original pattern was projected through a grid of medium-sized circles and another one in which the original pattern was projected through a grid of small circles. In all versions of the test, the same behavioral response was required. Performance was highest when the original pattern was provided as the cue to do a task. When the manipulated patterns were presented, the difference in performance was proportional to the amount of visual information being taken away by the pattern manipulation. As such, the pattern as projected through a grid of small circles provided the least visual information, and resulted in the lowest performance accuracy. Given that no significant difference in performance was seen between experiments using the original pattern and the pattern as projected through medium-sized circles, the rats do not seem to perform their behaviors based on a specific area of the visual stimuli.

Histological results

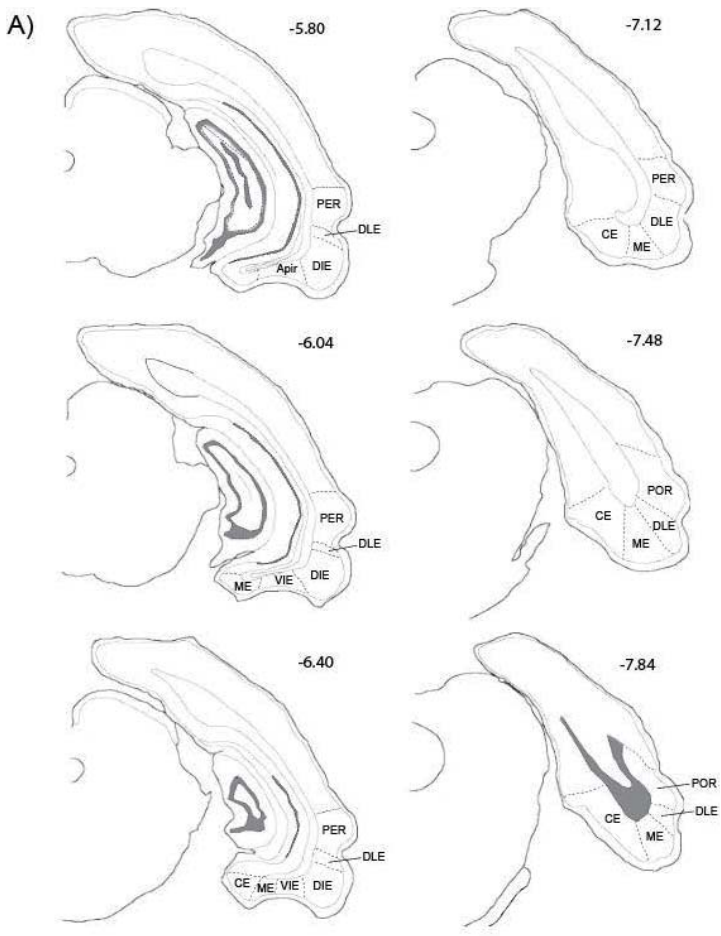
Classification of the lateral and medial divisions of the entorhinal cortex

To overcome various discrepancies from the conventional rat atlas, i.e. overall shape of brain section and nomenclature of subdivisions, I used a customized rat atlas made by my collogue (YounJin Kim) for the current study (**Figure 24**). Because an inconsistent nomenclature from the various literatures (e.g. regarding the specific layers or subdivisions of the entorhinal cortex) could cause a confusing interpretation, I used the same nomenclature as a previous study (Insausti et al., 1997) to avoid this issue. In this study, the EC was cytoarchitectonically subdivided into the medial and lateral divisions and distinguished from the adjacent brain regions as well.

In agreement with the previous study, the present study subdivided the EC into six sub-regions, Caudal entorhinal division (CE), Medial entorhinal division (ME), Ventral intermediate entorhinal division (VIE), Amygdalo-entorhinal transitional division (AE), Dorsal intermediate entorhinal division (DIE) and Dorsal lateral entorhinal division (DLE). **Figure 24** shows the segregated subdivisions of the EC including the PER and POR above the DLE. The CE, ME and VIE belong to the medial division of the EC whereas the lateral division of the EC contains the AE, DIE and DLE. In addition, each division of the EC generally comprises six layers, from the most superficial layer (layer I) to the deepest layer (layer VI). Furthermore, each division of the EC has the layer-specific differences, i.e. distribution, size and number of the neurons, or in the case layer IV, the presence of lamina. Layer II of the EC is most noticeable visually, as this layer is densely packed with big and darkly stained neurons (**Figure 25, 26**). Meanwhile, layer IV of the EC, known as lamina dissecans, is also easily discriminable as it contains fewer neurons than the other layers. When the customized-rat atlas was applied to the photomicrographs of the histological result of the current study, it fit more accurately than

the conventional rat atlas in terms of the overall shape and the locations of each sub-region (**Figure 24B**).

CE forms the ventral part of the caudal pole of the rat hemisphere and it has the longest ventral part along the medial side. CE also mediocaudally borders with the parasubiculum (paraSUB) while it is ventrolaterally separated from ME, in which the neurons of layer II are not continuous and are instead grouped into several islands. DIE, on the other hand, forms the ventrolateral part of the EC, and it extends along the rostrocaudal axis of EC, as seen in coronal section. DIE borders dorsally with PER and laterally with VIE, where it forms a narrower and very closely organized layer II.



A customized-rat atlas

Representative histological slide

Figure 24. Customized-rat atlas sections along the rostral-caudal axis. A) The subdivisions of the EC were divided into CE, ME, VIE, AE, DIE and DLE. The PER, POR and Apir were also marked. **B)** Comparison of the customized-rat atlas with the histology result sample (rat no. 362). The overall shape and the subdivisions of the EC of the customized-rat atlas (left) were well-fitted with the representative histological result. Note the cannula tip located in the DIE (right). Caudal entorhinal division, CE; Entorhinal cortex, EC; Medial entorhinal division, ME; Ventral intermediate entorhinal division, VIE; Amygdalo-entorhinal transitional division, AE; Dorsal intermediate entorhinal division, DIE; Dorsal lateral entorhinal division, DLE; Perirhinal cortex, PER; Postrhinal cortex, POR; Amygdalopiriform transition, Apir.

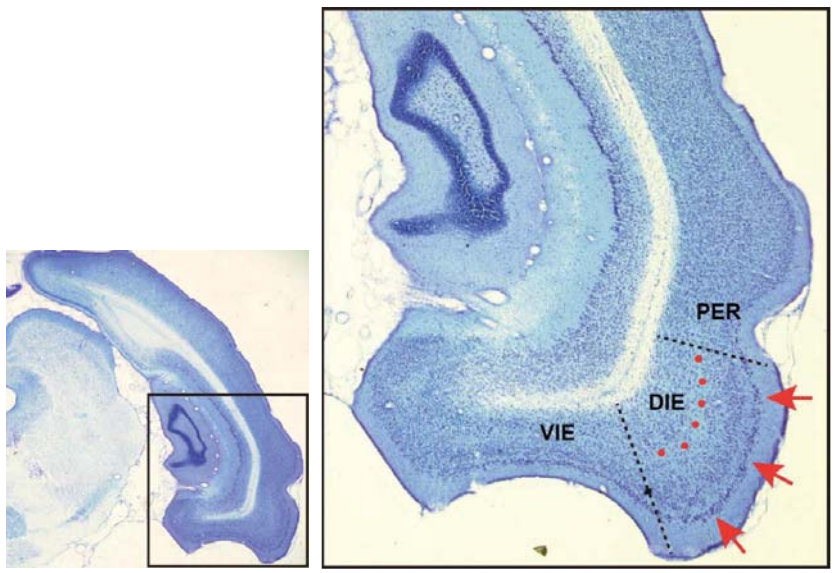


Figure 25. Distinct appearances of layer II and IV of the DIE. The photo on the left shows a representative brain section of right hemisphere of a rat at -6.40mm from bregma in coronal section. The right photo is an enlarged view of the area inside the black box from the photo on the left. Note the red arrows indicating layer II of the DIE, which consists of large and darkly stained neurons. Red dots indicate layer IV of the DIE, also known as lamina dissecans, which contains fewer neurons than the other layers. Black dashed lines divide the DIE from the PER and VIE. Dorsal intermediate entorhinal division, DIE; Perirhinal cortex, PER; Ventral intermediate entorhinal division, VIE.

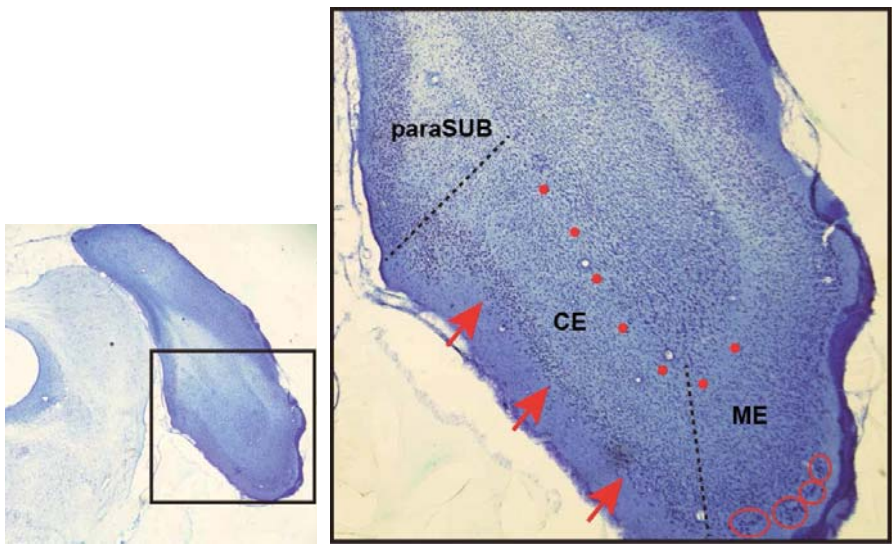


Figure 26. Characteristics of layer II and IV of the CE. The photo on the left shows a representative brain section of right hemisphere of a rat at -7.84mm from bregma in coronal section. The right photo is an enlarged view of the area inside the black box from the photo on the left. Note the red arrows indicating layer II of the CE, which consists of a continuous band of large, darkly stained neurons. Red dots indicate layer IV of the CE, which lacks neurons. Red circles on the bottom right show that neurons in layer II of ME are grouped into several islands, and they border medially with CE. Black dashed lines divide the CE from the paraSUB and ME. Caudal entorhinal division, CE; Medial entorhinal division, ME; Parasubiculum, paraSUB.

Verification of cannulae location

The locations of the cannula tracks were verified using photomicrographs (**Figure 27**). After completing the histological process, I marked the canuula tip positions for all subjects on the customized-rat atlas to verify whether the cannulae were accurately placed in the targeted brain areas. As described above in the methods sessions, the tip of the dummy cannula protruded 1mm from the tip of the guide cannula, and thus the location of each dummy cannula tip was marked on the customized-rat atlas (**Figure 28**). In the SSC task group, the black circles or dots represent the position of each tip on the lateral and medial divisions of the EC, respectively. In the SNSC task group, blue dots represent cannula tips located on more superficial layers of the LEC, while red dots represent tips located on deeper layers of the LEC. Black dots show tip positions for the MEC. In marking tip positions for all subjects on the customized rat atlas, I found that all tips targeting the LEC were located on the DIE, whereas those targeting the MEC were located on the CE.

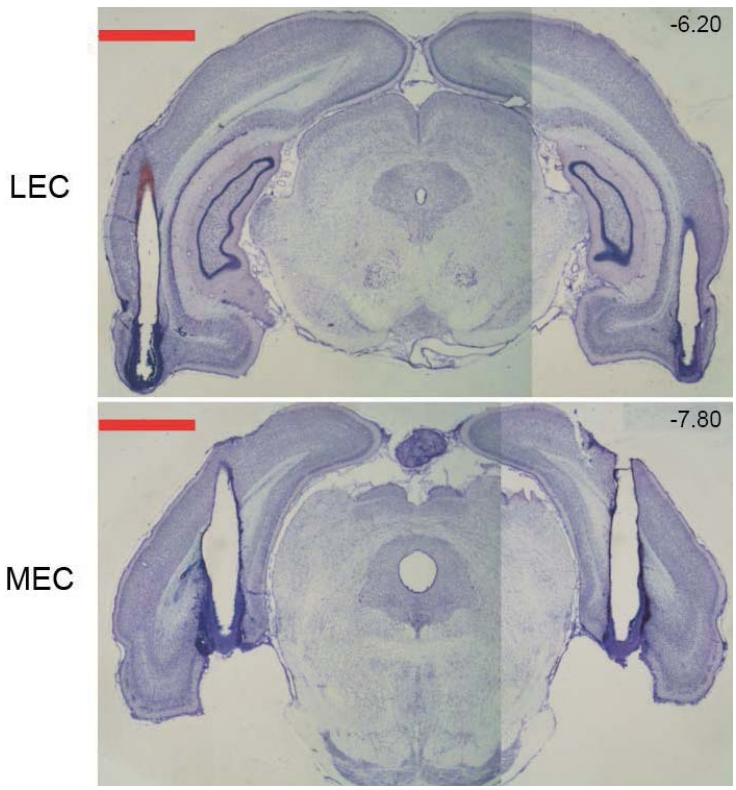


Figure 27. Representative photomicrographs showing cannula tracks. These photos show the representative photomicrographs of the brain section of the LEC and MEC, respectively, in coronal section. Note the cannula tracks bilaterally targeting the LEC and MEC. The red bars in the upper left corner represent 2mm. The numbers in the upper right corner indicate the distance from bregma (mm). Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC.

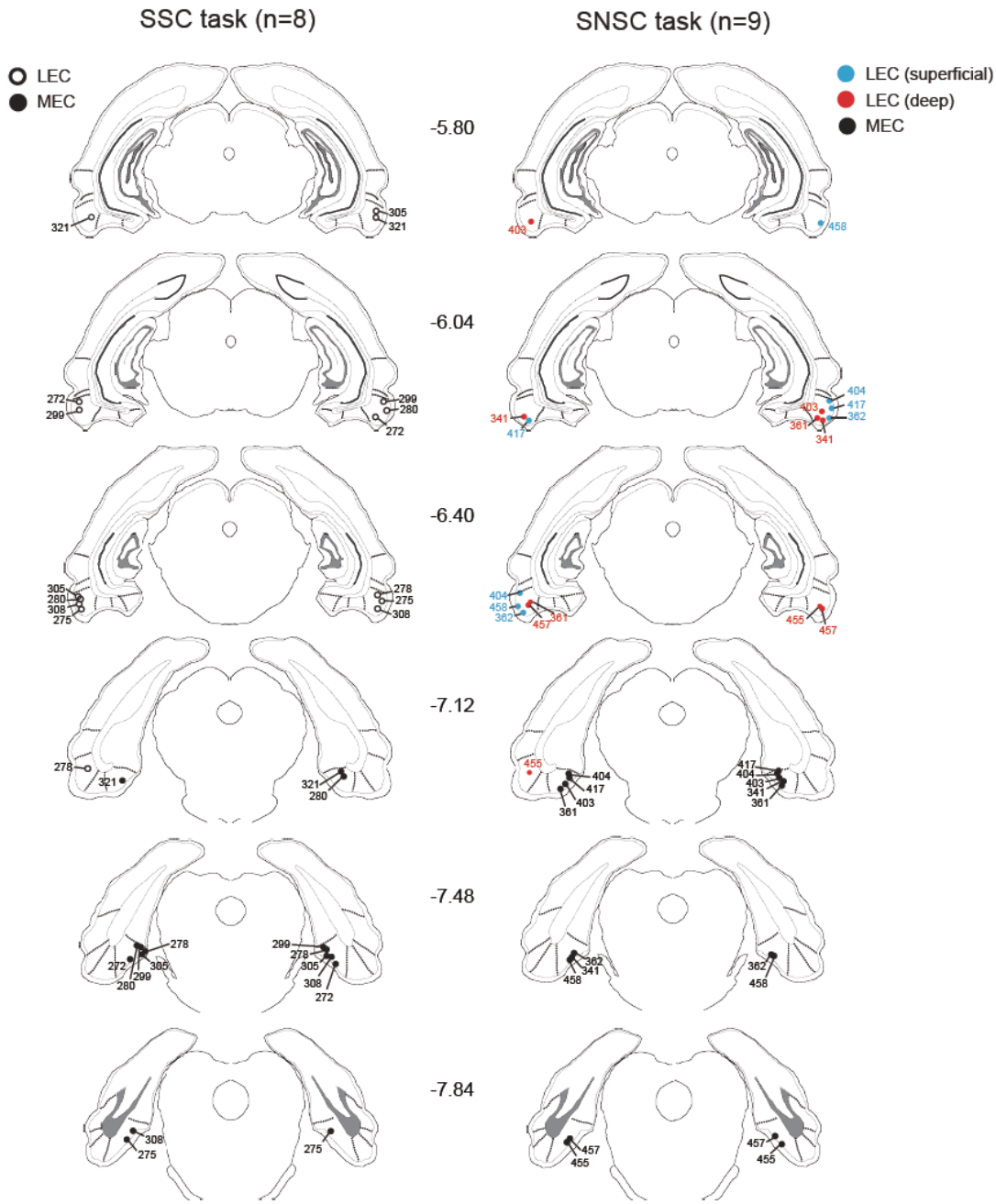

SSC task (n=8)
SNSC task (n=9)
LEC
MEC
LEC (superficial)
LEC (deep)
MEC
-5.80
-6.04
-6.40
-7.12
-7.48
-7.84

Figure 28. Verification of cannula tips for all rats involved in the SSC and SNSC tasks. Cannula tip positions for the SSC (left panel) and SNSC (right panel) task groups were marked on the regions where the cannula tips were placed. Furthermore, the cannula tips targeting the LEC in the SNSC task group were subdivided according to the tip positions located on more superficial (blue circles) or deeper (red circles) layers of the EC. The numbers in between the atlases of the two groups indicate the distance from bregma (mm). Entorhinal cortex, EC; Lateral entorhinal cortex, LEC; Scene-based non-spatial choice task, SNSC; Scene-based spatial choice task, SSC.

General discussion

As one of the major upstream structures of the hippocampus, the anatomical (Witter et al., 1989; Witter and Amaral, 1991; Witter et al., 2000a; Kerr et al., 2007; Agster and Burwell, 2013) and physiological characteristics of the EC (Hafting et al., 2005; Hargreaves et al., 2005; Knierim et al., 2006; Henriksen et al., 2010; Yoganarasimha et al., 2011; Ito and Schuman, 2012; Tsao et al., 2013; Hales et al., 2014) have been extensively studied. Although studies describing these anatomical and physiological properties may explain indirectly the functional roles of the EC, it is difficult to accept this research as an absolute theory unless the results cannot otherwise be explained by studies manipulating behavioral outcomes. Recently, the functional roles of the subdivisions of the EC have also been described in various behavioral paradigms (Steffenach et al., 2005; Morrissey et al., 2012; Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a; Hales et al., 2014). Nevertheless, a number of obstacles remain in attempts to identify the definite functions of those subdivisions.

One of the limitations of previous behavioral experiments is a result of the permanent damage caused by injecting an exitotoxic drug or by passaging a radio-frequency current into the LEC or MEC. Because the neuronal circuits, including the damaged region, are functionally related, this may cause neural circuit plasticity that could result in a confused interpretation of the observed results (Schenk and Morris, 1985). Furthermore, the permanent damage sustained by the subjects means these studies could not include the normal brain functions of those same subjects (Parron et al., 2004; Steffenach et al., 2005; Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a; Chao et al., 2016). On the other hand, temporarily inactivating a specific brain region using an inactivating agent, such as muscimol, can preserve normal brain function during testing. An advantage of preserving normal brain function is that it makes possible the comparison of normal brain function and inactivation mode of the same region within the same subjects, while lesion studies can make only the between-group comparisons.

Surprisingly, no previous studies have used reversible inactivation in discriminating the differential roles of the LEC and MEC in spatial versus non-spatial memory experiments.

Although the functional roles of the subdivisions of the EC have been described by previous studies, which characterize the LEC as playing a role in identifying an object (Hunsaker et al., 2013; Van Cauter et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a) and the MEC as processing spatial information and contextual memory (Steffenach et al., 2005; Hunsaker et al., 2013; Van Cauter et al., 2013; Hales et al., 2014), behavioral outcomes and external visual cues perceived by the subjects seem not to have been systematically controlled. Behavioral experiments using animal subjects are required to rigorously measure those subjects' behaviors with objective indicators, but some behavior measurement tools used in previous studies, such as time spent sniffing or rearing at a novel object, inevitably required subjective interpretation. Particularly in the task of observing objects freely, it is unclear whether or not a behavior is a response caused by the provided stimulus, and this may lead to a confused interpretation of the results of the previous studies. Testing animal subjects in tasks using consistent, simple visual cues which require different motor responses is the current study's method of solving this problem (i.e. specific spatial and non-spatial responses prompted by the same sets of background visual scenes). Looking at the body of previous research, I believe the current study provides, for the first time, evidence which clearly identifies the differential functions of the subdivisions of the EC in scene-dependent memory tasks by applying the reversible inactivation technique within the same animal subjects. In previous studies, the role of the MEC is described as associating visual-based contextual information with either egocentric or path-integrative information (Parron et al., 2004; Hafting et al., 2005; Hargreaves et al., 2005; Brun et al., 2008; Van Cauter et al., 2013), and the results of the SSC task in the present study may support these findings.

The present study verified the functional role of the LEC in disambiguating object-associated responses based on visual scene-dependent information, while the LEC did not play a key role when an object by itself was cued as a stimulus. These results also support

prior literature suggesting that damage to the LEC did not cause memory impairment in tasks that required either distinguishing an object itself or determining the location of an object, but did cause a performance deficit in tasks when objects were normally recognized in association to visual contextual information (Deshmukh and Knierim, 2011; Tsao et al., 2013; Wilson et al., 2013b; Wilson et al., 2013a). With this background in mind, the present study provides evidence that scene-dependent memory is not exclusively processed in the MEC, but in the LEC as well, and that the type of motor response elicited by a scene-dependent memory task is an important variable when determining the involvement of the LEC and MEC. Additionally, experiments requiring scene-dependent memory are meaningful in that they provide opportunities for functionally dissociating the subdivisions of the EC.

Given that the hippocampus interacts with several upstream structures, it is not surprising that various cognitive processes, such as temporal processing of events (Lee and Wilson, 2002; Eichenbaum, 2014; Allen et al., 2016), value-based decision making (Lee et al., 2012; Mizumori and Tryon, 2015; Palombo et al., 2015; Schumacher et al., 2016) and pattern completion and separation (Marr, 1971; Lee et al., 2004; Leutgeb et al., 2005), occur in the hippocampus. Furthermore, the functional role of the hippocampus in selecting specific behaviors based on scene-dependent information has also been identified in previous studies using rats (Kim et al., 2012; Delcasso et al., 2014; Lee et al., 2014). Considering that the superficial layer of the EC not only provides direct input to the hippocampus, but also receives back-projections from it (Witter et al., 1989; Witter and Amaral, 1991; Kerr et al., 2007; Agster and Burwell, 2013), it may be possible that scene-based information formed at the level of the hippocampus is transmitted to the deep layer of the LEC and MEC as well (**Figure 29**), and this information was used to solve the SSC and SNSC tasks used in the present study. Regarding the results of those tasks, the EC may selectively receive and process information from the hippocampus, which results in a certain type of behavioral outcome.

Therefore, when it comes to theorizing how episodic memory systems work in the

medial temporal lobe, it is hypothesized that (i) memory systems, especially regarding the relationship between the hippocampus and the EC, may be organized more as working reciprocally to each other, rather than in a strict hierarchy. The results of the SNSC task show that there are significant differences in performance according to the location of cannulae implanted in the superficial or deep layer of the LEC. Considering performance deficits shown only in the latter group, this result demonstrates that the LEC not only conveys anatomical input to the hippocampus via its superficial layer, but also receives input from the hippocampus to the its deep layer, suggesting the functional role of the deep layer of the LEC in associating scene-dependent information from the hippocampus with behavioral responses. Although anatomical connections and layer-specific properties of the EC were intensively investigated in prior studies (Witter et al., 2000b; Naber et al., 2001), differential roles of the LEC according to its superficial and deep layer were rarely examined in the behavioral experiments. The present study suggests that there are distinct roles according to the superficial and deep layers of the LEC, and this assumption was proved by testing rats in several behavioral tasks.

In the present study, temporarily inactivating the LEC or MEC severely impaired performance in tasks based on scene-dependent information requiring non-spatial or spatial responses, respectively, while the same inactivation of these structures produced no performance deficit when tactile cues or objects by themselves were presented as a signal to do a task. These results demonstrate that (ii) the LEC and MEC are specialized to associate scene-dependent information, but not other types of sensory information, with specific behavioral responses to form a mnemonic memory. Although it has been known that both the PER and POR convey different amount of external sensory inputs to the LEC and MEC (Kerr et al., 2007), the present study suggests that the cortical input from the POR to the LEC might be a key to explain the functional role of the LEC in processing scene-dependent stimuli. When the MEC was inactivated by injecting MUS during the SNSC task, no performance deficit was found, suggesting that the LEC might convey scene-dependent information to the hippocampus on behalf of the MEC. This result presumably demonstrates the functional role of the LEC in processing scene-dependent

stimuli, which has not been highlighted in previous studies.

Looking at previous studies, the present study appears to be the first behavioral experiment demonstrating a functional double dissociation between the LEC and MEC in scene-dependent memory. In conclusion, while previous studies support a simple “what” versus “where” dichotomy for the roles of the LEC and MEC, respectively, the present thesis suggests a more refined framework for their functional roles. Rather than simply assigning “What” and “Where” roles to the subdivisions, the present study demonstrates the LEC’s and MEC’s respective roles as “What should I do to this object in this context?” and “Where should I go from here in this context?”. Furthermore, although the present thesis reveals the functional differences between the LEC and MEC in the behavioral paradigms, the specific functions along the superficial and deep layer of those areas should be examined more. One of the effective ways to investigate layer-specific functions of those regions might be an optogenetic technique with retro- or anterograde tracing, which has the advantage of accurately targeting the desired brain region.

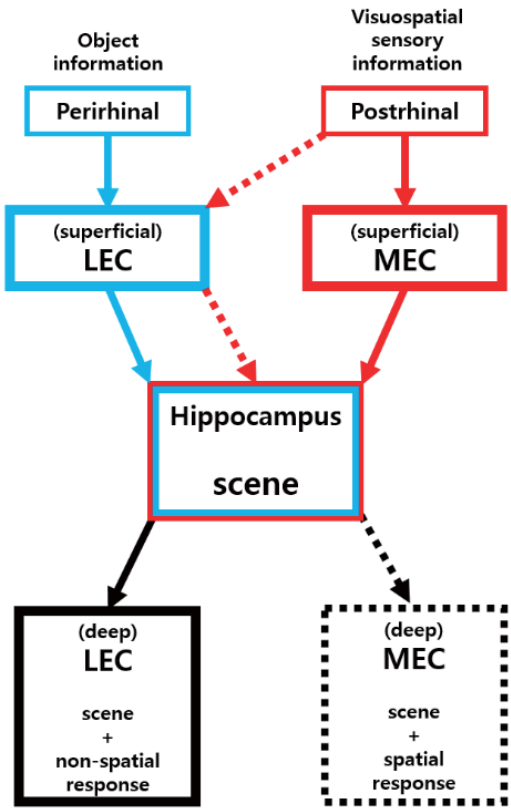


Figure 29. Schematic illustration of information processing stream of the medial temporal lobe including the superficial and deep layers of the LEC and MEC. Note the pathway from the postrhinal cortex to the LEC that may relay scene related information, and eventually, an integrative representation of scene from the hippocampus and non-spatial response might be combined in the deep layer of the LEC, which is proved by the results of the SNSC task. Although the layer-specific role of the MEC is not clearly proved from the present study, the results of the SSC task strongly demonstrate the involvement of the MEC in associating scene dependent information with egocentric spatial responses. Red and black dotted line indicate a presumable pathway. A square formed with black dotted line on the MEC deep layer indicate a hypothetical functional role of those region. Entorhinal cortex, EC; Lateral entorhinal cortex, LEC; Medial entorhinal cortex, MEC; Scene-based non-spatial choice task, SNSC; Scene-based spatial choice task, SSC.

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국문초록

장면 기억 의존적 행동 시 내측과 외측 내후각피질의 차별적 역할

유 승 우

우리는 살아가며 수많은 시각적 장면과 마주하며 특히, 익숙한 장면에 마주치게 될 경우 이 장면 자극을 떠올림으로써 어떤 행동을 결정한다. 해마를 포함한 내측두엽은 일화 기억을 형성하는데 중요한 뇌 영역이며, 선행 연구를 통해 장면 자극이 인간, 영장류, 설치류의 해마 기능을 활성화시키는 감각 정보 중 하나라고 알려졌다. 하지만, 장면 자극이 해마의 상위영역에서 처리되는 기전에 대해서는 직접적으로 연구된 사례가 없다. 이 논문은 장면 기억과제를 통해, 해마의 상위 영역인 외측, 내측 내후각피질의 차별적 역할에 대해 검증하였다. 특정 장면을 기억한 후, 이 장면 정보에 기반하여 왼쪽 혹은 오른쪽 공간을 선택하는 행동 과제를 수행 할 때, 약물 주입을 통해 내측 내후각피질이 비활성화된 쥐는 외측 내후각피질이 비활성화 된 쥐에 비해 현저하게 낮아진 과제 수행 능력을 보였다. 반면, 장면 정보에 기반하여 물체를 밀거나 혹은 물체 안에 들어있는 모래를 파는 비공간적 행동을 요구하는 과제에서는, 외측 내후각피질이 비활성화 된 쥐가

내측 내후각피질이 비활성화 된 쥐에 비해 현저하게 낮아진 과제 수행 능력을 나타내었다.

이 실험 결과는 외측 내후각피질과 내측 내후각피질은 동일한 장면 자극이 제시되더라도 서로 다른 형태의 행동적 반응을 도출하는데 특화되어 있다는 점과, 이러한 행동적 반응은 장면 기억과제에서 내측 내후각피질과 외측 내후각피질의 역할을 설명하는데 중요한 요소라는 점을 나타낸다. 더불어 기존 이론과는 달리 내측 내후각피질 뿐만 아니라 외측 내후각피질에서도 시각 장면 정보를 처리한다는 점과, 외측 내후각피질 내에서도 표면 층과 내면 층에 따라 각기 다른 역할이 있다는 점을 시사한다. 내측, 외측 내후각피질의 역할이 각각 '무엇' 혹은 '공간'에 대한 정보를 처리할 것이라고 여겨졌던 기존 이론을 넘어서서, 이 논문은 '이 맥락적 상황에서 제시된 물체에 어떤 반응을 할 것인지' 혹은 '이 맥락적 상황에서 어디로 이동을 해야 하는 것인지' 라는 개선된 이론을 제안한다.