INTEGRATION, NAVIGATION, AND ORGANIZATION: AN EXPLORATION OF TWO SPATIAL PROCESSING LOOPS USING ENVIRONMENTAL ENRICHMENT IN ADOLESCENT RATS

by

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Abstract

Spatial cognition and memory help to build understanding of an organism's environment. Understanding how one comes to perceive and navigate an environment can give insight into the order and disorder of environmental processing. In this thesis, c-FOS was used as a measure of neural activation in eight regions within two neural circuits involved with spatial processing. One neural circuit is thought to be involved with the processing of overall spatial scene, while the other is thought to be involved in processing object-location information. The two neural circuits of interest are begun with efferents from superficial medial entorhinal cortex (MEC) and superficial lateral entorhinal cortex (LEC), with MEC leading the loop processing overall spatial scene and LEC leading the loop processing object-location information. The MEC-led loop also involves proximal cornu ammonis 1 (CA1), distal subiculum, and deep MEC. The LEC-led loop involves distal CA1, proximal subiculum, and deep LEC. Environmental enrichment (EE) was used to study these loops, as EE is thought to cause activation in these regions. EE is the provision of stimulation using an environment containing toys, ramps, and platforms. Neural activation in the neural regions was manipulated through periodic EE /or a final EE session organized as a 2x2 factorial design, with four groups: No+No, EE+No, EE+EE, and No+EE (No/EE before + references periodic EE, No/EE after + references final EE). Four hypotheses were posed, with predictions of higher activation of just the No+EE group in superficial MEC, proximal CA1, distal subiculum, distal CA1, and proximal subiculum when compared to all other groups, and predictions of higher activation of the No+EE and EE+EE groups in superficial LEC, deep MEC, and deep LEC when compared to control groups (No+No and EE+No). Significantly higher neural activation was observed in the No+EE group in comparison to all other groups in superficial MEC, proximal CA1, and distal CA1 ($p = .042$, $p = .003$, and $p <$

.001, respectively). In superficial LEC, distal subiculum, proximal subiculum, deep MEC, and deep LEC, No+EE and EE+EE groups showed significantly higher activation than control groups (No+No and EE+EE) (p= .017, p = .007, p < .001, p = .002, and p = .006, respectively), with no significant difference between No+EE and EE+EE groups. The findings suggest neural processing in superficial MEC and LEC and proximal and distal CA1 that is in accordance with current research in the field. These results also suggest a more involved role of the subiculum in spatial processing and add to the growing body of evidence investigating the role of deep entorhinal cortex (EC) in providing contextual feedback to superficial layers.

Keywords: spatial representation, environmental enrichment, processing loops, c-FOS, entorhinal cortex, hippocampus, subiculum

Integration, Navigation, and Organization: An Exploration of Two Spatial Processing Loops using Environmental Enrichment in Adolescent Rats

Human perception of the environment happens in less than a moment. Every day we move through space, navigating through rooms, past doorways and up the stairs, but how does one come to understand and navigate through this space so effortlessly? Spatial navigation is underpinned by complex corticohippocampal microcircuitry (Figure 1). Regions involved in spatial processing are located in the medial temporal lobe of humans, which is a central region of the temporal lobe that sits just above the brainstem, behind the ears (Hariri, 2015). While rats do not have a defined medial temporal lobe, the regions of interest are located similarly in the rat brain. Using an animal model to explore spatial navigation and to distinguish integration within processing pathways can help us to understand how information is processed and integrated in the brain, providing insight into the nature of spatial cognition and memory. Furthermore, spatial navigation is involved in the formation of episodic memory, specifically related to space and time. Investigation into this topic can increasing understanding of dysfunction of these pathways in degenerative conditions like Alzheimer's disease (Vlček & Laczó, 2014). In this study, differences in neural activation elicited by single and/or periodic environmental enrichment (EE) sessions in two spatial processing pathways through the entorhinal cortex (EC), the subiculum, and cornu ammonis 1 (CA1) region of the hippocampus were explored using a rat model. Exploration of this topic elucidated how the brain filters spatial and nonspatial information about an environment based on whether or not there is prior experience with said environment.

Entorhinal Cortex

The EC is the link between cortical layers and the hippocampal formation. It can be broken into two distinct types of layers: superficial, which is composed of cortical Layers 2 and

3, and deep, which is composed of cortical Layer 5 (Knierim et al., 2013). The superficial layers of the EC receive incoming information about the environment, which is then transmitted to the hippocampus. The deep layers of the EC receive spatial information after it has been processed through the hippocampus and the subiculum, and send contextual information back to the superficial layers. Axons from deep layers that project to superficial layers can have both excitatory and inhibitory effects on superficial neurons (Canto et al., 2008). The feedback from deep layers is a continuous process as an animal interacts with its environment. The superficial layers are constantly providing novel information to the hippocampus, while simultaneously receiving contextual, processed information back from the deep layers (Nilssen et al., 2019).

Beyond its division into two groups of layers, the EC is divided into two distinct regions marked by differing cytoarchitecture and electrophysiological response: lateral EC (LEC) and medial EC (MEC). It was previously thought that MEC processed purely spatial information related to path integration, while LEC processed non-spatial, object-related cues from an environment. Hargreaves et al. (2005) performed single-cell recordings in MEC and LEC and found that while MEC neurons displayed high spatial specificity in firing, LEC neurons displayed little to no spatial specificity. Around the same time, a topographic neural map was found within MEC, composed of what become known as grid cells (Fyhn et al., 2004; Hafting et al., 2005). Grid cells fire in relation to an organism's current environment creating a mental map of the environment it occupies and lending to path integration and navigation. Correspondingly, head-direction cells (Sargolini et al., 2006) and boundary cells (Savelli et al., 2008) have also been found, which fire in relation to head orientation and environmental boundaries, respectively. These same cells were not found in LEC. There is ample evidence in support of LEC processing object-related cues, but mixed evidence regarding potential object-place

recognition in LEC. Wilson et al. (2013) found that LEC was required for object-context recognition and Yoganarasimha et al. (2011) found that LEC lacked spatial selectivity in both cue-deprived and cue-rich environments. In contrast, Tsao et al. (2013) found that when placed in a familiar environment, LEC neurons fired in relation to previous locations of objects, and Desmukh & Knierim (2011) and Cauter et al. (2013) found object location-related LEC firing as well. Another interesting finding was that MEC neurons incorporated allocentric information, meaning it was processed without regard to oneself, while information transmitted by LEC was egocentric, which means it was processed in regard to oneself (Wang et al., 2018; Rinaldi et al., 2020). As research progresses, more anatomical and physiological evidence is pointing towards the idea that LEC processes local cues regarding objects and their locations, while MEC processes global scene and the organism's location within its environment; however, the exact roles played and the method by which LEC processes incoming spatial information requires further exploration.

Hippocampus (CA1)

The hippocampus is the next step along the path of environmental processing. It can be divided into four regions; CA1, CA2, CA3, and dentate gyrus, with CA1 receiving the most substantial information from superficial EC and being the region of interest in this study. In correspondence with their difference in function, LEC and MEC neurons send signal to different areas of the CA1 region of the hippocampus. LEC sends efferents to the area of CA1 that is distal to CA2, and MEC sends efferents to the area of CA1 that is proximal to CA2 (Henrikson et al., 2010; Knierim et al., 2013). As a whole, the hippocampus plays a role in the formation of spatial and correspondingly episodic memory (O'Keefe, 1976; O'Keefe & Nadel, 1978). The EC contributes to this role with the information it provides regarding environment; CA1 is thought to

integrate object-context information from LEC and spatial information from MEC in order to build a cognitive map of the environment. It is home to place cells that fire in relation to an organism's location in its environment (O'Keefe & Dostrovsky, 1971). These place cells integrate information from the EC and fire with a high level of specificity regarding location. Initially, it was thought that these place cells fired only in relation to spatial information (O'Keefe & Nadel, 1978); however, it has since been determined that they fire in relation to a combination of both spatial and non-spatial cues (Knierim et al., 2006). It is still unclear, however, whether CA1 processes information only using an allocentric framework, as information relayed from MEC is allocentric, or if it also incorporates egocentric information (Suthana et al., 2009; Rinaldi et al., 2020), as studies have been unsuccessful in finding a role for CA1 in egocentric processing despite knowledge that LEC processes egocentric information (Wang et al., 2018). After processing, the proximal and distal CA1 send efferents to deep MEC and deep LEC and distal and proximal subiculum (in relation to CA1), respectively (Amaral et al., 1991). This maintained difference in afferent/efferent regions, combined with the differential information processed in LEC and MEC, contributes to the different processing pathways for spatial integration in the medial temporal lobe.

Subiculum

The subiculum is a much more mysterious region of the brain than its counterparts. The function of the subiculum is ill-defined. It is known to play a role in spatial processing, as explored in this study, but also to have implications in stress response (O'Mara, 2005). Electrophysiological recordings of subiculum neurons in a freely-moving rat show cells firing in relation to place similar to that of the hippocampus proper, but with much less specificity (O'Mara et al., 2000) and investigation of subicular firing in response to objects showed

alterations in object exploration behavior correlated to neuron firing (Anderson & O'Mara, 2004). While these findings do not mention specificity regarding distal or proximal regions, they are consistent with the involvement of the subiculum in the processing of both spatial and object-location related cues. Furthermore, the alterations in behavior are consistent with the knowledge that proximal and distal subiculum send efferents to deep LEC and deep MEC, respectively. The alterations in behavior seen can be attributed to contextual information reported back to superficial layers by the deep layers. Interestingly, Kapgal et al. (2016) suggests a lack of requirement for the subiculum in the processing of spatial information. In this study, impairments in spatial navigation and learning in rats with lesions to the subiculum were overcome through the use of a long-term rehabilitation program using EE and physical exercise. The lack of clarity regarding subicular function warrants further exploration into its role in the processing loops of spatial navigation.

Spatial Processing Loops

As touched upon in the previous sections, spatial navigation and processing in the medial temporal lobe can be broken into two feedback loops. The first loop begins with the superficial layers of MEC (Figure 2). MEC processes information regarding global scenes and an organism's location within its environment (Knierim et al., 2013). Axons from this region reach the proximal CA1 region of the hippocampus and distal subiculum (Henrikson et al., 2010). From proximal CA1, the spatial information is further processed before being sent to distal subiculum and directly to the deep layers of MEC (Amaral et al., 1991). Distal subiculum then sends efferents to deep layers of MEC as well, and deep MEC relays the processed information back to superficial MEC (Canto et al., 2008). It should be noted that proximal and distal CA1

refer to location in regard to CA2 and proximal and distal subiculum refer to location in regard to CA1.

The second loop begins with the superficial layers of LEC (Figure 3). LEC processes information regarding local cues and location of the organism in relation to objects (Knierim et al., 2013). Axons from this region travel to the distal CA1 region of the hippocampus and to proximal subiculum (Henrikson et al., 2010). From distal CA1, the spatial and nonspatial information is further processed before being sent to proximal subiculum and directly to the deep layers of LEC (Amaral et al., 1991). Proximal subiculum then sends efferents to deep layers of LEC, and deep LEC relays the processed information back to superficial LEC (Canto et al., 2008). It should again be noted that proximal and distal CA1 refer to location in regard to CA2 and proximal and distal subiculum refer to location in regard to CA1.

Rat Model

Rats are a popular model for the study of anatomy and physiology, particularly in neuroscience, because their brains have similar structure and connectivity to the human brain. This, combined with the ease of maintaining a rat colony and the freedom allowed to researchers to manipulate conditions, place electrodes, and sacrifice rats experimentally has led to extensive research regarding the rodent brain. Extensive research using the rat model has created an abundance of knowledge regarding cognition and memory in the rat. The structures examined in this study, in the medial temporal lobe, are highly conserved across rats and humans in terms of both organization and connectivity (Clark & Squire, 2013), and rats have been the model organism for many groundbreaking studies regarding spatial cognition and functionality of the EC, CA1, and subiculum (O'Keefe, 1976; Fyhn et al., 2004; Anderson & O'Mara, 2004).

The age of rats studied in experimentation should not be overlooked. Rats at postnatal day (pnd) 20-21 have the brain maturation of a 2–3-year-old human, while rats at pnd 35-49 reflect the brain maturation of a 12–18-year-old human (Semple et al., 2013). Age of experimentation should be picked to best suit the needs of a study (McCutcheon & Marinelli, 2009). As this study is investigating neural activation as an indicator of function, the study of late adolescent (pnd 49) rats is advantageous due to the malleable and formative nature of the brain during such time, while still allowing close to full structural development. The study of adolescent rats also provides increased control of potential environmental experiences and increases the likelihood that the effects seen are the result of experimental conditions and not due to other learned or experienced events.

Environmental Enrichment (EE)

EE is the provision of social or physical stimuli through the use of play, exploration, toys, etc. that differ from the home cage. EE has been used to investigate neural plasticity and learning and is a useful tool in understanding and investigating the response of the brain to novel or experienced stimuli. EE has been shown to improve learning and memory, increase dendritic branching, and influence neurogenesis (Simpson & Kelly, 2011). EE has also been shown to have impacts in the medial temporal lobe specifically. Berman et al. (1996) found increased dendritic spine density in the hippocampus following EE. Additionally, an increase in thickness of the EC has found to be associated with fitness and EE (Whiteman et al., 2016). Effects on the medial temporal lobe are not limited to physiological changes, they can be seen in functionality as well. When compared to rats housed in control housing, rats in EE housing performed better in the radial arm maze, which was designed to test both spatial working and reference memory, and in the Morris water maze, which was designed to test spatial learning and memory (Leggio

et al., 2005). EE was chosen for this study as it provides an environment in which there is a controlled global scene and varied objects and object locations. This provides an excellent paradigm for the investigation of spatial processing and navigation across experienced and novel environments.

c-FOS

The c-FOS protein is a common neural activity marker. The gene *c-fos* becomes activated in response to extracellular stimulation and is referred to as an immediate early gene (IEG) due to the rapid and transient response in activation (Chaudhuri, 1997). IEGs often encode inducible transcription factors (ITFs) that function to promote or repress gene expression. The c-FOS protein is the ITF encoded in response to the activation of the *c-fos* gene (Sagar et al., 1988). Essentially, *c-fos* expression and c-FOS protein occur in response to neural activation. It is a messenger system in place to control expression of other genes. This makes c-FOS a valuable tool in exploring activation of brain regions in response to stimulation. c-FOS has been used in the past to study neural activation in regions of the medial temporal lobe. VanElzakker et al. (2008) found that environmental novelty was correlated with increased c-FOS expression in the CA1 region of the hippocampus and in the deep layers of EC, with c-FOS expression increasing with novelty. Meanwhile, Jiménez-Díaz et al. (2006) examined c-FOS expression in the EC and subiculum in response to associative learning and Ionov et al. (2019) found expression of c-FOS in subiculum and LEC in response to antidepressants. Because of the ample evidence supporting both c-FOS as a neural activity marker and c-FOS expression in the regions of interest, c-FOS was chosen as the measure of neural activation in response to EE for this study.

Current Study

Given the lack of clarity regarding the specifics of processing and function within the two spatial processing loops of the medial temporal lobe, there was a need for research that investigates the two loops as separate entities. This study hoped to shed some light on the integration of information and the application of context in the LEC-led and MEC-led spatial cognition pathways. Specifically, it hoped to elucidate differences in activation between the loops in processing an enriching environment with or without prior experience in the environment.

A 2x2 factorial was used to create four EE paradigms, consisting of a history of EE and a final enriching experience to examine neural activation in Long Evans rats. The neural activation studied reflects brain response to the final session of EE or lack thereof experienced before death. The tissue from this study was stained to display c-FOS protein and digital microscopic images were made of superficial MEC, superficial LEC, proximal CA1, distal CA1, distal subiculum, proximal subiculum, deep MEC, and deep LEC. These images were used to take counts of active, i.e. c-FOS positive, neurons across all regions for all conditions.

In this study, the cage used for enrichment remained the same across all enriching experiences, but the objects within the cage and the location of said objects varied across sessions in order to study object related LEC processing. The regions looked at were part of one of two loops of spatial processing. The first progresses through superficial MEC to proximal CA1, then distal subiculum, then deep MEC and back to superficial MEMC. The second progresses through superficial LEC to distal CA1, then proximal subiculum, then deep LEC and back to superficial LEC.

The first hypothesis of this experiment was that superficial MEC would exhibit significantly higher neural activation in the group that received only a final enriching experience

prior to death (No+EE) when compared to all other groups (No+No, EE+No, EE+EE). Because MEC processes information regarding global scenes and an organism's location within its environment, superficial MEC should have significantly less incoming information to process regarding the environment in the group that had already experienced this overall scene in previous enrichment sessions. In contrast, it was predicted that in superficial LEC there would be significantly higher activation in groups that received a final enriching experience before death, regardless of whether they had a history of enrichment (No+EE and EE+EE), when compared to groups that did not receive a final enriching experience (No+No and EE+No), with no significant difference found between No+EE and EE+EE groups. This is because objects and their relative locations varied due to cage set up and rat play across both periodic sessions and the final enriching session, causing an abundance of incoming object-related information regardless of whether there was a history of enrichment.

Beyond the EC, the second hypothesis was that there would be significantly higher activation of only the No+EE group when compared to all other groups (No+No, EE+No, EE+EE) in proximal CA1 and in distal CA1. This is the result of the abundance of information there is to integrate in a novel environment. The hippocampus is thought to be the location within the spatial processing loops where context is applied and incoming environmental information from the superficial layers of EC is integrated with other sensory information. Groups without a history in an environment have a large amount of processing to do regarding all aspects of spatial environment, and since CA1 is thought to be the major integration center of the spatial processing loops a novel environment involves an overall higher amount of information to integrate. This will override the initial lack of difference seen between No+EE and EE+EE groups in superficial LEC and will instead show significantly higher activation in

only the No+EE group in distal CA1. The higher activation of No+EE will be maintained from superficial MEC to proximal CA1 for the same reason.

Because of evidence regarding the subiculum as more of a relay center for spatial processing than an area of integration, the third hypothesis predicted that the higher activation of the No+EE group when compared to all other groups seen in CA1 regions of both LEC-led and MEC-led loops will be maintained through both the proximal and distal subiculum. There is no evidence to suggest that any organization or contextual application occurs in this region.

The fourth and final hypothesis predicted that in both deep LEC and deep MEC there will be significantly higher activation in both No+EE and EE+EE groups when compared to controls (No+No and EE+No), with no significant difference between the two. This is because in deep layers, there is a large amount of contextual information to be relayed back to superficial layers in the EE+EE group. During the final enriching experience in the EE+EE group, deep layers are inhibiting superficial layers to prevent superficial layers from processing unnecessary information. It is the predicted higher activation in deep MEC in the EE+EE group that will cause the predicted lower activation of superficial MEC seen in this same condition. Because the information regarding objects and their locations is novel in both conditions, there is less substantial a need for inhibitory feedback from deep LEC in the EE+EE group; however, there is still need for contextual information regarding the novel environment, which causes the activation seen in deep layers. The higher activation seen in the No+EE group in deep LEC and MEC is the result of the constantly updating nature of the feedback loop. Even though there is no context to provide regarding previous experiences in this environment, there is context regarding the last second of exploration, and the second before that, etc.

This study provided insight into the way brains process information regarding space and the navigation of space, particularly in reference to whether or not one has an experience in said space. This can aid in understanding of the nature of cognition and memory and be used in the investigation of disorders that result in a decline in memory and navigation skills. It is suspected that differences will be seen across regions based on whether or not there was a history within the environment and on the location of the region in either the MEC-led or LEC-led processing loop of spatial cognition.

Materials and Methods

Experimental Design

Twenty Long-Evans rats (10 male, 10 female) were randomly assigned to a group in a 2x2 factorial design (periodic enrichment x final enriching experience) (Figure 4). Rats were housed in the College of Arts and Sciences animal care facility at Appalachian State University. Prior to and between experimentation, rats were housed in a standard shoebox cage with bedding and two other same-sex rats and experienced a 12 h light and dark cycle. During an enriching experience, rats were moved to a larger enclosure containing ramps, platforms, toys, and bedding, along with their cagemates and other rats (Figure 5). Toys included objects of a variety of shapes, colors, and sizes, including objects that hung from the ceiling of the enclosure. The enrichment enclosure remained the same across all enriching experiences, but the toys present and their locations varied across sessions. It was ensured that toys were kept separate between sexes. Half of the rats received periodic enrichment during 20, 90-min sessions between postnatal day (pnd) 22 and 48. Rats that did not receive periodic enrichment were picked up and put down twice with a 90-min increment in between on 20 days to control for handling. Rats that were to receive a final enriching experience received a single 90-min enrichment session on pnd

49. They were then moved to a solitary cage in the dark for 90-min prior to sacrifice. Rats that did not receive a final enriching experience were placed in a solitary cage in the dark for 90-min prior to sacrifice. All rats were sacrificed on pnd 49. The 90-min session of quiet and dark provided ample time for c-FOS protein synthesis, to ensure that c-*fos* gene expression was in response to the final enriching experience.

To summarize the experimental design and manipulations, the 2x2 factorial design created four groups. The first group was the pure control group, which received no periodic enrichment or final enriching experience (No+No). The second group controlled for just periodic enrichment, as it received periodic enrichment with no final enriching experience (EE+No). The third group was an experimental group, which received periodic enrichment with a final enriching experience. The fourth group was the second experimental group, which received no periodic enrichment with a final enriching experience. Experimental design was approved by the Institutional Animal Care and Use Committee at Appalachian State University (Protocols #15-02 and #19-12).

Sacrifice and Perfusion

After 90 min in the quiet and dark, rats were injected with a lethal dose of sodium pentobarbital (≥100 mg/kg b.w., ip). When corneal and tail reflexes were absent, rats were perfused intracardially with phosphate-buffered saline (PBS) until color was lost from lungs and paws. Rats were then perfused with phosphate-buffered 4% paraformaldehyde. The head was removed using a guillotine, and the brain was removed from the skull. Each rat brain was post-fixed individually in 10% sucrose-2% paraformaldehyde for one week then transferred to 10 mM phosphate buffer with a 0.02% sodium azide.

Immunohistochemistry and Staining

Prior to sectioning, rat brains were cut blocked into left and right hemispheres. Each hemisphere was then cut into 50 μm sagittal sections using a Vibratome and the sections were stored in a 24-well culture plate in 10 mM phosphate-buffer. Tissue was selected for floating section immunohistochemistry and stained for c-FOS by using a stereomicroscope to determine a range of sections containing the desired structures. On the first day of immunohistochemistry, tissue was rinsed in PBS before being incubated in a 12.5% goat serum (Vector Labs) with 0.2% Triton-X solution for 60 min. Tissue was then transferred to the primary antibody solution, anti-c-FOS (made in rabbit, 1:1000, Cell Signaling Technologies) with 0.2% Triton-X and 0.2% goat serum, for 42-45 h. On the second day of immunohistochemistry, tissue was removed from the anti-c-FOS solution and rinsed six times in PBS, at 10 min per rinse. Following the rinses, tissue was transferred to secondary antibody solution, biotinylated goat anti-rabbit (1:300, Vector Labs) and incubated for 60 min. Tissue was removed from secondary antibody following incubation and rinsed three times for 10 min in PBS prior to a 60-minute incubation in an Avidin-Biotin Complex (ABC, Vector Labs). Tissue then underwent two more 10 min rinses in PBS before the addition of VIP enzyme substrate (Vector Labs), which was allowed to react with sections for a minimum of 2 minutes. Following the reactions, tissue sections were transferred to distilled water for a minimum of 15 min before being mounted onto gel-coated slides, air dried, dehydrated, cleared, and cover slipped.

Alternate sections were mounted onto gel-coated slides and air dried. These sections were then dehydrated in graded ethanols, rehydrated, stained with thionin, differentiated, dehydrated, cleared with toluene, and coverslipped. The alternate sections provided examples of the brain's cytoarchitecture through sections containing structures of interest in this thesis.

Microscopy

Digital images were made of structures of interest (superficial LEC, superficial MEC, distal CA1, proximal CA1, distal subiculum, proximal subiculum, deep LEC, and deep MEC) using a Nikon Eclipse microscope with a Plan 10 objective and a 1.3-megapixel Firewire camera (Figures 6-11). Digital images were opened in Adobe photoshop and a scaled 200 x 200 μm grid was overlayed. The counting tool was used to label activated neurons in each section of interest (Figure 12). A counting protocol was used in which only neurons that reached a certain pigmentation with discrete, not blurred, edges were counted. Three 200 x 200 μm boxes were counted from each structure. Boxes were chosen in order to capture random, yet representative sampling, in which boxes were chosen randomly from the image, but it was ensured that the boxes chosen were not an outlier when compared to the general activation level seen across the image. It was ensured that neurons appearing on the bottom and left borders were not counted, to avoid double counting. Each count was performed by two separate observers and an average was taken for use in statistical analyses. Neural activation was compared across groups in each structure using a two-way ANOVA and an eta squared value was calculated as a measure of effect size.

Results

Superficial Entorhinal Cortex

The first hypothesis regarding activation in superficial MEC and superficial LEC was supported. In superficial MEC, there was significantly higher activation in the No+EE group when compared to all other groups (EE+EE, EE+No, and No+No) ($F(1, 16) = 4.90$, $p = .042$) (Figure 13; Table 1). This interaction effect was moderate size with $\eta^2 = .08$. While not hypothesized, the main effect of last EE experience was also statistically significant with higher activation in both the EE+EE and No+EE groups when compared to control groups (EE+No and

No+No) ($F(1, 16) = 26.0$, $p < .001$, $\eta^2 = .45$) In superficial LEC, there was significantly higher activation in No+EE and EE+EE groups when compared to control groups (No+EE and EE+No) ($F(1, 16) = 7.11$, $p = .017$) (Figure 14; Table 2), which supported the first hypothesis. The main effect was large ($\eta^2 = .28$) with no significant difference in activation between No+EE and EE+EE groups.

CA1

The second hypothesis regarding activation in distal CA1 and proximal CA1 was supported. In proximal CA1, there was significantly higher activation in the No+EE group when compared to all other groups (EE+EE, EE+No, and No+No) ($F(1, 16) = 12.0$, $p = .003$, $\eta^2 = .18$) (Figure 13; Table 3). Similar to superficial MEC, the main effect of last EE experience was also statistically significant with higher activation in both the No+EE and EE+EE groups in proximal CA1 when compared to control groups (EE+No, and No+No) ($F(1, 16) = 31.4$, $p < .001$, $\eta^2 = .45$). In distal CA1, there was significantly higher activation in the No+EE group when compared to all other groups (EE+EE, EE+No, and No+No) ($F(1, 16) = 29.8$, $p < .001$, $\eta^2 = .22$) (Figure 14; Table 4), which support the second hypothesis. Like proximal CA1, there was also significantly higher activation in the No+EE and EE+EE groups together in distal CA1 when compared to control groups (EE+No and No+No) ($F(1, 16) = 58.3$, $p < .001$, $\eta^2 = .44$).

Subiculum

The third hypothesis regarding activation in proximal subiculum and distal subiculum was not supported. In distal subiculum, there was also significantly higher activation in both the No+EE and EE+EE groups when compared to controls (EE+No, and No+No) ($F(1, 16) = 9.58$, $p = .007$) (Figure 13; Table 5), which did not support the third hypothesis. The main effect was large ($\eta^2 = .34$) with no significant difference in activation between No+EE and EE+EE groups.

In proximal subiculum, there was significantly higher activation in both the No+EE and EE+EE groups when compared to control groups (No+EE and EE+No) ($F(1, 16) = 43.1$, $p < .001$) (Figure 14; Table 6), which did not support the third hypothesis. The main effect was large ($\eta^2 = .64$) with no significant difference in activation between No+EE and EE+EE groups.

Deep Entorhinal Cortex

The fourth hypothesis regarding activation in deep LEC and deep MEC was supported. In deep MEC, there was significantly higher activation in both the No+EE and EE+EE groups when compared to control groups (EE+No and No+No) ($F(1, 16) = 13.3$, $p = .002$) (Figure 13; Table 7), which supported the fourth hypothesis. The main effect was large ($\eta^2 = .45$) with no significant difference in activation between No+EE and EE+EE groups. Unlike in any other region, activation of EE+EE in deep MEC was actually higher than activation of No+EE, although the difference was not significant (EE+EE $z = 1.5$; No+EE $z = 1.3$) In deep LEC, there was also significantly higher activation in both the No+EE and EE+EE groups when compared to control groups (EE+No and No+No) ($F(1, 16) = 10.3$, $p = .006$) (Figure 14; Table 8), which supported the fourth hypothesis. The main effect was large ($\eta^2 = .28$) with no significant difference in activation between No+EE and EE+EE groups.

Discussion

This study explored brain response to a final EE based on whether there was or was not a history of experience in EE. The objective of this study was to examine the regions of the medial temporal lobe involved in two processing loops of spatial navigation to help understand the functions performed both by individual regions and by each loop as a whole. This information can give insight into the nature of spatial cognition and memory and aid understanding of disorders that exhibit spatial deficits, like Alzheimer's disease (Vlček & Laczó, 2014).

Superficial MEC and LEC

The first hypothesis of this study was in relation to activation in superficial layers of LEC and MEC. It predicted that there would be significantly higher activation of the No+EE group when compared to all other groups in MEC and there would be significantly higher activation of both No+EE and EE+EE groups when compared to control groups in LEC, with no significant difference between the two. This prediction was made on the basis that if MEC processes overall environment, it would see less activation in the EE+EE group as the overall environment had already been processed during previously enriching experiences (e.g. Knierim et al., 2013). In LEC, if it is true that LEC processes information in relation to objects and their locations, higher activation would be seen in both No+EE and EE+EE groups, as object and their locations vary across sessions and during play (e.g. Desmukh & Knierim, 2011; Cauter et al., 2013; Tsao et al., 2013; Knierim et al., 2013). The results of this study supported this hypothesis. There was significantly higher activation in the No+EE group in superficial MEC when compared to all other groups (p=0.042) and significantly higher activation in both No+EE and EE+EE groups in superficial LEC when compared to controls (p=0.017), with no significant difference between the two. This in turn supports the notion that LEC and MEC are processing different types of incoming information related to space based on whether or not there is experience in an environment. Further, these results support the conclusion made by Knierim et al. (2013) that MEC processes spatial information regarding the overall spatial scene, while LEC processes both nonspatial and spatial information regarding objects in an environment and the organism's location in reference to them. Additionally, these findings support the conclusions by Tsao et al. (2013), Desmukh & Knierim (2011), and Cauter et al. (2013) that LEC is not just processing non-spatial information, but spatial information as well. If LEC was processing only object

presence in this experiment, there would likely be significantly diminished activation in the EE+EE group when compared to the No+EE group as object novelty would be diminished. Correspondingly, if MEC was processing information related to object location, it would likely not have the seen result of significantly diminished activation in the EE+EE group when compared to the No+EE group.

Proximal and Distal CA1

The second hypothesis of this experiment predicted that there would be significantly higher activation of both proximal CA1, which receives afferents from superficial MEC, and distal CA1, which receives afferents from superficial LEC, in the No+EE group when compared to all other groups. This hypothesis was made knowing that CA1 is thought to integrate spatial and nonspatial information coming from different sensory pathways in order to create an episodic memory about an environment (Knierim et al., 2006). The results showed significantly higher activation in the No+EE group when compared to all other groups in both proximal (p=0.003) and distal (p<0.001) CA1, which supported the hypothesis. While it is commonly accepted that CA1 integrates allocentric information regarding space (Suthana et al., 2009), it is unclear what role CA1 plays in egocentric integration. Wang et al. (2018) found that superficial LEC processes information primarily with egocentric bearing. Distal CA1 is the primary recipient of afferents from superficial LEC, so it was thought that this egocentric information must be relayed in some fashion to distal CA1. Conflictingly, Rinaldi et al. (2020) found no significant activation in CA1 in egocentrically trained mice. The findings of this study support the notion that CA1 encodes allocentric information, but provide limited information regarding CA1 processing of egocentric information. It was initially thought that the significantly higher activation in No+EE groups in both proximal and distal CA1 would be the general result of mass

novelty, but it is also possible that this result is a reflection of mass novelty stemming specifically from increased novel allocentric information coming from superficial MEC (as significantly higher activation was seen in No+EE in this region). In a study investigating response of proximal and distal CA1 with conflicting global and local cues, Desmukh (2020) found that there was not a difference in spatial selectivity between proximal and distal CA1, with distal CA1 showing spatial selectivity in situations where LEC was not. The present study supports these findings with the significantly higher activation of just the No+EE group in both distal and proximal CA1 and with the different activation pattern seen between LEC and distal CA1 (distal CA1 did not show the same lack of difference between No+EE and EE+EE groups that was seen in LEC). This shows a disconnect between LEC and distal CA1. Wang et al. (2018) suggested that LEC receives egocentric information and processes them to be incorporated into the allocentric framework used in the hippocampus. If this is true, it is possible that the disconnect between LEC and distal CA1 in this study is the result of LEC processing egocentric information into an allocentric framework for distal CA1. This could also account for disconnect and lack of spatial selectivity difference seen by Desmukh (2020).

Distal and Proximal Subiculum

The third hypothesis of this study predicted that there would be significantly higher activation in the No+EE group when compared to all other groups in distal and proximal subiculum. Interestingly, the hypothesis was not supported for distal or proximal subiculum. In both regions, the No+EE and EE+EE groups were significantly higher than control groups (distal: p=0.007, proximal: p<0.001), with no significant difference between the two. This indicates that having previous experience in an environment causes activation in regions of the subiculum involved in both MEC-led and LEC-led spatial processing loops that is comparable to

the activation seen when in a novel environment. Further, this differs from the trend seen in proximal and distal CA1, which suggests that some level of processing and integration is occurring in the subiculum. This is inconsistent with previous findings that struggle to find a role for the subiculum, and instead pose that it is likely a relay center for spatial processing (Kapgal et al., 2016; O'Mara, 2005). Instead, it is in line with the suggestion by Matsumoto et al. (2019) that the subiculum is modifying information coming from upstream targets, to then be organized and sent to specific regions downstream. The findings in the current study show subicular activation that mirrors that of deep layers of the EC. Distal and proximal subiculum receive afferents directly from superficial MEC and LEC, respectively, along with afferents from proximal and distal CA1, respectively (Amaral et al., 1991; Henrikson et al., 2010). It is possible, given this knowledge and the findings of this study, that the subiculum reintegrates information from superficial layers that was reorganized or directed elsewhere when going to or in CA1 (for example, egocentric information from LEC) so that deep EC can then send relevant information back to superficial layers.

Deep MEC and LEC

The fourth hypothesis of this study predicted that deep layers of both MEC and LEC would have significantly higher activation in No+EE and EE+EE groups when compared to control groups, with no significant difference between the two. This hypothesis was supported for both regions (deep MEC: $p = .002$; deep LEC: $p < .001$). While the function of deep EC layers is poorly understood, the supposed function of deep layers of EC is to provide contextual information back to superficial layers to either inhibit or excite them (Nilssen et al., 2019) as well as relay processed information back to various regions of the cerebral cortex (Knierim et al., 2013). If this hypothesized function is correct, the need to relay contextual inhibitory information

to superficial layers in EE+EE groups likely causes the activation of EE+EE that is comparable to that of No+EE in this region. The high activation of the No+EE group, in turn, is the result of the need to send excitatory efferents to superficial layers, to encourage exploration of a novel environment in which new information is constantly needed (e.g. Canto et al., 2008; Nilssen et al., 2019). Although the finding was not significant, it is possible that the higher activation of EE+EE than that of No+EE in deep MEC is a reflection of the particular need for inhibition of superficial layers in this pathway, due to repetition of global cues from previous enriching experiences. As discussed in the presentation of this hypothesis, it is likely the inhibition from deep MEC in the EE+EE group that caused the lesser activation seen in the EE+EE group in superficial MEC.

Future Directions

While the findings of this study posed some interesting questions, there is significant need for further investigation into each of these regions. In the superficial EC, different function and efferent specificity has been found between layer 2 and layer 3 (Nilssen et al., 2019). It would be beneficial to conduct a study in which activation of layers 2 and 3 is looked at individually in order to parse out differential activation in these regions. Secondly, it would be beneficial to perform a study in which the presence or position of objects in the enriching environment is manipulated. This would help to investigate the role of superficial LEC in processing objects and their locations (e.g. Desmukh & Knierim, 2011; Cauter et al., 2013; Tsao et al., 2013). Correspondingly, it would be beneficial to perform a study in which the overall scene differs between periodic EE and the final EE. This would provide greater insight into the role of MEC in processing global cues and help to verify if the findings of this study are actually

the result of the maintained global scene between periodic EE and the final EE (e.g. Fyhn et al., 2004; Hafting et al., 2005; Knierim et al., 2013).

In proximal and distal CA1, there is a need for further investigation in the form of both lesion-based behavioral tasks and electrophysiological studies to examine processing differences between these two regions (e.g. Desmukh 2020). While this study provided support for some previous findings (Wang et al., 2018; Desmukh, 2020), the findings were too vague to extend beyond speculation. Further research should focus on the type of information coming to distal CA1 from LEC, along using behavioral studies like that of Rinaldi et al. (2020) to better determine whether egocentric information is processed in this region. It would also be a worthy endeavor to provide a similar experiment to this one, but to include CA3 and dentate gyrus, as these have connections with both superficial EC and CA1 regions (Knierim et al., 2013).

The subiculum is the most mysterious region of the medial temporal lobe and was previously looked over in favor of studying the hippocampus. The findings of this study and other recent reviews (e.g. Matsumoto et al., 2019) provide intriguing evidence for future exploration. This study provided support for a more involved role of the subiculum in spatial processing. The findings, however, were again too vague to extend beyond speculation. Future research should first focus on lesion-based behavioral tasks that target neurotransmitters or receptors relating to subicular neurons to parse out possible functions of the subiculum (e.g. Kapgal et al., 2016). Research could then move into electrophysiological studies regarding neural connectivity and firing in this region (e.g. O'Keefe & Dostrovsky, 1971; Fyhn et al., 2004). Future research is also needed to determine the role of efferents from superficial layers to the subiculum (e.g. Amaral et al., 1991; Henrikson et al., 2010) and to parse out how distinct distal and proximal subiculum really are from each other.

Finally, there is an abundance of knowledge regarding connectivity of deep EC, but much less known regarding function. The results of this study are in accordance with previous findings that suggest that deep layers provide feedback to superficial layers (e.g. Canto et al., 2008; Nilssen et al., 2019). There is a need for future research to focus on lesion studies examining behavioral and activation alterations in rats, specifically regarding deep layers of EC. These findings could not only provide information regarding the role of deep EC, but could help to uncover the type of information processed and relayed by the subiculum.

Conclusion

This study examined two processing loops in the medial temporal lobe, one led by superficial MEC and thought to process information regarding global scene and path navigation and the other led by superficial LEC and thought to process information regarding objects and their locations (Amaral et al., 1991; Canto et al., 2008; Henrikson et al., 2010; Knierim et al., 2013). These loops were explored using EE and c-FOS expression in order to determine how neural activation differed in each region based on whether there was or was not a history in the environment. This study found support for superficial MEC processing global scenes and superficial LEC processing local cues (e.g. Knierim et al., 2013). There was also evidence that suggests that LEC may process egocentric information into an allocentric framework before providing it to distal CA1 and that proximal and distal CA1 integrate information to create episodic memory (e.g. Wang et al., 2018; Rinaldi et al., 2020; Desmukh, 2020). Furthermore, this study found evidence for a more involved role of the subiculum in modifying information to be relayed to deep EC and supported the supposed function of deep EC in providing contextual excitatory or inhibitory information to superficial layers (Canto et al., 2008; Nilssen et al., 2019). Despite the intriguing findings of this study, no significant conclusions can be made without

further research in the form of environmental manipulations, lesion studies, and electrophysiological data. Once these findings have been fleshed out, research into this region could help us to better understand the nature of spatial cognition in humans and how this cognition becomes disordered. This study followed through the two spatial processing loops of the medial temporal lobe as individual entities using a history vs. no history approach and provided results that pose some intriguing and novel questions into the roles of EC, CA1, and subiculum in spatial navigation and processing.

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Table 1

Neural activation in superficial MEC

	M*z*	**SD**	**n**
No+No	0.0	0.9	5
EE+No	-0.5	0.4	5
EE+EE	0.8	0.6	5
No+EE	3.5	1.5	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 2

Neural activation in superficial LEC

	M*z*	**SD**	**n**
No+No	0.0	0.9	5
EE+No	-0.8	0.6	5
EE+EE	0.7	0.8	5
No+EE	0.5	0.6	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 3

Neural activation in proximal CA1

	M*z*	**SD**	**n**
No+No	0.0	1.0	5
EE+No	0.3	0.9	5
EE+EE	1.6	0.5	5
No+EE	6.2	2.3	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 4

Neural activation in distal CA1

	M*z*	**SD**	**n**
No+No	0.0	1.0	5
EE+No	0.1	0.6	5
EE+EE	1.1	0.5	5
No+EE	6.0	1.3	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 5

Neural activation in distal subiculum

	M*z*	**SD**	**n**
No+No	0.0	0.8	5
EE+No	0.8	3.0	5
EE+EE	2.7	2.4	5
No+EE	4.8	1.9	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 6

Neural activation in proximal subiculum

	M*z*	**SD**	**n**
No+No	0.0	0.8	5
EE+No	0.0	1.0	5
EE+EE	1.9	1.0	5
No+EE	4.6	1.4	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 7

Neural activation in deep MEC

	M*z*	**SD**	**n**
No+No	0.0	0.9	5
EE+No	-0.4	0.9	5
EE+EE	1.5	0.7	5
No+EE	1.3	1.1	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Table 8

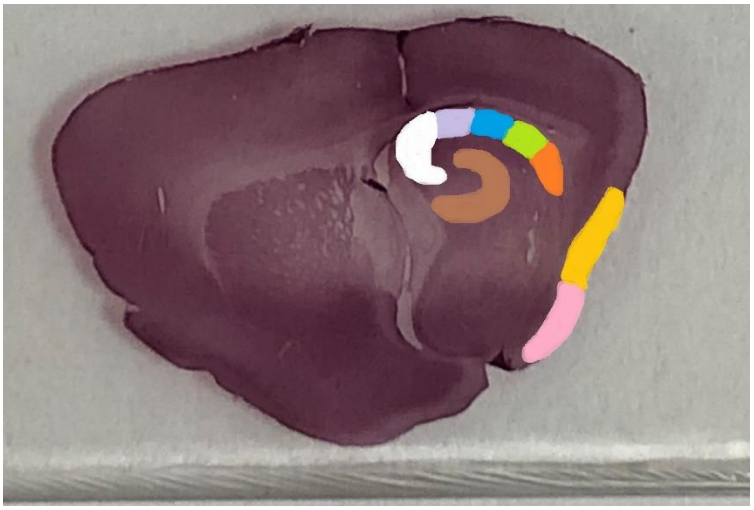
Neural activation in deep LEC

	M*z*	**SD**	**n**
No+No	0.0	0.9	5
EE+No	-1.1	0.4	5
EE+EE	0.0	0.5	5
No+EE	1.1	0.8	5

Note. The z-scores in this table are standardized against the baseline No+No group.

Figure 1

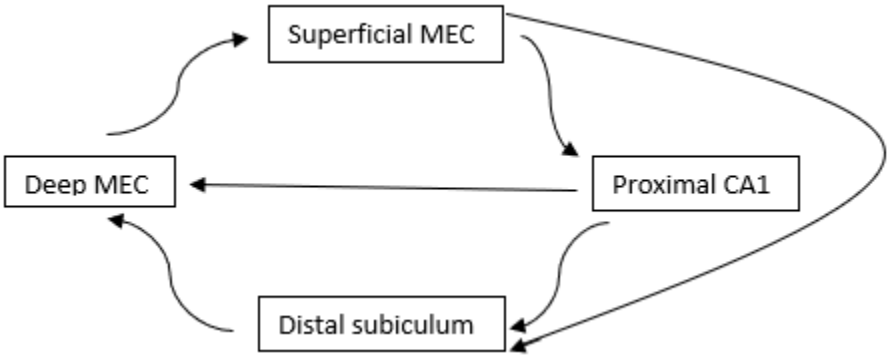
Anatomical connectivity of corticohippocampal regions



Note. This figure displays anatomical connectivity of corticohippocampal regions on a sagittal section of a rat brain. LEC is in pink, MEC is in gold, distal subiculum is in orange, proximal subiculum is in green, distal CA1 is in blue, proximal CA1 is in purple, CA2 and CA3 are in white, and dentate gyrus is in brown.

Figure 2

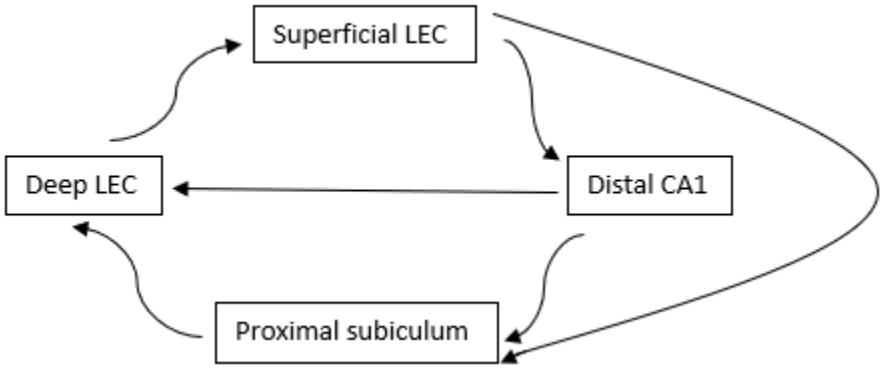
Spatial processing loop of MEC.



Note. This figure displays connectivity in the MEC-led loop of spatial processing in the medial temporal lobe. This graphic is simplified, as there are other parts of the brain not included in this study that connect with the regions shown.

Figure 3

Spatial processing loop of LEC



Note. This figure displays connectivity in the LEC-led loop of spatial processing in the medial temporal lobe. This graphic is simplified, as there are other parts of the brain not included in this study that connect with the regions shown.

Figure 4

Layout of the 2x2 factorial experimental design.

Final Enriching Experience	Periodic Enrichment: No	Periodic Enrichment: Yes
No	No+No	EE+No
Yes	No+EE	EE+EE

Figure 5

Example set up of an environmental enrichment cage.

Note. This figure displays a sample set up of one female environmental enrichment cage. As seen, there are a variety of toys, ramps, and shelves for rats to play with.

Figure 6

Digital image of activated neurons in superficial (B) and deep (A) MEC.

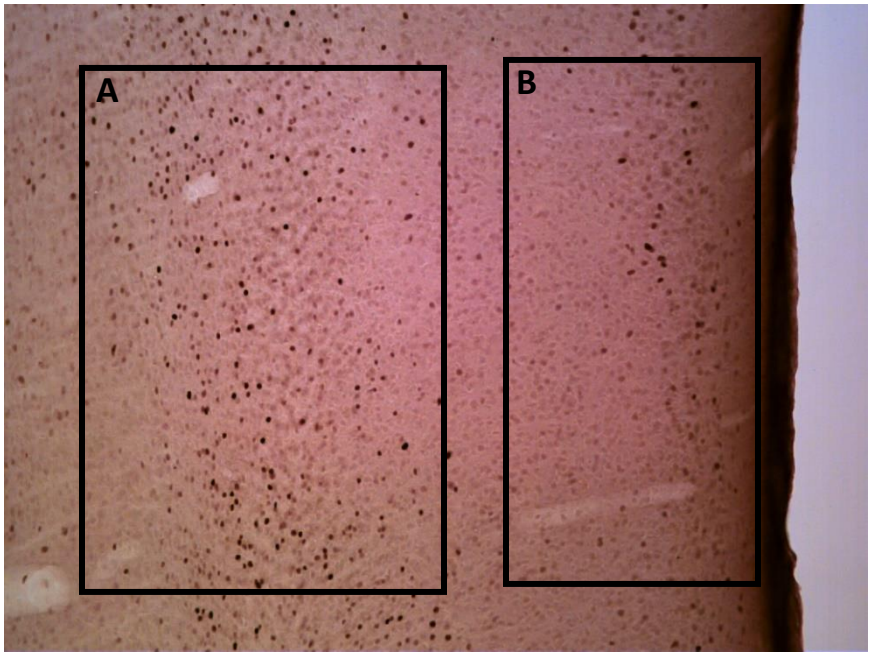


Figure 7

Digital image of activated neurons in deep (A) and superficial (B) LEC.

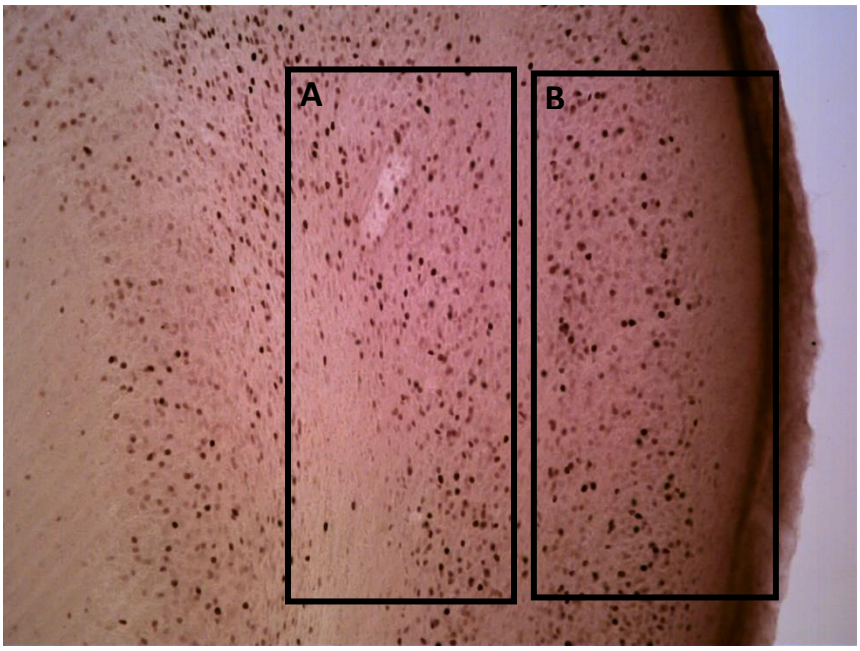


Figure 8

Digital image of activated neurons in proximal CA1.

Figure 9

Digital image of activated neurons in distal CA1.

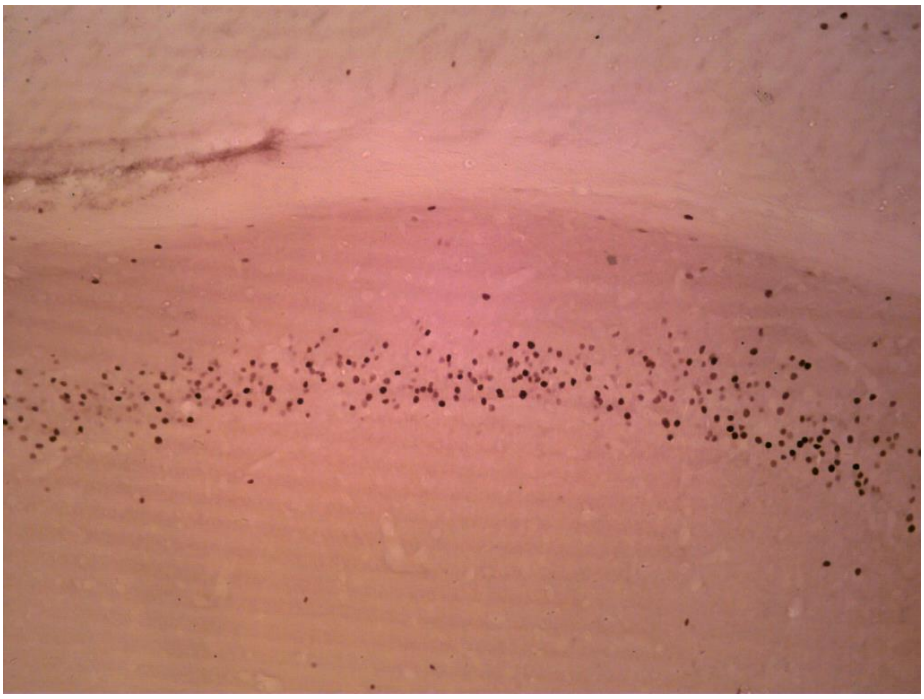


Figure 10

Digital image of activated neurons in distal subiculum.

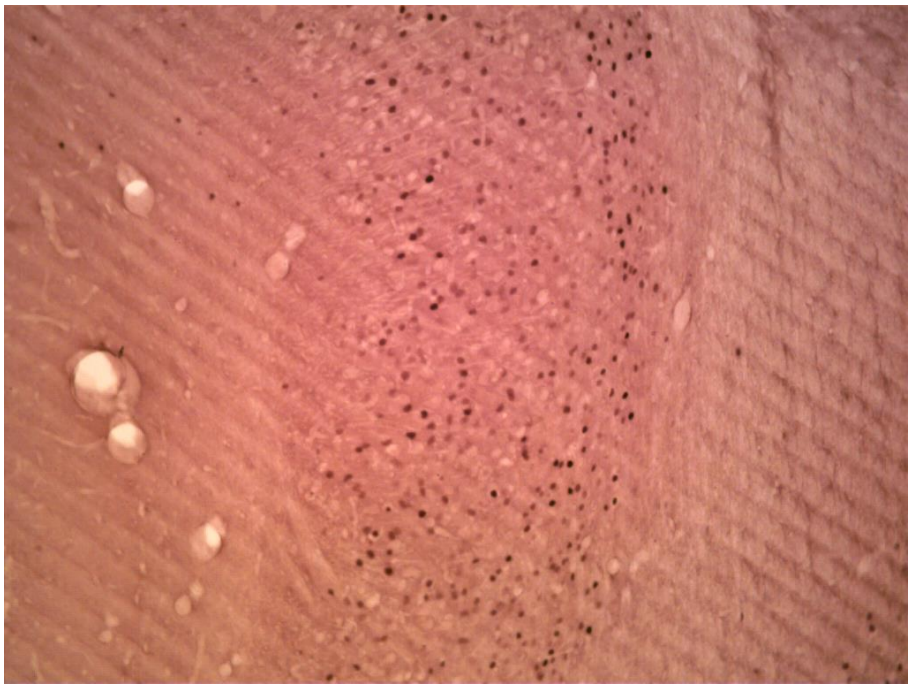


Figure 11

Digital image of activated neurons in proximal subiculum.

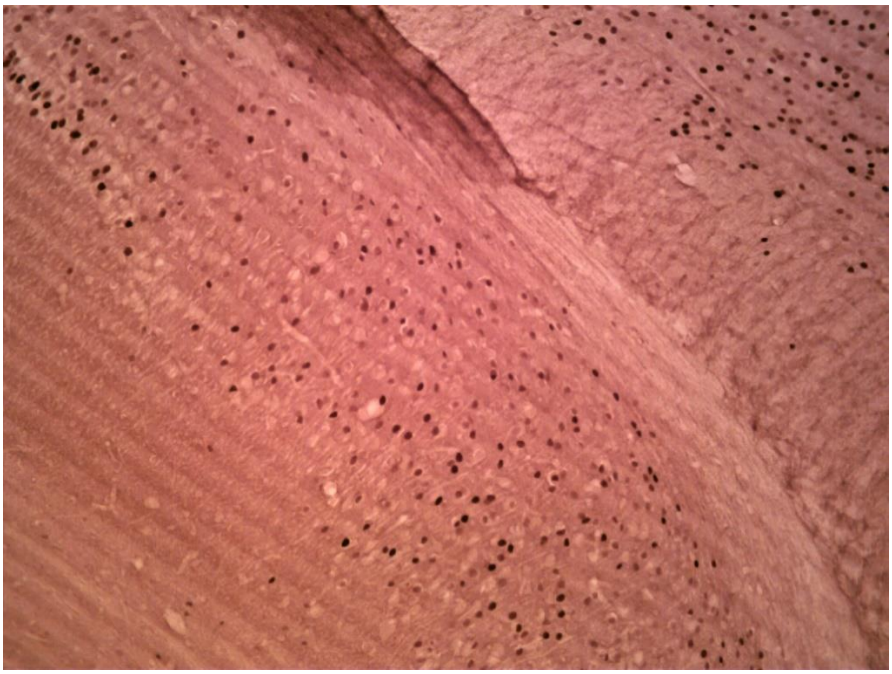
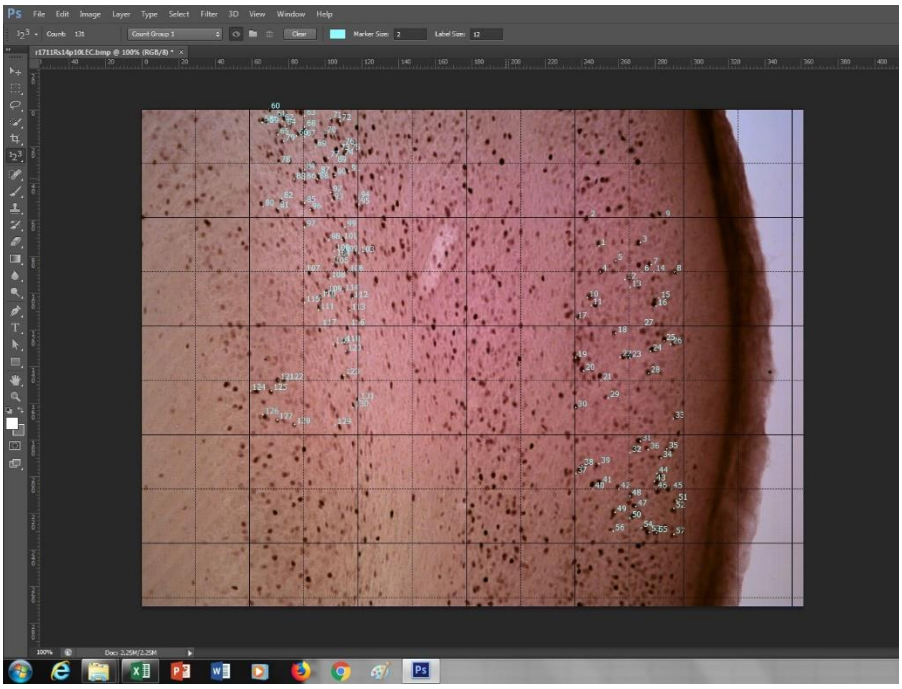


Figure 12

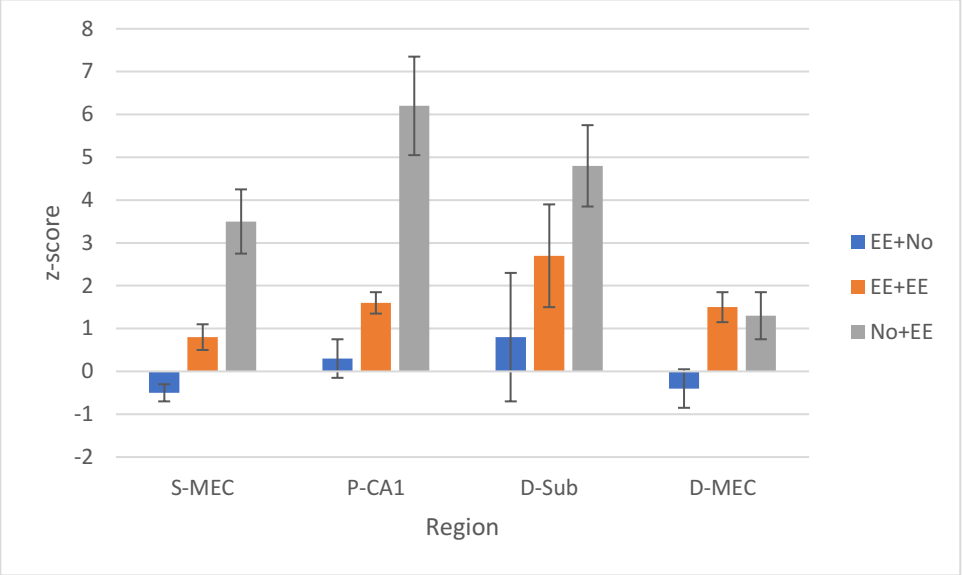
Example image of neural counts in superficial and deep LEC.



Note. This figure displays a sample image of neural counting procedure in superficial and deep LEC. The left half of the screen shows deep LEC and the right half shows superficial LEC. As shown, three 2x2 boxes were counted, with care taken to count neurons with defined edges and pigmentation.

Figure 13

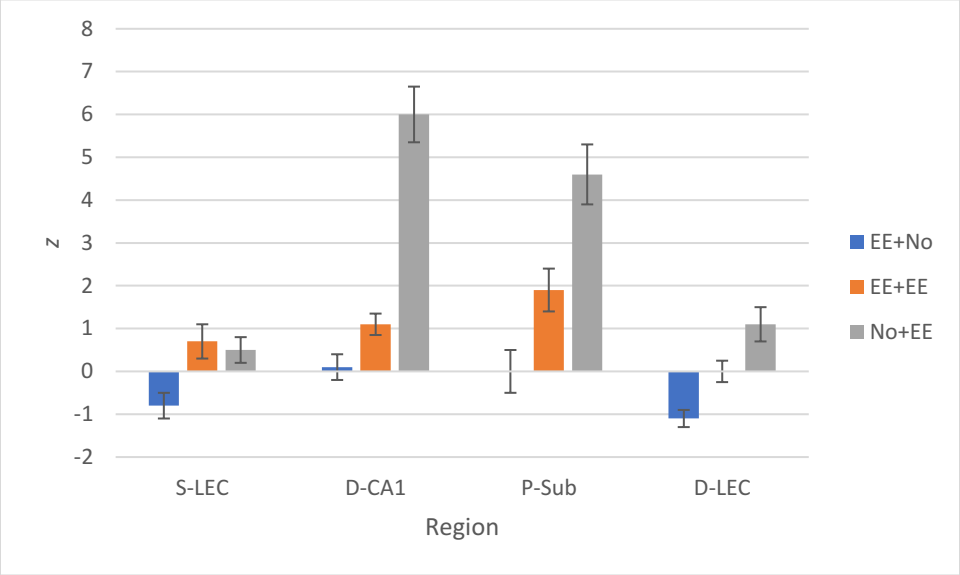
Neural activation in MEC-led spatial processing loop



Note. This figure displays the z-scores of EE+No, EE+EE, and No+EE groups across superficial MEC, proximal CA1, distal subiculum, and deep MEC, which are standardized against the No+No group. Error bars depict the standard deviation of the data.

Figure 14

Neural activation in LEC-led spatial processing loop



Note. This figure displays the z-scores of EE+No, EE+EE, and No+EE groups across superficial LEC, distal CA1, proximal subiculum, and deep LEC, which are standardized against the No+No group. Error bars depict the standard deviation of the data.