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Implications of Environmental Enrichment on Neural Activation in the Entorhinal Cortex

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Abstract

Environmental enrichment (EE) allows for informal learning in an environment containing stimulating objects and other rats. It promotes spatial, social, and object learning in rats and has profound effects on the brain by increasing neuroplasticity in brain regions dedicated to learning and memory such as the hippocampal formation (HF) and entorhinal cortex (EC). The six layers of the EC are a relay center for the HF. EC Layers II and III relay neural signals to HF, and EC receives HF output in Layer V. We examined EE effects on EC activation using the neural activity marker c-FOS. We used four groups of young adult rats **(***n*=7 each). Two had a history of EE with one having an EE experience just before sacrifice (EE EE) and one not (EE No). The other groups had no EE history with one having an EE experience just before sacrifice (No EE) and the other not (No No). Activated neurons in EC Layers II, III, and V were counted in digital images of tissue processed with immunohistochemistry to visualize c-FOS. While EE EE, EE No, and No No groups had similar levels of activity, only No EE rats showed enhanced levels of activity in EC Layers II and III (+371%, $p<0.01$). In contrast, EC Layer V showed 200% more neural activation in EE EE and No EE groups than in groups without a final enrichment experience. The activity increase in the input layers of No EE indicate that input is influenced by a final experience only when the experience is novel. However, for output areas, an increase was observed in No EE and EE EE. This indicates that input is heavily influenced by history and output is heavily influenced by final experience. History of EE suppresses neural activation by current experiences and the output layers are activated during an experience regardless of history. The results suggest that a history of particular experience may suppress input to the memory system via the EC; however, any current events seem to contribute to processed information contributing to memory consolidation regardless of an animal's history with similar experience (i.e., EC Layer V activity).

Keywords: Adolescence, Environmental Enrichment, Entorhinal Cortex

1. Introduction

The role of memory formation and storage is extremely vital to learning about and interacting with our environment. To understand memory formation and how external factors may impact it, we must examine the brain structures and neural circuits involved in memory and learning. Rodents are commonly used as models for exploring the effects of different environmental conditions on gross and fine brain structure and behavior. Previous studies suggest that even slight differences in environmental exposure and experiences can significantly influence both neuroanatomy and neurochemistry in the rodent brain[9]. Experimental evidence has found that enriched rodents exposed to novel and stimulating environments have an increase in brain weight and size when compared to non-enriched rodents[13]. Exposure to an enriched environment has also been found to promote both neuroplasticity through increased dendritic branching and gene expression for neurotrophins which support neuron development and function[9]. The changes that

occur structurally and functionally in the brain can often impact cognition, as can be observed in behavioral and problem-solving tasks comparing rodents with exposure to different rearing conditions[4].

The effects of external environment factors on neural development and behavior can be observed in multiple neural circuits, including those responsible for the formation and consolidation of memory. The temporal lobe memory circuit, comprised of the hippocampus and entorhinal and perirhinal cortices, is vital to general memory consolidation as well as spatial learning and navigation[2]. The effects of enrichment on the temporal lobe memory circuit have been widely examined in rodents, which provides appropriate models of the circuit and can be implemented in a variety of conditions to induce anatomical and functional changes in the brain. Changes induced through enrichment are often observed in the hippocampal formation (HF) and are considered indicative of the memory circuit's functionality. The entorhinal cortex (EC), which functions as a relay center between the hippocampus and certain cortical regions of the brain, can also indicate how efficiently the memory circuit is functioning. However, the effects of enrichment on the EC are not studied as extensively as effects on the hippocampus[12] In this study, the effects of a single and/or history of enriching experiences on function of the EC were examined.

1.1 Environmental Enrichment

Environmental enrichment (EE) is frequently used to examine the effects of novelty and experiences on brain structure and function in animals[1]. During a typical EE session, an animal is exposed to an environment containing numerous stimulating objects that often vary in texture, shape, and other physical properties as well as other animals[1]. Evidence suggests the profound impacts of EE on brain structure, brain function, and behavior. Some of the anatomical changes observed in the rodent brain following EE included increased spine density, dendritic branching and length, and number of granule cells[1,9]. EE also increased rates of neurogenesis and synaptogenesis in the brain[13]. These structural changes can have a significant impact on cognitive function in rodents exposed to EE, particularly in areas associated with memory and learning, such as the HF[7]. These effects may be partially due to the increase in synaptic connections that promote neuroplasticity and allow neural circuits to function more efficiently[1,8]. When compared with non-EE rodents, EE rodents tend to perform better on memory and spatial learning tasks, indicating an improvement in function of regions dedicated to memory formation and spatial navigation[13] such as the hippocampus and its input source and output target, the entorhinal cortex. This cortical region, which is involved in memory and learning, is of interest in the study of anatomical and neurochemical effects of EE.

1.2 Entorhinal Cortex and The Temporal Lobe Memory Circuit

The EC is an anatomical structure located within the medial temporal lobe that relays information between the cortical regions of the brain and the hippocampus[12]. The EC in combination with the hippocampus and perirhinal cortex form a neural circuit referred to as the medial temporal lobe memory circuit, which is found in a variety of mammalian species[11]. This neural circuit is considered vital to the formation, storage, and recollection of memory and is likely involved in spatial learning as well[6].

Although the rodent brain lacks a medial temporal lobe, many of the structures in the temporal memory circuit are present[6]. The EC is located anterior to the hippocampus and functions as a relay center, transmitting input information from the cerebral cortex to the hippocampus and output information from the hippocampus to various regions of the cerebral cortex through monosynaptic connections[12]. The EC is comprised of 6 layers, or bands, that project to different areas of the hippocampus; the more superficial layers serving as afferent and the deeper layers serving as efferent pathways[6]. The superficial layers (I, II, III) of the EC receive input from cortical regions of the brain and transmit the information to the dentate gyrus and CA1, CA2, and CA3 neurons of the hippocampus[12]. The deeper layers (V/VI) of the EC receive output from the subiculum and CA1 neurons of the hippocampus and project the information to multiple cortical areas of the brain, although this mostly appears to occur via Layer V[5]. Through the examination of activity occurring within layers II, III, and V of the EC, the functionality of the neural circuit can be observed; although further research is necessary to fully understand the significance of the EC in regards to memory formation and spatial learning[5]. There is suggestive evidence that the efficiency of the temporal memory circuit can be influenced by environmental factors. For example, exposing rodents to EE has been found result in increased dendritic branching and spine growth in the hippocampus[7], and increased thickness of the EC and higher production levels of the neurotransmitter glutamate have also been observed in rodents exposed to enrichment[9]. Most importantly for the present study, unique or unusual experience, such as one or more EE exposures, can produce enhanced activity in perirhinal cortex, which like EC, is connected with the hippocampus and important for memory and learning.

The purpose of the current study was to examine the effects of exposure to an enriching environment on the EC to help understand the influence that enrichment may have on the temporal lobe memory system in adolescent rats. Adolescent rats were selected for the study because experiments looking at EE typically use adult rats as subjects[9] and less is understood about the implications that EE has on the adolescent brain. In this study, we focused on the activity occurring within the areas of the EC receiving cortical input, Layers II and III, and those layers receiving hippocampal output (Layer V) as indicative of the neural circuit's overall function. In this exploratory study, we assumed a single exposure and/or a history of EE would alter neural activity across layers of the EC.

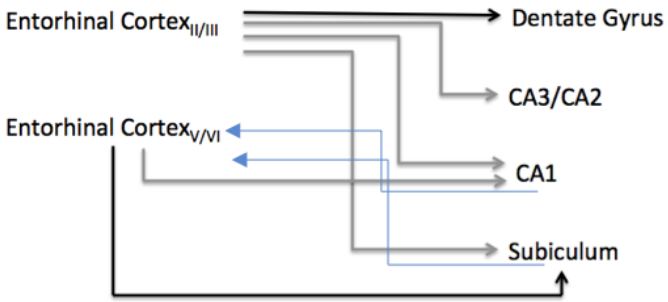

Figure 1. Diagram depicting the major and minor pathways in the temporal lobe memory circuit (black: major, gray: minor).

2. Materials and Methods

2.1 Subjects

Long Evans Hooded rats (*n*=27, 13 male, 14 female) were used in this experiment. They were housed in plastic tubs in a humidity and temperature controlled vivarium. Subjects were kept in groups of three and given food and water *ad libitum.* Subjects were kept on a 12:12 hour light-dark cycle. Subjects were bred and cared for by the Arts and Sciences Animal Facility at Appalachian State University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University.

2.2 Environmental Enrichment

Enrichment was used to create a 2x2 factorial design with the 2 variables being history and final experience. From this, four study conditions were created (Fig 2). The four study conditions were coded with "EE" to indicate EE and "No" to indicate no enrichment.

		Last Exposure	
		-- EE	**-- No**
History	**EE --**	EE EE	EE No
	No --	No EE	No No

Figure 2. The table indicates the varying amounts of enrichment subjects received, with the first (leftmost) code in the series relating to history, and the second (rightmost) code relating to final experience.

Half of the subjects (*n*=13, 6 male, 7 female) had a history of enrichment and half did not (*n*=14, 7 male, 7 female). Half of the subjects were given a final enriching experience (*n*=14, 7 male, 7 female) on the day of their sacrifice and half were not (*n*=13, 6 male, 7 female). EE consisted of placing the rats in enclosures with various platforms, ramps, and varied objects for 90 minutes a day from postnatal day (pnd) 30 to pnd 60.

Subjects were enriched using a cage with a 45.7 x 48.3 x 78.7 cm (w x d x h) wooden frame and 1/2-in. hardware cloth cage with multiple platforms to climb. Platforms were located at 14.0, 24.8, 43.2, and 61.0 cm above the floor of the cage and were accessible via hardware cloth ramps (Fig. 3). Toys and objects were varied each day. The placement of these things was repeated each week in a five day cycle. The subjects were enriched in same-sex groups of six for 60 minute intervals. In the enrichment cage, subjects had the opportunity to interact with rats not from their home cage, and therefore were less familiar. Together the experimental subjects were able to interact with novel situations, objects, and spaces.

A control condition included the subjects that did not receive a history of enrichment (*n*=14, 7 male, 7 female). These subjects were picked up and handled for 30 seconds then placed back into their home cage to control for any stimulation the experimental group received from handling to and from the home cage to the enrichment cage.

Figure 3. Photo of the female enrichment cage including a random assortment of toys and other objects.

The photograph above displays an example of the female enrichment cage set up in one of the five arrangements, which includes a foam football, a mug, PVC pipe, fake fruit, several objects hung by string or chain links, and objects of similar nature. The male cage (not shown) was set up in the exact same arrangements on the same days, but is a mirror image of the female cage. Subjects were not given any food or water while in these cages.

2.3 Immunohistochemistry

All subjects were perfused between pnd 75 and 78. Subjects that had a final experience (group No EE and EE EE) were placed in the enrichment cage for 90 minutes on the day they were sacrificed. Those that did not have a final experience (group EE No and No No) did not partake in an enrichment experience before sacrifice. All subjects were placed in a quiet and dark space for ~90 minutes before they were sacrificed. This allowed for the expression of the c-FOS protein from the c-*fos* gene. Subjects were then given a lethal injection of sodium pentobaribtol and the researchers waited until they no longer responded to tail and blinking reflexes. The subjects were perfused intracardially with a phosphate buffered saline solution (PBS) until their circulatory system was clear. The subjects were then perfused with 4% paraformaldehyde in 10 mM phosphate buffer. Once the brains were cured, they were removed from the skull and placed in 4% paraformadehyde and 10% sucrose. After one week in this solution, the brains were transferred to PBS and kept at 4 °C. The brains were later cut into 50 µm sections using a Vibratome Series 1000 and floated in individual PBS wells while kept at 4 °C. Sections with a clear and complete image of the EC and lateral EC were selected to be processed by floating section immunohistochemistry. This allowed for the researchers to collect data on neural activation by looking at the amount of expression of c-FOS.

On Day 1 of immunohistochemistry (ihc), sections were rinsed in PBS (2x5 minutes), then rinsed in 0.5% hydrogen peroxide in water for 15 minutes. They were rinsed two additional time in PBS (2x5 minutes). Sections were then floated in rat anti-c-FOS made in rabbit (Santa Cruz, SC-52) at 4 °C for approximately 40 hours on an variable speed oscillator orbital shaker.

On Day 2 of ihc, sections were rinsed in PBS and exposed to the VIP enzyme substrate from Vector Labs for two minutes. Sections were removed from the enzyme and floated PBS until they were mounted onto gel-coated slides. Once mounted, sections were dehydrated in a graded series of ethanol solutions, cleared with toluene, and cover-slipped using Permount glue (Fisher).

2.4 Data Collection

Activated neurons were quantified in EC regions using digital microscopy and stereological technique. Once the staining was complete, digital pictures of the EC were taken using a Nikon Eclipse microscope and PixeLink digital camera, which allowed for the pictures to be seen and taken from a computer. The images were taken at plan 10 and rotated to maximize the amount of the EC displayed. The activated neurons are indicated because they are darker than other neurons due to the c-FOS staining. The researchers created a template to decide the how dark a cell needed to be for it to be counted. Using a photoshop program, grid squares were superimposed on to the images. Each solid grid line was a 100 µm x 100 µm section. Three grid squares were sampled from Layers II/III and three from Layers V/VI by counting the activated neurons (Fig. 4). Active neurons included on the top and right borders were included in the sample, and neurons on the bottom and left border were not included in the sample so as to not double count active cells.

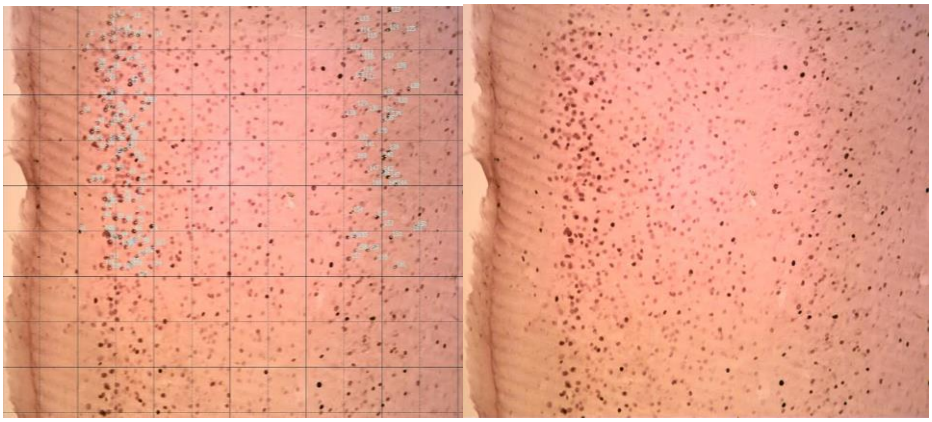

Figure 4. Example from the EE EE condition of the original image taken with the PixeLink digital camera (left), and final image with grid squares and activated neurons counted (right).

After the active cells were counted, they were averaged across all the photoshop boxes sampled from for the enrichment condition specific to that tissue. The means, standard deviation, sum of squares, and f-values were all calculated. The ratios of activated neurons between Layers II/III and V/VI and between each of the four enrichment conditions were analyzed.

3. Results

Looking at the EC over all four study conditions (Fig. 5), there are two notable differences. First, the No EE condition shows a large spike in neural activity in Layers II/III and V/VI. Secondly, in the conditions that had a final experience (No EE and EE EE), Layers V/VI show an increase in activity.

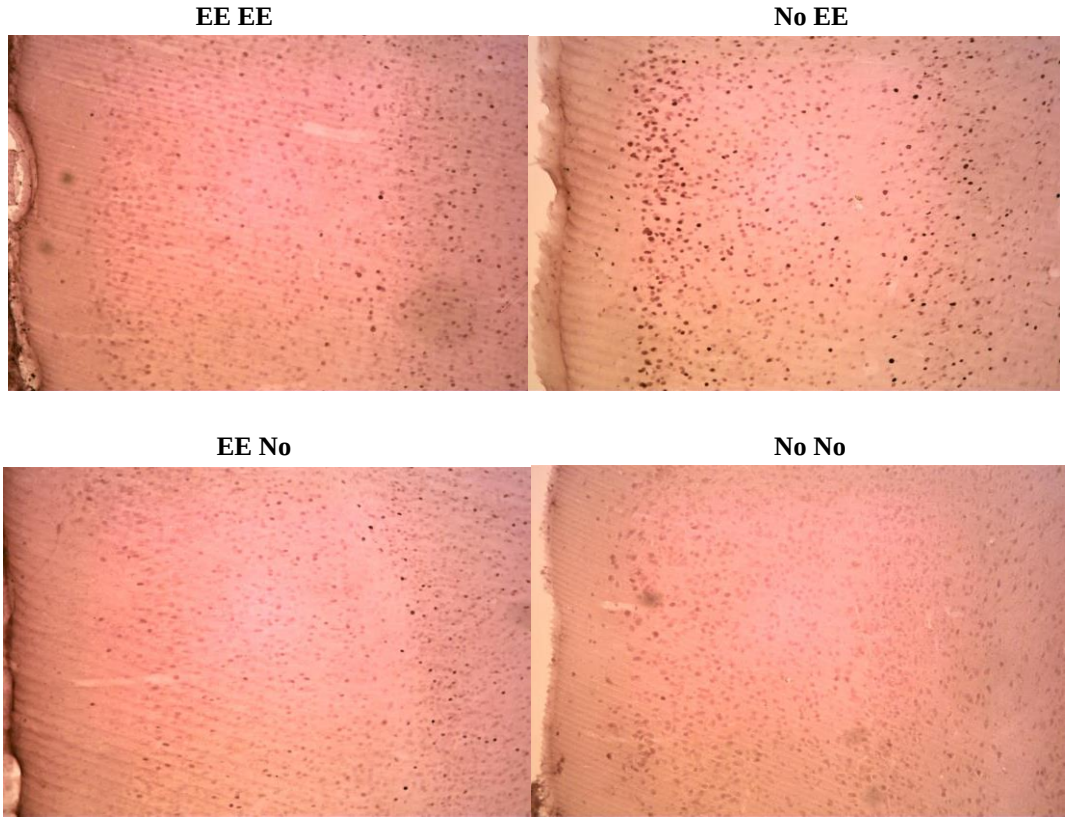

Figure 5. Example photos from all four study conditions.

In the input areas of the EC (Layers II/III), only those with a final experience and no history (No EE condition) showed a 317% increase neural activation, $f(1)=25.63$, $p < 0.01$, over the mean of the other three study conditions ($\mu=1.4$). In the output areas of the EC (Layers V/VI), the subjects with a final experience, with or without history, (No EE and EE EE conditions, $\mu=2.7$) showed a 200% increase, $f(1)=25.96$, $p < 0.01$, over the conditions with no final experience ($\mu=0.9$). The means and standard deviations (Fig. 6) reflect the same proportion of neural activity shown in the photos above.

Layers II and III

	-- EE	-- No
EE --	1.1 (1.31)	6.6 (1.63)
No --	1.2 (0.70)	1.8 (0.83)

Layers V and VI

	-- EE	-- No
EE --	2.2 (1.18)	0.7 (0.27)
No --	3.2 (1.12)	1.1 (1.18)

Figure 6. Table of the means and standard deviations for the input (II/III) and output (V/VI) layers of the EC within the four study conditions.

These two main differences were observed qualitatively (Fig. 5) and quantitatively in the means of Fos+ neurons (Fig. 6). Differences in Layers II/III between the EE EE, EE No, and No No conditions were not statistically significant. Differences in Layers V/VI between the No No and EE No conditions, as well as the EE EE and No EE were not statistically significant, but the difference between the averages (-- EE $\mu=2.7$, -- No $\mu=0.9$) of the final experience group (EE EE and No EE), and the no final experience (No No and EE No) groups were.

4. Discussion

4.1 Layers II and III

The hypothesis, predicting a single exposure or a history of EE would alter neural activity in the EC, was supported. The large increase in neural activity in the afferent pathways of the EC (Layers II/III), specifically in the condition in which the rodents had no history but did have a final experience (No EE), suggests a history of EE in adolescence suppresses neural activity. This is because the history of exposure to EE makes the subject's brain more efficient when processing novel information. Previous research has shown repeated activation of input areas to the EC enhances its ability to store and recall information[11]. From this, the researchers concluded that the subjects with a history of EE were better able to adapt to new arrangements in their environment and other stimuli. This is because the conditions with a history of EE showed less neural activity than the conditions without a history of enrichment.

4.1 Layers V and VI

The increase in activity in the efferent areas of the EC, Layers V/VI, suggest that neural activation is highly influenced by final experience, regardless of whether or not the experience is novel. This is contradictory to the results observed in Layers II/III of the EC, and indicates that in afferent areas, final experience dictates the amount of neural activation. However, in efferent areas, history dictates the amount of neural activation. In the output areas of the EC, a history of EE does not suppress neural activation as much because the subjects' ability to interact with that environment still requires neural effort. This means that even if their brain does not have to work as hard to sense or adapt to their environment (because they have a history of enrichment), the brain still has to work hard to interact with the environment.

4.3 Implications and Limitations

If this rodent indicator is a model of the human brain, it can be concluded that humans devote massive amounts of processing power to understanding unique and novel experiences, even informally, as was the case with the subject's learning environment. This can be concluded because the subjects that had a final experience (No EE), had activation in the input areas of the brain when they experienced a completely new environment for the first time. The brain becomes more efficient at processing environments it is used to. The results from the output areas of the brain suggest that humans connect new experiences to everything they know, regardless of whether or not those experiences are novel. This conclusion was drawn from observing that the output areas of the brain showed high levels of activation because the EC was pulling past memory from the cerebral cortex and linking it into the temporal lobe memory circuit so the subject can better understand and predict their environment. These conclusions are significant because they imply that exposure to a particular environment can suppresses neural activity in an area of the brain important for relaying signals into circuits necessary for consolidation of many forms of long term memory. Because the EC receives processed information back from hippocampus, the results of our study can be extrapolated to comment on the HF. It seems that neural faculties are either dedicated to understanding an environment when it is new or dedicated to interacting with that environment when it is known. While the EC is not responsive to sensory information about known things in known environments, the hippocampus is likely sifting through the input provided by EC to pick out some information about known things from known environments and recommitting them to the EC.

Future studies in this area could include studying the EC in adulthood as well as adolescence. The brain may be less able to process new information if the final experience is had in adulthood. Olfactory sensation could also impact this study, because the EC is closely related to olfaction and the different smells between the home cage, the enrichment cage, and subjects from other home cages may impact this area of the brain. Because the efferent parts of the EC (Layers V/VI) are related to motor neurons, this part of the brain could be seeing a higher influx of neural activation because the enrichment cage is set up to allow for physical development and motor stimulation. In addition, the overall increase in oxygenation to the brain because of physical activity may increase activity in the brain over all. The researchers plan to collect data from the lateral EC, dentate gyrus, and subiculum to investigate any differences and similarities between the results found in this study and other brain structures within the temporal lobe memory circuit and HF.

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