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One Enriching Experience Enhances Neural Activity in the Adolescent Hippocampal Formation

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Abstract

By fostering physical and social stimulation with interactive floor plans, unique objects, and conspecifics, environmental enrichment (EE) sessions can enhance neural activity in hippocampus that provides at least some of the basis for exploratory and preference behavior as well as and learning and memory. While EE manipulations usually occur over time, the present research was conducted to examine the effect of a single enriching experience on neural activity in hippocampal formation (HF) of 12 adolescent Long-Evans rats. Prior to sacrifice, half of the rats experienced EE in a multi-level enclosure with objects and other rats, and the other six rats served as not enriched controls. Immunohistochemistry was used to process brain tissue for the neural activity marker c-FOS, and microscopy was used to measure the density of c-FOS positive neurons in HF structures. Mean densities of activated neurons were greater in HF internal processing regions CA3 (+172%), CA2 (+136%), and CA1 (+183%) and in the subiculum (+164%), an output area, of rats exposed to EE than in controls (all $p<.05$). However, no significant difference within the dentate gyrus, a primary input zone for hippocampus, of enriched and control rats was observed ($p>.05$). Results suggest an enriching experience does not necessarily increase input to HF, but it does increase recruitment of neuron populations in sequence through the hippocampus. The enhanced output of HF observed following one enrichment session implies this experience may significantly impact learning and memory, albeit informal, which could change future behavior. For adolescents, such experiences may help them to adapt to physical and social novelty in new situations.

Keywords: Immediate Early Gene, Brain Development, Learning & Memory

1. Introduction

Adolescence is a time of great transition within an individual, marked with instances of discovery, independence, rebellion, and identification. During this critical time of maturation, experiences play a large role in one's progression of self. Adolescents who fail to receive the appropriate social and physical environments that foster these necessary experiences are at a higher risk for underdevelopment in adulthood. One study's findings reported that social isolation within adolescent rats resulted in increased behavioral inhibition, anxiety-like behaviors and latency for social approach and contact within those affected[1]. In fact, Lukkes and his colleagues found that these consequences were so ingrained within the rats that even instances of resocialization did not reverse their anxiogenic profiles leading into adulthood[1]. Another study focused on the implications of impoverishment on the developing brain showed that adolescents in poverty exhibit atypical structural development in critical areas of the brain including their frontal and temporal lobes, hippocampus, as well as a decrease in total gray matter[2]. In addition to a healthy living environment, another serious aspect concerning physical stimulation is exercise. Adolescents who lack the ability to engage in

adequate amounts of physical exercise run the risk of under-developing critical cognitive functioning such as attention levels, behavioral inhibition, and memory[3,4,5,6]. Thus, adolescence may serve as a prominent timeframe for studying the outcomes that experiences have on the developing brain both structurally and behaviorally.

1.1 Environmental Enrichment

Environmental enrichment (EE) is the term used when laboratory animals are exposed to housing that cultivates greater levels of social and/or physical stimulation than they would be under standard housing conditions. The social aspect of EE involves the placement of animals into larger groups, rather than with basic littermates or in isolation.The physical aspect of EE involves the manipulation of floor-plans, toy exposure, and other items that increase exercise or play. In one study focused primarily on physical EE the introduction of a running wheel was manipulated between groups as either a primer for EE or not[7]. Their findings revealed that this simple act of physical exercise prompted neurogenesis within the DG of the HF in both groups, however when it was followed by exposure to EE it amplified its beneficial cell-surviving effects within the adult rat[7].

As a follow-up to these findings another study was done attempting to tease out the differential effects that physical and social EE have separately[8]. In their study, Brenes and his colleagues created two experimental groups representative of a physical EE and a social EE but excluded the inclusion of a running wheel in both conditions. Instead, other forms of exercise were included in the physical EE such as cage tests, place object recognition tests and open field tests[9,10,11]. Results from their experiment supported the previous study's findings that the physical EE proved to have better effects on the neuronal activity in the DG of the HF as compared to the social condition[8]. However, their study found that there was varied increases in the cell proliferation of the social conditions based on the number of animals they had in the cage. Brenes and his team suggested that this may be indicative of the effects that social exercise such as rough-and-tumble play has on the animals[8]. With the two of these studies in mind, our team thought it best to include both physical and social aspects into our EE in order to promote a more well-rounded and impactful experience. Both of these aspects contribute to the overall influence that EE has on an individual and their brain.

In addition to those affects, various other studies have found that EE has a profound influence on a range of both structural and behavioral responses within the brain of adolescent rats; including neurogenesis, increased dendritic branching and cell size, a general improvement in learning and memory as well as a reduction in anxiety through improvements in novel experiences[12].

Neurogenesis refers to the growth, proliferation and survival of nervous tissue. This is a structural change observed in the brain that can have profound effects on the physical and social aspects of one's life. Van Praag and his team of researchers evaluated the effects of laboratory rats that had been placed in an enriched environment and participated in activities associated with said environment[13]. They observed an increase in cell proliferation and recruitment of new neurons in the hippocampal dentate gyrus[13]. They rationed that although novel experiences may appear different to each rat, they affect the same molecular pathway with similar anatomical changes.

In addition to neurogenesis, researchers have also found positive effects on dendritic branching and cell size in the brains of laboratory rats exposed to enriched environments. Mora and team studied these effects in 10-12 rats that had been exposed to an enriched environment for a prolonged period of time[14]. They were successful in finding increased dendritic branching, new synaptic formation and increased brain weight in areas such as the cerebral cortex and the basal ganglia[14]. They questioned whether aging could have similar effects on these areas of the brain, as aging is presumably correlated with increased novel experience.

Besides the brain's structural effects observed from EE, laboratory animals have also expressed improvements in behavior such as learning and memory. Forgays & Forgays made a discovery that associated problem-solving and perceptual learning with environmental experience[15]. They observed the differences in free-environmental vs. restricted-environmental laboratory rats and the amount of time it took them to complete a maze. It was found that the free-environmental rats, those who'd been previously exposed to a greater variety of environmental scenarios, completed the maze at a faster pace than the restricted-environmental rats[15].This goes on to suggest problem solving and perceptual learning, more broadly learning and memory, is positively associated with novel and enriched environments.

In addition to learning and memory, there are other behavioral effects observed from exposure to enriched environments, such as a reduction in anxiety. Lopes and colleagues made strides in the research of EE's effects on anxiety-like behavior[16]. They found that rats exposed to an enriched environment for one week showed decreased anxiety-like behavior and panic-response in comparison to those rats held in standard housing. Decreased anxiety-like behavior and panic-responses were measured by the percentage of those rats who spent time on the elevated T-maze

and climbing the staircases, more broadly identified as fear-provoking activities[16]. These responses associated increased EE with a reduction in anxiety. Researchers confirmed this finding could promote anxiolytic therapy in humans.

Most of the current literature on EE is focused on prolonged exposure, which is what allows for these long-term impacts on the brain to be recorded. White included in his master's thesis an experiment that sought to pull apart the effects that an acute exposure of EE had on animals previously engaged in EE and those without a prior history to pinpoint the technique's long-term outcomes[17]. His findings supported that acute experiences with EE produced significantly higher levels of neuronal activity in the brain, and yet interestingly enough when a rat had a prior history of EE those increases plateaued and eventually ended in a lower basal activation. This suggested that with prolonged exposure to EE the circuit affected becomes more optimized at sensing and processing information[17]. This research left our team with one question: does one single exposure to EE without the manipulation of prior enrichment affect the adolescent brain, and if so, how?

1.2 *c-fos* and c-Fos expression

We sought to answer this question using a neuronal protein marker known as c-Fos. This protein marker allowed us to map functional activity in the hippocampal formation of our laboratory rats, exposing areas where neurons were or were not active. Active neurons were associated with a dark color, due to c-Fos accumulation. Inactive neurons were associated with a light color, due to lack of c-Fos accumulation. This protein marker is derived from the immediate early gene (IEG), *c-fos.* Chaudhuri describes the signal transduction sequence that affirms the production of the c-Fos protein[18]. This sequence is most commonly initiated by a calcium (Ca^{2+}) influx into the cell, which commonly occurs through the NMDA receptor. From here, different enzyme systems are activated, which in turn activates proceeding transcription factors. The IEG is transcribed into *c-fos* mRNA, which is translated into the c-Fos protein[18]. This neuronal protein marker has been found effective in a number of studies identifying neuronal activity, and in turn is what was also used in the current investigation.

1.3 Current Investigation

Learning and memory primarily take place in the brain's limbic system. It is understood that this process falls into a circuit between the cerebral cortex, the entorhinal cortex and the HF. The entorhinal cortex (EC) acts as an anatomical gateway for information processing between the cerebral cortex and the HF. Once the information is transmitted through the EC and into the HF, it begins its journey in the dentate gyrus, which acts as the circuit's main input center[19]. From here, the information is sent to the cornu ammonis (CA) regions, beginning at CA3 and CA2, and then being filtered to CA1. The information is then directed to the subiculum (SUB), which acts as the circuit's main output center[19]. This is a persistent cycle that efficiently contributes to the procedure of learning and memory.

VanElzakker, and his team looked at the effects of EE in this circuit of the HF. He and his team of researchers were successful in finding that laboratory rats who were exposed to an enriched environment over a prolonged period of time showed significant increases in neural excitation with altered hippocampal neural activity in the CA1 and subiculum structures[20]. This could be observed by the activation of Fos protein in the neurons of the HF in those environmentally enriched rats[20].

Similar to VanElzakker's study, the current experiment focuses on the effects of EE in each section of the hippocampal formation, including the dentate gyrus, CA3, CA2, CA1, and the subiculum. In this case, however, the primary interest was only that of a single enriching experience, rather than prolonged exposure. One central hypothesis was tested: the number of *c-fos* positive neurons in these five brain structures of rats that receive one session of EE would be significantly greater than those rats who remained in regular housing.

2. Materials & Methods

2.1 Subjects

Twelve long-evans rats were obtained from the breeding colony at Appalachian State University. The rats were adolescents at postnatal day (PND) 39. The rats lived in four groups of three in regular shoebox housing. Regular

housing involved the implementation of a 12-hour day-night light rotation system, very few toys, and minimal human interaction. At PND 39, the rats were split up into two groups of experimentals and controls.

2.2 Experimental Groups

1. *Single Exposure (NOEE)*: Rats were housed in regular conditions until PND 39. At PND 39, six rats were randomly selected to be housed in an enriched environment for 90 minutes. After those 90 minutes were over, they were placed into the quiet and dark for an additional 90 minutes, allowing their neural protein marker a chance for full expression.
2. *Controls (NONO):* Rats remained in the regular housing, until they were sacrificed at day 39. Immediately before their sacrifice, they were placed into the quiet and dark to allow a chance for their neural protein marker to fully express. These rats were picked up and put down for 15-20 seconds to account for the handling of the other group.

2.3 Environmental Enrichment Procedures

The enriched environment was the same for all six rats selected for the NOEE group. These environments were structured with multiple level cages, colorful toys hanging from the ceiling, and the inclusion of a larger group of playmates. Other toys, such as a foam football, a tennis ball, and a coffee mug were placed onto the varying levels of the cage. The items in the cages remained unchanged during the 90-minute enriching session unless otherwise moved by the rats themselves.

2.4 Immunohistochemical/Counting Procedures

The tissue prepared on each slide was stained with c-Fos, an excellent neural protein marker. c-Fos is an immediate early gene (IEG) which is derived from the protein, *c-fos*. This c-Fos protein marker allowed for the visibility of active neurons when using light microscopy techniques. The counting of the c-Fos enriched neurons was accomplished using a Nikon Eclipse microscope and a PixeLink digital camera. Each structure of the HF was counted for the total number of darkened neurons, based off of a subjective light/dark threshold agreed upon by the team.

3. Results

The mean number of activated neurons located within each region of the HF circuit for the experimental group was greater than that of the control group, which supports the idea that EE impacts the brain in ways that allow for a higher level of neuronal activation as when compared to rats in the control condition. The differences between groups were measured in terms of percent ch
ange as a way to relate the extent to which the amount of *c-fos* positive neurons intensified under the presence of EE. These differences were deemed statistically significant for the CA3, CA2, CA1, and SUB regions with p-values under 0.0500. The difference between groups in the DG region were reported to be not significant with a p-value of 0.0526.

Table 1. Physical data for the number of activated neurons in the HF circuit. This was collected in the lab at Appalachian State University during the year of 2018.

Hippocampal Formation Cell Count Data					
	DG	*CA3*	*CA2*	*CA1*	*SUB*
NOEE	MEAN = 6.26 SD = 2.10	MEAN = 8.99 SD = 2.02	MEAN = 10.35 SD = 3.47	MEAN = 23.53 SD = 8.15	MEAN = 17.43 SD = 4.15
NONO	MEAN = 3.05 SD = 1.64	MEAN = 3.33 SD = 2.44	MEAN = 4.40 SD = 2.47	MEAN = 8.27 SD = 5.74	MEAN = 6.64 SD = 4.27
% Changes	+105%	+172%	+136%	+183%	+164%
p-values	0.0526	0.0117	0.0314	0.0222	0.0110

4. Discussion

The results of our study showed that adolescent rats exposed to EE had significantly greater means of *c-fos* positive neurons in all regions of the HF circuit except for the DG. However, these results did not fully support our central hypothesis that all five regions would exhibit a significantly greater mean, and thus we rationed that this slight error could be attributed to two possibilities: one being that between groups, the information that is being input at the DG is processed in a much different way in the rats exposed to EE than those in regular housing; or perhaps this could be the result of too small a sample size.

After revisiting previously discussed literature on the effects of EE, our team thought it best to evaluate the HF circuit because of its close relationship to learning and memory. It has also been observed that the HF is the region most associated with EE from the existing literature, which only emphasized our choice[5,12]. While most of our findings supported our hypothesis, it was of special note that one region (the DG) did not. Drawing from past research, it was odd to see this in our results, but perhaps this only illustrates just how efficient the HF circuit becomes once under exposure to EE. We ration this because if the initial information being input to the DG is the same in both groups, and yet only the EE group experiences statistically higher means of c-fos positive neurons throughout the remainder of its circuit, that its efficiency level has been raised appreciably in comparison to the control group. This could warrant future research in order to explore the differences EE has between each individual region of the HF and its potential impacts separately as well as combined.

Another probable reasoning behind this minor error could be accredited to our relatively small sample size of just twelve rats. We reason this because the difference between the groups was increased by almost double and accounted for a 105% change which indicates that there was change. However, this change fell just under the bar to be deemed statistically significant at a p-value of 0.0526. We believe that a larger sample size would result in a more pronounced difference that would reflect significance.

In addition to narrowing the scope on how exactly EE affects the brain and where within the brain these effects are most noticeable, there are numerous behavioral implications our study proposes. Given that EE has been associated with neurogenesis, increased dendritic branching and cell size, a general improvement in learning and memory as well as a reduction in anxiety through improvements in novel experiences as stated earlier, means that these effects can translate into a variety of improvements within the behavior of adolescents exposed to enriching experiences[1,4,12]. As levels of anxiety in novel experiences decrease, the ability to adapt quicker and more easily allow for a greater exploration of one's environment, which could then lead to a healthier development of one's coping strategies. Increasing the instances of neurogenesis in turn could encourage better performance in tasks of focus or learning, which could lead to fewer errors in general, and more patience. The ability to form better connections between thoughts and their corresponding matches may in turn strengthen memory recall, which could occur through the increasing size of cells or dendritic branching between neurons. Our study adds the important facet of a single EE

exposure to the large body of literature mainly focused on prolonged EE exposure and its wide range of effects that it may have on brain functioning, and thus should serve helpful for future research in this growing field.

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