ON THE NATURE OF NEURAL ACTIVITY IN THE HIPPOCAMPAL FORMATION:
EFFECTS OF PERIODIC AND/OR ACUTE ENVIRONMENTAL ENRICHMENT ON
C-FOS EXPRESSION IN ADOLESCENT RATS

by

Meghan N. Pavelka

Honors Thesis

Appalachian State University

Submitted to the Department of Psychology
and The Honors College
in partial fulfillment of the requirements for the degree of

Bachelor of Science

May 2018

Approved by:

_____

Mark C. Zrull, Psychology, Ph.D., Thesis Director

_____

Andrew C. Bellemer, Ph.D., Second Reader

_____

Andrew R. Smith, Ph.D., Departmental Honors Director

_____

Jefford B. Vahlbusch, Ph.D., Dean, The Honors College

Abstract

ON THE NATURE OF NEURAL ACTIVITY IN THE HIPPOCAMPAL FORMATION:
EFFECTS OF PERIODIC AND/OR ACUTE ENVIRONMENTAL ENRICHMENT ON
C-FOS EXPRESSION IN ADOLESCENT RATS

Meghan N. Pavelka

Chairperson: Mark C. Zrull

The interaction with novel objects and same-sex conspecifics, known as environmental

enrichment (EE), provides a lasting impact on the brain and behavior of mammalian species.

Specifically, EE can alter hippocampal networks associated with spatial learning and

memory. This study investigates the influence of periodic and/or acute EE exposure on

activation in the hippocampal formation (HF) of adolescent Long Evans rats. One group (EE-

No; n=6) received twenty, 90-min EE exposures from postnatal day (PND) 25-48, while a

different group (EE-EE; n=6) received this same periodic EE, along with an acute 90-min

exposure before perfusion. A third group (No-EE; n=6) solely received a single 90-min EE

exposure before perfusion. A control group (No-No; n=6) did not receive any EE exposure.

Following perfusion, brain tissue was processed using immunohistochemistry to visualize c-

FOS protein expression, a marker of neural activation, and densities of c-FOS+ neurons were

quantified via microscopy. Relative to non-enriched controls, an acute EE exposure produced

more c-FOS+ neurons in HF internal processing regions, CA3 (+172%), CA2 (+136%), and

CA1 (+183%), and in subiculum (+164%), a major output region of the HF (all p<.03). In

dentate gyrus (DG), a major input region of the HF, rats that experienced a final, acute EE

exposure exhibited 105% more c-FOS+ neurons compared to No-No rats ($p=.053$).

Compared to acutely enriched (No-EE) rats, those that received both periodic and acute EE (EE-EE) exhibited significant reductions in c-FOS expression among CA3 (-43%), CA2 (-47%), CA1 (-64%), and subiculum (-41%) structures (all $p<.03$). The results suggest that an EE history in adolescence suppresses activation as a signal moves sequentially throughout the HF pathway. Additionally, a single EE exposure may increase recruitment of neuronal populations in sequence of the HF, without necessarily increasing input to this pathway.

Acknowledgements

I would like to thank Mark C. Zrull for his invaluable support and mentorship during the past three years and specifically through the completion of this project. I would also like to thank Andrew C. Bellemer and James C. Denniston for their guidance and mentorship. Thank you to the Animal Care Facility of Appalachian State University for their efforts in caring for and providing subjects for this research. Lastly, I would like to thank my fellow undergraduate research assistants, including Charles Fennell, Erica Turner, Makayla Wood, Paige Clayton, and Samara Santiago, who made the execution of this endeavor possible.

Table of Contents

List of Tables

List of Figures

On the Nature of Neural Activity in the Hippocampal Formation: Effects of Periodic and/or

Acute Environmental Enrichment on c-FOS Expression in Adolescent Rats

Meghan N. Pavelka

Appalachian State University

Abstract

The interaction with novel objects and same-sex conspecifics, known as environmental enrichment (EE), provides a lasting impact on the brain and behavior of mammalian species. Specifically, EE can alter hippocampal networks associated with spatial learning and memory. This study investigates the influence of periodic and/or acute EE exposure on activation in the hippocampal formation (HF) of adolescent Long Evans rats. One group (EE-No; n=6) received twenty, 90-min EE exposures from postnatal day (PND) 25-48, while a different group (EE-EE; n=6) received this same periodic EE, along with an acute 90-min exposure before perfusion. A third group (No-EE; n=6) solely received a single 90-min EE exposure before perfusion. A control group (No-No; n=6) did not receive any EE exposure. Following perfusion, brain tissue was processed using immunohistochemistry to visualize c-FOS protein expression, a marker of neural activation, and densities of c-FOS+ neurons were quantified via microscopy. Relative to non-enriched controls, an acute EE exposure produced more c-FOS+ neurons in HF internal processing regions, CA3 (+172%), CA2 (+136%), and CA1 (+183%), and in subiculum (+164%), a major output region of the HF (all p<.03). In dentate gyrus (DG), a major input region of the HF, rats that experienced a final, acute EE exposure exhibited 105% more c-FOS+ neurons compared to No-No rats (p=.053). Compared to acutely enriched (No-EE) rats, those that received both periodic and acute EE (EE-EE) exhibited significant reductions in c-FOS expression among CA3 (-43%), CA2 (-47%), CA1 (-64%), and subiculum (-41%) structures (all p<.03). The results suggest that an EE history in adolescence suppresses activation as a signal moves sequentially throughout the HF pathway. Additionally, a single EE exposure may increase recruitment of neuronal populations in sequence of the HF, without necessarily increasing input to this pathway.

On the Nature of Neural Activity in the Hippocampal Formation: Effects of Periodic and/or

Acute Environmental Enrichment on c-FOS Expression in Adolescent Rats

The influence of early sensory stimulation on brain development and cognitive

performance has long been of interest to psychologists and researchers (Forgays & Forgays,

1951; Hymovitch, 1952). Particularly, the exposure to complex environments and same-sex

conspecifics, known as environmental enrichment (EE), has been investigated for its

beneficial effects on neural development and behavior in a number of mammalian species

(Mesa-Gresa, Pérez-Martinez, & Redolat, 2013; Simpson & Kelly, 2011; van Praag et al.,

2000). EE is characterized by the opportunity to explore and interact with novel

environments, as well as opportunities for physical and social stimulation that are provided in

supplement to standard housing conditions (Simpson & Kelly, 2011; van Praag et al., 2000).

As a result, several animal facilities and laboratories have begun to employ enrichment into

their guidelines of care.

While the parameters and duration of EE exposure often vary among studies, the

standard definition of an enriched environment includes a combination of same-sex

conspecifics, generally in larger groups than the standard home cage, and inanimate objects,

such as tunnels, toys, and nesting materials (Simpson & Kelly, 2011; van Praag et al., 2000).

Many studies include opportunities for physical exercise with the provision of running

wheels (Li et al., 2013; Novokovic, Heumann, & Manahan-Vaughan, 2015). However, the

beneficial effects of an enriched environment have also been demonstrated in the absence of

voluntary exercise (Birch, McGarry, & Kelly, 2013; Dhanushkodi, Bindu, Raju, & Kutty,

2007; Mármol, Sánchez, Torres, & Chamizo, 2017; Neidel et al., 2016; White, 2013).

Ubiquitously across protocols, EE allows for the interaction with a complex, stimulatory environment.

In addition to physical and social stimulation, EE provides an opportunity for the exposure to novelty. Many EE protocols include the novel arrangement of objects, as well as opportunities to interact with novel same-sex conspecifics. Additionally, EE is typically provided on a schedule of regular, time-limited exposures (e.g. Pavelka, Salinas, & Zrull, 2017; White, 2013). Some protocols that feature continuous EE, in which subjects are housed in enrichment cages throughout the entirety of the experiment, often employ a variety of objects that are routinely placed in novel spatial arrangements (Bennett, McRae, Levy, & Frick, 2006; Hullinger, O'Riordan, & Burger, 2015). However, given the amount of time in which subjects may habituate to the EE cage, protocols of continuous EE may be less effective at providing opportunities for novelty exposure.

Enrichment Effects

During EE, the exposure to novel experiences provides an opportunity for informal learning and explicit memory consolidation. Thus, several studies have reported improved learning and memory retention in various behavioral tasks, such as fear avoidance conditioning, novel object recognition, open field, object-in-place, radial maze, and Morris water maze tasks (Cobb, 2015; Hullinger et al., 2015; Lu, Zhong, Yan, Tian, & Shen, 2017; Mármol et al., 2017; Mesa-Gresa et al., 2013; Soares et al., 2017). Additionally, a history of EE has been shown to enhance problem solving abilities (Forgays & Forgays, 1951; Hymovitch, 1952) and social exploration, as it reduces stress and alters emotional responses in enriched animals (Mesa-Gresa et al., 2013; van Praag et al., 2000).

The behavioral changes that occur in response to EE may be explained by global

changes that occur in the brain following enrichment, including increased synaptogenesis,

cortical thickness, glial density, dendritic branching, and neurogenesis, especially in the

dentate gyrus (DG) region of the hippocampus (Ali, Wilson, & Murphy, 2009; Birch,

McGarry, & Kelly, 2013; Eckert & Abraham, 2013; Mohammed et al., 2002). EE may

impact levels of certain neurotrophic factors, immediate early genes (i.e. *c-fos*), and

hypothalamic-pituitary-adrenal (HPA) axis activity, which may promote plasticity and

neurogenesis (Simpson & Kelly, 2011; van Praag et al., 2000). More specifically, EE

enhances levels of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF),

which promote cell survival and functions associated with synaptic plasticity (Mohammed et

al., 2002; Simpson & Kelly, 2011; van Praag et al., 2000). Studies of the HPA axis, which is

activated by stress, indicate that EE may also provide an attenuating effect on HPA

reactivity. This phenomenon may explain the alteration of emotional responses, such as

reduced stress and increased sociability in enriched animals (Simpson & Kelly, 2011; Mesa-

Gresa et al., 2013). Thus, EE may promote a variety of adaptive changes in the brain that

may impact certain aspects of behavior.

In a study conducted by Hirase and Shinohara (2014), EE was shown to promote

increased brain volume, enhanced neurogenesis, and increased neuropil complexity,

indicative of enhanced computational capability of pre-existing circuits. Continuous exposure

to enrichment during late adolescence and early adulthood has been shown to promote

increased brain weight, thicker and longer cerebral cortices, increased size of neuronal

perikarya and nuclei in the cortex, as well as more proliferative glial cells (Mohammed et al.,

2002). While many neural changes that occur in response to EE have been observed in the

absence of voluntary exercise, the inclusion of physical stimulation in enrichment protocols has been shown to produce altered choline uptake in the hippocampus and cortex, as well as increases in noradrenaline and serotonin, which have been linked to learning, plasticity, and neurogenesis in the adult brain (van Praag et al., 2000). It may be suggested that EE promotes global changes in the brain, including experience-dependent plasticity of pre-existing circuits.

In summary, the effects of EE on neuroanatomical and neurochemical parameters, which in turn, impact certain aspects of behavior (i.e. alteration in emotional responses and more efficient processing of information), may promote the overall wellbeing of enriched rats. While enrichment has been widely shown to produce lasting, global impacts on the brain, researchers have been particularly interested in the effects of EE on the structure and function of hippocampal circuits due to the opportunity for spatial learning and memory during enrichment (Hirase & Shinohara, 2014). In the current study, the influence of periodic and/or acute EE exposure on activation of neurons in the hippocampal formation (HF) is investigated.

Hippocampus

The hippocampus plays a significant role in many aspects of learning and memory, and it contributes to spatial awareness and novelty detection (Andersen, Morris, Amaral, Bliss, & O'Keefe, 2007; Lee, Hunsaker, & Kesner, 2005). The HF, a major excitatory pathway of the hippocampus, is involved in several memory processes, such as encoding, consolidation, and retrieval of spatial information. The HF pathway receives input from the entorhinal cortex (EC), which serves as the major interface between the neocortex and the hippocampus (Price, 2016). EC projects to DG, which provides input to *Cornu Ammonis*

(CA) 3 (CA3), then to CA2 and CA1, which project to the subiculum (Sub) output region of this pathway (Andersen et al., 2007; Riedel & Micheau, 2001). Axons from EC synapse with DG granule cells, which then synapse on CA pyramidal cells (Andersen et al., 2007).

The five HF structures investigated in the current study have been shown to have differential roles in hippocampal-dependent memory processes, as well as differential responses to EE (Andersen et al., 2007; Eckert et al., 2010; Riedel & Micheau, 2001). DG plays an important role in the encoding of novel spatial relationships. CA1 and CA3 are both involved with the initial formation and consolidation of spatial memory, as well as the processing and consolidation of novel information (Andersen et al., 2017). Subiculum (Sub) receives input from CA1, and it serves as a major output region of the pathway, sending efferents to the septal complex, nucleus accumbens, anterior thalamus, and mammillary nuclei (Andersen et al., 2007; Riedel & Micheau, 2001). Sub also plays an important role in the processing of spatial information, or the consolidation of information regarding the physical aspects of an environment (Andersen et al., 2007).

Thus, as a signal progresses through the HF pathway, the five structures of interest in the present study are sequentially activated (from DG to CA3 to CA2 to CA1 to Sub). A simplified schematic diagram of the connections within the HF is shown in Figure 2. Activation of the HF pathway serves as a neural substrate for the processing and encoding of environmental cues, such that activation in the earlier structures of the pathway (i.e. DG and CA3) allows for the recognition of novelty, whereas sequentially later structures of the HF (i.e. CA1 and Sub) process and consolidate novel information (Andersen et al., 2007; Cobb, 2015; Riedel & Micheau, 2001).

EE promotes a variety of neurochemical and neuroanatomical changes, including increased neurogenesis, enhanced synaptic transmission and plasticity, increases in neurotrophic factors, and the alteration of excitability in the HF (Eckert & Abraham, 2013; Mohammed et al, 2002; Ramírez-Rodríguez et al., 2013). Enrichment has been shown to promote increased neural activation in CA1 and layer five of the lateral EC, which receives and integrates cortical inputs from CA1 and Sub, indicating that EE may promote changes in the response to novelty carried out by output structures of the HF (Ali et al., 2009; Price, 2016; VanElzakker, Fevurly, Breindel, & Spencer, 2008). Additional studies have demonstrated an increase in intrinsic excitability of the CA1 region, which accompanied significant enhancements in hippocampal-dependent learning and memory following short-duration (<40 days) exposures to EE (Makara, Losonczy, Wen, & Magee, 2009; Valero-Aracama, Sauvage, & Yoshima, 2015). It seems that the up-regulation of intrinsic excitability in CA1 pyramidal cells may be explained by enhanced synaptic transmission and increased spine density, neurogenesis, and BDNF levels, suggesting changes to the structure and function of hippocampal connectivity following EE (Valero-Aracama et al., 2015). EE may therefore serve to enhance the structure, function, and efficiency of hippocampal circuits.

Importantly, the increase in CA1 excitability following EE was not observed in studies that featured longer durations (>8 weeks) of continuous enrichment exposure (Duffy, Abel, & Nguyen, 2001; Eckert, Bilkey, & Abraham, 2010). One study in particular demonstrated that continuous, long-term (3-5 months) EE exposure showed no effect on basal synaptic transmission; however, long-term EE did lead to subtle changes in synaptic plasticity within CA1 pyramidal cells, but not DG granule cells (Eckert et al., 2010). In these studies, the minimal physiological changes produced in response to long-term EE may be

explained by the animal's habituation to novel experiences due to the continuity of EE exposure. Prior research also suggests that neural activation may be altered differently between DG and CA1 structures in response to novel environment (Nitz & McNaughton, 2004). Thus, the impact of EE on the structure and function of the HF pathway may be attenuated by the animal's habituation to novelty, and differential effects may be produced between the major input and output structures of the HF.

Another study demonstrated that in response to periodic enrichment throughout adolescence, overall activation of the HF pathway was suppressed (Pavelka et al., 2017). The repeated activation of the HF during EE may have allowed rats to utilize this pathway more efficiently over time. In line with this theory, it is possible that fewer neurons in the HF pathway were activated to produce the same physiological response. This may be attributed to an increase in the density of post-synaptic receptors, allowing for more efficient synaptic transmission (Huang & Thathiah, 2015). However, this effect was only significant in the major output structures of the HF (CA1 and Sub), supporting the hypothesis that EE may differentially impact the input and output structures of the HF. Since DG and CA3 are activated early in the HF pathway, relative to CA1 and Sub, it is possible that the sequence of activation in the HF may explain the less robust changes observed in these two structures.

Contrastingly, short-term EE exposures (3-6 weeks) have been shown to promote more robust effects on early neuronal survival and synaptogenesis in DG granule cells (Birch et al., 2013). Thus, this discrepancy may suggest that the modulation of some physiological properties in response to enrichment may be dependent upon the duration and continuity of EE exposure. Short-lasting EE effects may be due to the animal's initial reaction to novelty, which may decrease over time as the animal habituates to novelty exposure. The animal's

initial response to EE has often been characterized by an increase in activation of

hippocampal networks, potentially due to an increase in post-synaptic potentials (i.e. as

indicated by an increase in c-FOS expression) that may reflect the animal's informal learning

and processing of novel information (Pavelka et al., 2017; White, 2013). In paradigms of

long-term enrichment exposure, however, activation is often suppressed, while behavioral

changes are strengthened over time (Cobb, 2015; Valero-Aracama et al., 2015). Further

research is therefore required to elucidate the mechanisms for these long-lasting behavioral

effects.

Adolescence

While the outcomes of EE are most commonly examined in adult (Ali et al., 2009;

Neidl et al., 2016; Stein, O'Dell, Funatsu, Zorumski, & Izumi, 2016) and infant animal

models (Lu et al., 2017; Soares et al., 2017; Valero-Aracama et al., 2015), several researchers

have studied EE across the lifespan as well (Buschler & Manahan-Vaughan, 2017;

Hymovitch, 1952; Mora-Gallegos et al., 2015). These studies suggest that there may be a

developmental period during which EE is most beneficial, but it has not been specified when

this critical period likely occurs. It may be suggested, however, that this period could occur

during adolescence since early exposure to EE can provide a more lasting impact on

problem-solving behaviors and spatial memory, as compared to later EE exposure during

adulthood (Hymovitch, 1952; Mora-Gallegos et al., 2015).

The few studies of enrichment among adolescents also suggest that the neurochemical

effects of EE may differ between adolescent and adult rats (Mora-Gallegos et al., 2015; van

Praag et al., 2000). While EE may serve as a protective experience against age-related

deficits in hippocampal-dependent learning and memory (Wilson, Gallagher, Eichenbaum, &

Tanila, 2006), greater EE effects are typically observed in younger, versus older, rats (Mora-Gallegos et al., 2015). Additionally, enhancements in the efficiency of synaptic transmission, as a result of EE, may be more robust during critical neurodevelopmental periods, including adolescence (Mora-Gallegos, 2015).

Adolescence is marked by an increase in risk-taking behaviors, which include increased exploration and a greater preference for novel, potentially threatening aspects of the environment (Steinberg, 2007). In rats, exploratory behaviors typically heighten during adolescence, in order to prepare the rat to leave the nest and survive in the wild by seeking potential food sources (Lynn & Brown, 2009). Adolescence may therefore serve as an especially relevant timeframe for the study of enrichment effects, particularly on the neural substrates of learning and memory. This includes learning and memory of social interactions, environments, and object placement within those environments. For this reason, the current study focuses on enrichment effects in adolescent rats.

c-FOS

In response to growth factor stimulation, the *c-fos* immediate early gene is rapidly and transiently expressed in many tissues (Sagar, Sharp, & Curran, 1988). The *c-fos* gene encodes for c-FOS protein, which often serves as an indirect measure of neural activation, since *in vitro* FOS levels rapidly increase following depolarization or stimulation with cholinergic agonists. While c-FOS is normally expressed in the rat brain, its expression transiently increases in response to neural activation (Sagar et al., 1988). Particularly relevant to the current study, c-FOS expression has been shown to increase in response to novel situations, such as an enriched environment, within the CA1 and subiculum regions of the HF (White, 2013). However, over the course of many EE sessions, c-FOS expression in the HF

may become suppressed as the animal habituates to opportunities for novelty exposure

(Pavelka et al., 2017). Thus, c-FOS expression may serve as an appropriate measure of

changes in neural activation following acute and/or periodic EE exposures. In the current

study, c-FOS immunoreactivity serves as an indicator of neural activation within the HF,

such that increased c-FOS protein expression (or an increase in the number of c-FOS+

neurons) serves as a correlative measure for areas of greater activation.

Current Study

The purpose of the current study is to elucidate the effects of both periodic and/or

acute EE exposures on neural activation within the HF of adolescent rats. Based on relevant

research (van Praag et al., 2000; Pavelka et al., 2017; White, 2013), it is hypothesized that

periodic and acute exposures to EE will both produce differences in the level of neural

activation (determined by relative c-FOS expression) observed in five HF structures of

interest, including DG, CA3, CA2, CA1, and Sub, as compared to non-enriched control rats

(Hypothesis 1).

Given the results of previous studies in our lab (Pavelka et al., 2017; White, 2013), it

is posited that a history of EE during adolescence will reduce activation in the major output

regions of the HF, including CA1 and Sub (Hypothesis 2). Less robust reductions may be

exhibited in the major input areas, DG and CA3, since these structures are activated

relatively early in the HF pathway (cf. Nitz & McNaughton, 2003).

Since previous literature (e.g., Valero-Aracama et al., 2015; White, 2013) suggests

that short-lasting EE effects may reflect the animal's initial reaction to novelty, it is

hypothesized that rats exposed to a single, acute enrichment session prior to sacrifice will

exhibit increased activation within the HF, relative to rats that did not experience an acute EE

exposure (Hypothesis 3). More specifically, rats that experienced an acute EE exposure, but not a history of periodic enrichment, (No-EE) may exhibit an increase in activation of the HF, relative to non-enriched control (No-No) rats.

Since changes in the excitability of HF structures may decrease over time as the animal habituates to novelty exposure, it is predicted that rats with a history of enrichment during adolescence may exhibit reduced activation in HF structures, relative to rats that did not experience periodic enrichment exposures (Hypothesis 4).

Materials and Methods

Subjects

Adolescent Long-Evans hooded rats (12 male, 12 female), housed in plastic shoebox cages in a humidity-controlled vivarium (12:12 h light:dark cycle), were used as subjects. Food and water were supplied *ad libitum*. Subjects were bred by Charles Rivers and cared for in 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 (Protocol #15-02).

Experimental Design

Subjects were randomly assigned to one of four groups, using a 2 X 2 (periodic enrichment X acute enrichment) experimental design (see Figure 1). One group (EE-No) received 20, 90-minute exposures to an enriched environment from postnatal day (PND) 25 to PND 48, while a different group (EE-EE) received this same periodic enrichment from PND 25 to 48, along with an acute 90-minute exposure before perfusion on PND 49. A third group (No-EE) was housed in standard home cages throughout life before receiving a single

90-minute EE exposure prior to perfusion on PND 49. A control group (No-No) was held in standard home cages throughout life and did not receive any EE exposure.

Environmental Enrichment

Between PND 25 and 48, a group of enriched rats (*n*=12, 6 male, 6 female) received a total of 20, 90-minute EE sessions. EE took place in a 45.7 X 48.3 X 78.7 cm (w X d X h) wooden frame and 1/2-in. hardware cloth cage. Objects of varying size, shape, and color were arranged on the floor and platforms of the cage, as well as hung from the cage ceiling. 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. The arrangement of objects was varied daily, according to four different set-ups that were alternated throughout the 20 EE sessions. EE sessions were given in groups of 6 same-sex conspecifics from multiple home cages to allow for the interaction with familiar and unfamiliar rats. EE provided opportunities for novelty exposure, as well as physical and social stimulation.

A group of age-matched control rats (*n*=12, 6 male, 6 female) did not receive enrichment between PND 25 and 49. Instead, they were handled twice on each EE day to control for possible confounding effects of handling, since enriched rats were regularly handled by experimenters during the transfer between cages.

Prior to sacrifice, half of the control non-enriched (*n*=6, 3 male, 3 female) and half of the periodically enriched rats (*n*=6, 3 male, 3 female) received a single 90-minute EE session on PND 49. This session represented an acute exposure to EE immediately prior to sacrifice.

Histology

Subjects were placed in quiet and dark conditions for 60 to 90 min, prior to receiving a lethal injection of sodium pentobarbital (100 mg/kg b.w., ip). Upon the absence of tail and

corneal reflexes, subjects were perfused intracardially using 10 mM sodium-potassim phosphate buffer, 7.2 to 7.4 pH, with 0.9% sodium chloride (PBS), followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB). Brains were extracted from the skulls and stored in 4% paraformaldehyde and 10% sucrose at 4 °C for one week, then transferred to PB solution with 0.1% sodium azide and stored at 4 °C until tissue was cut.

Sagittal sections (50 μm) were cut from individual hemispheres using a Vibratome® Series 1000 Sectioning System and floated in PBS. Selected sections were later processed using immunohistochemistry (ihc) to visualize the expression of c-FOS protein, a marker of neural activation (Sagar et al., 1988).

On Day 1 of ihc, sections were rinsed in PBS (2 X 5 min), followed by 0.5% hydrogen peroxide in water for 15 min. After two additional rinses in PBS (2 X 5 min), sections were incubated in 15% goat serum with 0.25% Triton-X for 60 min. Sections were then floated in anti-c-FOS made in rabbit (1:1500, Cell Signaling Technologies) at 4 °C for approximately 40 h.

On Day 2 of ihc, sections were rinsed in PBS (6 X 10 min) and incubated in biotinylated goat anti-rabbit secondary antibody (1:300, Vector Labs) for 60 min, followed by additional rinses in PBS (3 X 10 min). Sections were incubated in peroxide-labeled avidin-biotin complex (ABC, Vector Labs) for 60 minutes and rinsed again in PBS (2 X 10 min). Lastly, sections were exposed to the VIP enzyme substrate (Vector Labs) for at least 2 min. Sections were then removed from the substrate and floated in cold distilled water until they were mounted onto gel-coated slides. Once mounted and air dried, sections were dehydrated in a graded series of ethanol solutions, cleared with toluene, and cover-slipped

using Permount (Fisher). Alternate sections were similarly mounted, air dried, and processed for cell body staining to be assessed in future studies.

Microscopy and Data Analysis

Neural FOS expression densities, defined by the number of c-FOS positive (c-FOS+) neurons within a series of 200 X 200 X 50 µm sampling frames, were quantified using digital microscopy to compare levels of activation between groups. Microscopy was conducted using a Nikon Eclipse microscope and PixeLink digital camera. Images were viewed at a Plan 10 objective and 1024 X 768 pixel image size. Structures of the HF were identified using atlases of the rat brain (Paxinos & Watson, 1998; Pellegrino, Pellegrino, & Cushman, 1979).

Prior to counting, experimenters identified the darkest cell and the "lightest dark" cell within a sampling frame to use as markers. Individual markers for each section were used to reliably characterize cells as dark, versus light or medium, in appearance. Darker cells generally indicated a greater presence of c-FOS protein. The relative darkness of cells was used to categorize the level of neural activation, such that a larger proportion of dark (c-FOS+) cells within a sampling frame was used to identify areas of greater activation. For the purpose of this study, only the number of dark cells present in a sampling frame was included in the final dataset.

Prior to counting, experimenters took digital images (1024 X 768 pixels) of each structure of interest for each tissue section. Three 50 µm sagittal sections from each subject were analyzed in order to obtain an average neural density value for all five structures of interest within each subject. Using Adobe Photoshop, digital point markers were used to

mark individual dark c-FOS+ nuclei, which were presumed to be darkened in appearance relative to the image's background.

Cell counts were obtained by marking individual neurons that were present within a series of sampling frames (200 X 200 µm) through the depth of a tissue section (50 µm). Cells present on the top and/or right borders of the frame were included in the cell counts, while cells present on the lower and/or left borders of the frame were excluded. The number of dark cells in each sampling frame was recorded and averaged for each section. A total of four representative 200 X 200 X 50 µm sampling frames were chosen and counted by two individual experimenters for images that depicted DG, CA3, CA1, and Sub structures; for images that depicted CA2, only two representative sampling frames were analyzed and averaged due to the size of this structure. The cell counts obtained by each experimenter were averaged together to provide greater reliability of data. Inter-rater reliability analyses were conducted to compare the results obtained by the two individual experimenters. Neurons that appeared light or medium in coloration relative to the section's background, and were indicative of slight activation, were excluded from all analyses.

The above procedure was repeated for all HF structures of interest, including DG, CA3, CA2, CA1, and Sub, to obtain an average density of c-FOS+ neurons for each structure. Usable neural densities from regions of the HF were obtained for each experimental group, and a database of cell counts was compiled using Microsoft Excel. For each HF structure, the density of c-FOS+ neurons per section was compared between groups using a two-way analysis of variance (ANOVA) and an independent samples t-test.

Results

Inter-Rater Reliability

The densities of c-FOS+ neurons in each of the five HF structures of interest were independently evaluated by two raters. Neurons that were light or medium in coloration (indicating slight to moderate activation) were excluded from analysis. Light or medium-stained neurons were identified by comparing individual markers for each section (i.e. "lightest dark" and "darkest dark" cells), which were used to reliably characterize cells as dark, versus light or medium, in appearance. Three representative brain sections (200 X 200 X 50 μm tissue samples) were analyzed for each brain, yielding a total of 18 sections per experimental group and a total of 72 sections/observations per HF structure of interest. The results of reliability analyses revealed strong inter-rater reliability between the two raters for each HF structure: DG, $r(72) = .874$, $p<.001$; CA3, $r(72) = .747$, $p<.001$; CA2, $r(72) = .716$, $p<.001$; CA1, $r(72) = .747$, $p<.001$; Sub, $r(72) = .801$, $p<.001$. The descriptive statistics for each HF structure of interest between the two raters are provided in Table 1.

Although each experimental group originally consisted of six subjects (for a total of 24 subjects), six subjects in total were excluded from experimental analysis due to errors in perfusion and/or tissue staining, as well as due to the sequence in which tissue from individual hemispheres of rat brains were cut and processed. These procedural errors yielded two experimental groups that consisted of four subjects each (No-No and No-EE) and two experimental groups that consisted of five subjects each (EE-No and EE-EE). For some rat brains in each of the experimental groups (2 brains in No-No and No-EE groups and 1 brain in EE-No and EE-EE groups), both individual hemispheres were cut, processed, and analyzed to yield a total of 72 sections/observations per HF structure.

Acute Enrichment

Comparisons using independent samples t-tests revealed that activation in the internal processing regions, CA3, CA2, and CA1, as well as in Sub, a major output structure of the HF, significantly differed between the No-EE and No-No groups (all $p<.05$). Average neural densities of c-FOS+ neurons were greater in CA3 (+170%), CA2 (+135%), CA1 (+185%), and Sub (+163%) in the No-EE group, compared to the No-No controls. Thus, Hypothesis 1, which predicated that a final, acute exposure to EE would produce differences in HF activation compared to non-enriched (No-No) control rats, was supported for all HF structures of interest with the exception of DG (see Figure 4). This finding supports the results of previous studies that have reported an increase in c-FOS expression following a single enrichment exposure (Ali et al., 209; VanElzakker et al., 2008; White, 2013).

On average, rats that experienced a final EE exposure exhibited a 163% increase in activation of these four structures, compared to No-No control rats. In DG, a major input structure of the HF, the No-EE group exhibited 105% more c-FOS+ neurons per 200 X 200 X 50 μm sampling frame than the No-No control group; however, this difference was not statistically significant, $t(5) = 2.41$, $p=.053$. Thus, Hypothesis 2, which predicted that less robust reductions would be exhibited in the major input areas, DG and CA3, was partially supported. This finding supports the results of previous studies that have suggested differential effects of enrichment in the major input and output structures of the HF (Eckert et al., 2010; Valero-Aracama et al., 2015). The descriptive statistics of c-FOS+ neural densities, for each HF structure of interest, among the four experimental groups are provided in Table 2.

A significant interaction was observed between an acute, final EE exposure and activation in all five HF structures of interest, including DG ($F(1,14) = 10.74$, $p<.01$), CA3

($F(1,17) = 14.81$, $p<.002$), CA2 ($F(1,17) = 7.37$, $p<.02$), CA1 ($F(1,17) = 11.68$, $p< .01$), and

Sub ($F(1,17) = 24.69$, $p<.01$). Since subjects who experienced an acute EE exposure, but not

a history of periodic enrichment (No-EE), exhibited an increase in HF activation relative to

non-enriched controls (No-No), Hypothesis 3 was supported. Further evidence for these

findings is provided by qualitative differences in the observed activation within tissue

samples of No-EE and No-No groups (see Figures 5-9).

Periodic Enrichment

While periodic enrichment produced significant differences in the activation of four

HF structures (i.e. CA1, CA2, CA3, and Sub) between No-EE and EE-EE groups (all $p<.03$),

with EE-EE rats exhibiting significantly less activation than No-EE rats, significant group

differences were not observed between the EE-No and No-No control rats. In the EE-No rats,

activation was only slightly reduced in CA1 (-30%), CA2 (-5%), CA3 (-6%), DG (-6%), and

Sub (-32%) compared to the No-No rats, and these differences were not statistically

significant (all $p>.05$). Thus, Hypothesis 1 could not be supported by these results, such that

periodic enrichment did not produce significant effects on HF activation, compared to non-

enriched control rats. Importantly, however, it appears that periodically enriched rats

continued to exhibit a slight suppression of activation compared to the non-enriched rats,

although statistically insignificant.

In the EE-EE group, mean densities of c-FOS+ neurons were significantly reduced in

CA3 (-43%), CA2 (-47%), CA1 (-64%), and Sub (-41%) regions (all $p <.03$) compared to the

No-EE group. This group comparison suggests a significant reduction of neural activation in

response to periodic enrichment throughout adolescence. However, in the DG region, neural

activation was only slightly reduced (-17%) in the EE-EE group compared to the No-EE

group, and this difference was not statistically significant, $t(5) = 0.90$, $p=.20$. These findings support Hypothesis 2, which predicted that periodic enrichment throughout adolescence would significantly impact activation in the major output structures of the HF (i.e. CA1 and Sub) and would produce less significant effects on the activation of HF input structures, such as DG (see Figure 4). Additionally, these findings support the results of previous studies, which suggested that less robust effects are produced in HF input regions, particularly DG, in response to periodic enrichment (Pavelka et al., 2017; White, 2013).

There was a significant interaction between a history of periodic EE throughout adolescence and activation for three of the five HF structures of interest, including CA1 ($F(1,14) = 13.28$, $p <.01$), CA3 ($F(1, 17) = 4.72$, $p<.05$), and Sub ($F(1,17) = 9.03$, $p <.01$). However, this interaction was not significant for CA2 ($F(1,17) = 4.18$, p = .06) and DG ($F(1,17) = .76$, $p = .397$) regions. The descriptive statistics of c-FOS+ neural densities for each HF structure of interest are provided in Table 2. With the exception of CA2 and DG structures, Hypothesis 4 was supported, such that rats with a history of enrichment during adolescence (EE-EE) exhibited suppressed activation in HF structures, relative to rats that did not experience periodic enrichment exposures (No-EE). The group differences that support Hypothesis 4 can be observed both quantitatively (see Table 2) and qualitatively, given observable differences in activation among representative tissue samples from each experimental group (see Figures 6, 8, and 9).

Discussion

The wealth of previous literature on EE has provided substantial evidence to suggest a number of beneficial effects on the brain and behavior of several mammalian species (Mesa-Gresa et al., 2013; Mohammed et al., 2002; van Praag et al., 2000). Importantly, EE has been shown to improve aspects of spatial learning and memory and problem-solving

skills, as it promotes several adaptive changes in the brain (Mohammed et al., 2002; Simpson

& Kelly, 2011; van-Praag et al., 2000). These changes occur both generally in the brain and

specifically within regions involved in the processing of novel spatial information, including

the hippocampus (Stein et al., 2016). Since it is believed that memory storage occurs as a

result of changes in synaptic strength and efficiency, previous studies have suggested that

alterations in synaptic plasticity may partly explain the cognitive benefits of EE (Eckert et al.,

2010; Stein et al., 2016). However, the variability of EE protocols has produced mixed

results regarding this explanation. Importantly, EE effects have been shown to vary

depending on the age of subjects, the duration of EE exposures (and whether it is continuous

or interrupted), as well as the brain regions assessed (Bennett et al., 2006; Eckert et al., 2010;

Mora-Gallegos et al., 2015).

The current study sought to elucidate the effects of both periodic and/or acute EE

exposures on the activation of five major structures in the HF pathway, specifically within

early adolescent rats. The study examined the individual effects of experiencing consistent,

periodic EE exposures during adolescence, a single EE exposure prior to sacrifice, or both,

by comparing the relative levels of c-FOS expression from subjects who had been

periodically and/or acutely enriched to non-enriched subjects.

Hypothesis 1

Hypothesis 1 proposed that both periodic and/or acute enrichment experiences would

promote differences in the level of HF activation compared to non-enriched subjects, since

previous literature has demonstrated a variety of changes that occur in the brain as a result of

EE (Mohammed et al., 2002; van Praag et al., 2000). This hypothesis was partially supported.

Subjects who received periodic enrichment throughout adolescence, but not a final acute EE

exposure (EE-No), did not display significant differences in HF activation compared to non-

enriched (No-No) control rats. This finding was unexpected given that a previous study in

our lab has shown significant reductions in HF activation following periodic EE exposures during adolescence (Pavelka et al., 2017).

However, it may be suggested that the lack of significant differences between HF activation in EE-No and No-No groups could be attributed to the similarity of their final experiences prior to sacrifice. Unlike acutely enriched rats, both EE-No and No-No rats were housed in standard shoebox cages on the day of sacrifice; thus, these two groups exhibited similar levels of activation since the HF pathway was not being readily utilized during their final experience. Additionally, subjects who received both periodic and acute EE exposures did not display significant differences in HF activation compared to No-No controls. Subjects who solely received a final acute EE exposure (No-EE) exhibited significantly greater levels of activation in all HF structures of interest, with the exception of DG. Thus, in comparison to non-enriched controls, significant group differences in HF activation were solely produced by a final, acute EE exposure and were not shown in either periodic EE condition.

Previous literature on the benefits of periodic enrichment suggests that cognitive and behavioral EE effects are produced by improved efficacy of neural circuits responsible for environmental processing (Birch et al., 2013; Buschler & Manahan-Vaughan, 2017; Hirase & Shinohara, 2014). In the current study, it is unclear whether periodic EE throughout adolescence promoted more efficient circuits responsible for the processing of spatial information. The absence of activational differences between EE-No and No-No rats could be interpreted by a lack of alteration in synaptic efficiency of the HF pathway; however, this finding would contradict the results of former studies, which reported significant changes in the structure and function of hippocampal and cortical circuits in response to EE (Hirase & Shinohara, 2014; Makara et al., 2009). It is important to note that in these studies, synaptic

and dendritic plasticity were measured using a variety of methods that did not include the assessment of c-FOS expression.

Thus, it is possible that any improvement in spatial and working memory following periodic enrichment was not effectively shown by relative levels of c-FOS expression used in the current study. An increase in the number of activated neurons, measured by c-FOS expression, may not have been appropriately reflective of potential changes in the efficiency of that particular HF structure (Buschler & Mahanan-Vaughan, 2012; White, 2013). Perhaps, further research may employ alternative methods to more effectively assess plasticity in response to periodic and/or acute EE sessions during early adolescence.

Hypothesis 2

Hypothesis 2 suggested that a history of EE during adolescence would reduce activation primarily in the major output regions of the HF, including CA1 and Sub. It was proposed that in response to periodic EE, less robust reductions would be shown in the major input areas of the HF, including DG and CA3 (Pavelka et al., 2017). This hypothesis was partially supported. Significant group differences were observed in the activation of CA3 between EE-EE and No-EE groups. In comparison to No-No control rats, the CA3 region of periodically enriched (EE-No) subjects showed a minimal increase in activation, similar to that of DG and CA2. Previous studies regarding the effects of periodic EE on synaptic plasticity in CA3 have shown mixed results (Eckert et al., 2010; White, 2013). It has been suggested that CA3 may store the patterns of activity produced by DG; this information may then be projected to CA1, stratum radiatum, and stratum oriens, but whether these pathways play different roles in learning and memory is poorly understood (Eckert et al., 2010). Thus, further research is needed to fully elucidate the mechanisms of EE effects and the differential

roles that individual HF structures, and their associated projections, may play during an enriching experience.

Among all group comparisons using independent samples t-tests, significant differences in the activation of DG granule cells were not observed. This finding supported the results of former studies (Nitz & McNaughton, 2003; Pavelka et al., 2017), which suggested that less robust EE effects may be observed in structures that are activated relatively early in the HF pathway. According to Nitz & McNaughton (2003), neural activation may be differentially altered between DG and CA1 structures in response to novel environments, such that neural activation may be increased in DG and suppressed in CA1 following periodic enrichment. This discrepancy may likely be attributed to the sequence in which these structures are activated in the HF pathway, as well as their differential roles in the processing and consolidation of novel spatial information (Nitz & McNaughton, 2003).

The absence of significant group differences in the activation of DG granule cells between periodically enriched and non-enriched subjects was expected, given the results of previous studies (Eckert et al., 2010; Hirase et al., 2014). Following long-term enrichment (3+ months), basal synaptic transmission was unaffected in DG granule cells (Eckert et al., 2010). The lack of robust EE effects in regards to DG granule cell activation, compared to the activation of other HF structures, may be explained by the specific inputs that each structure receives (Andersen et al., 2007). DG primarily receives input from EC layer II, whereas CA3, CA2, CA1, and Sub, all receive input from at least two structures in the HF pathway (Wilson et al., 2006).

Additionally, the functional role of DG in identifying novel spatial relationships may partly influence this discrepancy as well. According to Nitz & McNaughton (2003),

differences in activation rates of DG and CA1 interneurons may bias the type of information (i.e. landmark vs. self-motion information) that is identified and processed during the development of a cognitive map of the novel environment.

Since the activation of DG is specifically associated with the recognition of novel aspects in the environment, it may be suggested that ability to identify novelty did not differ between periodically enriched and non-enriched rats (VanElzakker et al., 2008). However, structures that play a greater role in the processing and encoding of novel spatial information (i.e. CA1 and Sub) showed more robust group differences in activation. In other words, periodically enriched and non-enriched subjects may have differed in the processing and encoding of novel information, but subjects likely did not differ in their abilities to recognize novel, versus familiar, aspects of the environment (Van Elzakker et al., 2008). Since periodically enriched animals showed slight reductions in the activation of HF output structures, it may be suggested that the processing and encoding of novel information was accomplished more efficiently, requiring fewer neurons to become activated in order to accomplish the same result of memory storage (Pavelka et al., 2017). Further research utilizing more appropriate measures of plasticity and neurogenesis may be useful in corroborating this explanation.

Hypothesis 3

Hypothesis 3, which proposed that rats exposed to a single, acute enrichment session prior to sacrifice would exhibit increased HF activation, relative to rats who did not experience an acute EE exposure, was supported. Average neural densities were significantly greater in subjects who solely received an acute EE experience (No-EE), compared to non-enriched (No-No) controls. This finding was significant for all HF structures of interest, with

the exception of DG, and it supported the findings of previous studies (i.e. Valero-Aracama et al., 2015; White, 2013). It is suggested that the increase in HF activation following an acute exposure may be interpreted by the subject's initial recognition, processing, and encoding of novel spatial information (Pavelka et al., 2017; VanElzakker et al., 2008).

Interestingly, however, no group differences were observed between the HF activation levels of subjects who received periodic and acute EE exposures (EE-EE) versus non-enriched controls. It is possible that a history of periodic enrichment throughout adolescence greatly attenuated the effect of an acute EE exposure, since this final EE session was identical to all previous EE sessions and periodically enriched subjects likely habituated to the experience of novelty exposure (Pavelka et al., 2007; White, 2013). Thus, in the final, acute EE session, periodically enriched animals (EE-EE) were much less reactive to novel aspects of the environment, compared to those who were exposed to EE for the first time prior to sacrifice (No-EE). This may explain the presence of significant group differences between No-EE and No-No rats, as well as the absence of group differences between EE-EE and No-No rats (Pavelka et al., 2007). Essentially, both conditions of periodic enrichment (EE-No and EE-EE) produced statistically similar activation levels to the control condition (No-No).

Acute enrichment provided a significant increase in the level of HF activation, reflecting the subjects' initial learning and memory processes that occur following a single, short-term EE exposure (VanElzakker et al., 2008). This finding supports the notion that EE provides an opportunity for informal learning and memory through novelty exposure, in addition to physical and social stimulation (van Praag et al., 2000). However, over time, this

stimulation may become less impactful as the animal habituates to the enrichment experience.

Hypothesis 4

Hypothesis 4, which anticipated that subjects who were enriched throughout adolescence would exhibit reduced HF activation, relative to subjects who were not periodically enriched, was supported. Subjects who experienced both periodic EE exposures and an acute EE experience prior to sacrifice (EE-EE) exhibited significantly fewer c-FOS+ neurons in all HF structures of interest, with the exception of DG, compared to those who solely experienced a final, acute EE exposure (No-EE). This group comparison provides evidence for the attenuating effect of periodic enrichment on HF activation, whereas the group comparison of EE-No and No-No groups did not yield significant differences.

This discrepancy also supports the results of a previous study (White, 2013), which reported no significant group differences between periodically enriched and non-enriched control rats. EE-EE and No-EE groups exhibited significant differences in activation in both the current study and White (2013). In these groups, a final EE experience may serve as a challenge stimulus, which allows us to determine the influence of a history of periodic EE. As previously discussed, it may be suggested that an acute EE experience prior to sacrifice amplifies the impact of an enrichment history during adolescence.

Future Directions

In the current study, an increase in neural activation, measured indirectly by levels of c-FOS expression, may not have appropriately reflected changes in the efficiency of synaptic transmission or the plasticity of HF structures. Further research may employ alternative methods to more effectively assess changes in plasticity in response to periodic and/or acute

EE sessions during adolescence. For example, electrophysiological recordings and biochemical assays may serve as more appropriate measures for investigating long-term changes in synaptic plasticity. Employing these methods in supplement to measuring behavioral changes following EE (i.e. alterations in object recognition, novelty preference, and performance on spatial learning and memory tasks) may provide a useful investigation of how the structure and function of hippocampal circuits are affected by both periodic and/or acute EE.

Summary and Conclusions

A final, acute enrichment experience prior to sacrifice promotes an increase in neural activation of the HF pathway. However, this effect is attenuated among subjects who have experienced a history of periodic enrichment throughout adolescence, since this final experience closely resembles former EE exposures. In subjects who are exposed to EE for the first time briefly prior to sacrifice, a large increase in HF activation may reflect the subjects' initial reaction to novelty exposure (VanElzakker et al., 2008). In response to a new, enriching environment, subjects employ hippocampal circuits responsible for the recognition, processing, and encoding of novel spatial information, which may explain the large activational increases following their first enrichment exposure (Lu et al., 2017). However, over the course of several periodic EE sessions, subjects may habituate to the experience of novel situations and may become less reactive to novelty (Pavelka et al., 2017).

A history of periodic EE during adolescence may attenuate activation in the HF pathway, primarily in the major output regions (i.e. CA1 and Sub). This effect was amplified in the presence of a final, acute EE exposure prior to sacrifice. The reduction in c-FOS expression, a marker of neural activation, following periodic enrichment may be explained

by the improved efficiency of circuits responsible for processing spatial information (Eckert & Abraham, 2013). Thus, fewer neurons in a given structure, particularly in CA1 and Sub, must be activated in order to process novel spatial information. This increased efficiency may be attributed to enhanced synaptic transmission; for example, an upregulation in post-synaptic receptors may allow for more efficient synaptic transmission, and thus, more efficient information processing (Huang & Thathiah, 2015).

DG, which operates relatively early in the HF pathway and primarily receives input from EC layer II, reliably shows less robust activational changes in response to periodic and/or acute enrichment (Andersen et al., 2007; Pavelka et al., 2017). This finding supports the notion that EE may promote differential effects between major input and output structures of the HF pathway, including a greater level of neurogenesis in input structures (particularly DG) and a greater level of synaptic plasticity in output structures (Eckert et al., 2010).

In summary, EE may provide lasting, adaptive changes to the structure and function of neural circuits, including more efficient processing of novel information. These effects are particularly critical during adolescence, a period marked by an increased frequency of exploratory and risk-taking behaviors (Lynn & Brown, 2009). Thus, the diversity of experiences in early adolescence may promote neural changes that lead to enhanced cognitive performance and resilience against age-related cognitive declines throughout later adolescence and adulthood (Soares et al., 2017).

References

Ali, A. E. A., Wilson Y. M., & Murphy M. (2009). A single exposure to an enriched

environment stimulates the activation of discrete neuronal populations in the brain of

the fos-tau-lacZ mouse. *Neurobiology of Learning and Memory, 92*(3), 381-90. doi:

10.1251/bpo128

Andersen, P., Morris, R., Amaral, D., Bliss, T., & O'Keefe, J. (2007). *The Hippocampus

Book*. New York: Oxford University Press.

Bennett, J. C., McRae, P. A., Levy, L. J., & Frick, K. M. (2006). Long-term continuous, but

not daily, environmental enrichment reduces spatial memory decline in aged male

mice. *Neurobiology, Learning, & Memory, 85*(2), 139-152.

Birch, A. M., McGarry, N. B., & Kelly, A. M. (2013). Short-term environmental enrichment

in the absence of exercise, improves memory, and increases NGF concentration, early

neuronal survival, and synaptogenesis in the dentate gyrus in a time-dependent

manner. *Hippocampus, 23*, 437-450.

Buschler, A., & Manahan-Vaughan, D. (2017). Metabotropic glutamate receptor, mGlu5,

mediates enhancements of hippocampal long-term potentiation after environmental

enrichment in young and old mice. *Neuropharmacology, 115,* 42-50.

Cobb, D. E. (2015). *Environmental enrichment promotes adaptation to environmental

rearrangement in younger but not older adolescent rats.* (Undergraduate Honors

Thesis). Retrieved from Appalachian State University Library Database. LD175 .E5

No.1140.

Dhanushkodi, A., Bindu, B., Raju, T. R., & Kutty, B. M. (2007). Exposure to enriched

environment improves spatial learning performances and enhances cell density but

not choline acetyltransferase activity in the hippocampus of ventral subicular-lesioned rats. *Behavioral Neuroscience, 121*(3), 491-500.

Duffy, S. N., Craddock, K. J., Abel, T., & Nguyen, P. V. (2001). Environmental enrichment modifies the PKA-dependence of hippocampal LTP and improves hippocampus-dependent memory. *Learning & Memory, 8*(1), 26-34.

Eckert, M. J., & Abraham, W. C. (2013). Effects of environmental enrichment exposure on synaptic transmission and plasticity in the hippocampus. *Current Topics in Behavioral Neurosciences, 15,* 165-187.

Eckert, M. J., Bilkey, D. K., & Abraham, W. C. (2010). Altered plasticity in hippocampal CA1, but not dentate gyrus, following long-term environmental enrichment. *Journal of Neurophysiology, 103*(6), 3320-3329.

Forgays, D. G., & Forgays, J. W. (1951). The nature of the effect of free-environmental experience in the rat. *Journal of Comparative Physiolgical Psychology, 45*(4), 322-8.

Hirase, H., & Shinohara, A. Y. (2014). Transformation of cortical and hippocampal neural circuit by environmental enrichment. *Neuroscience, 280,* 282-298.

Huang, Y., & Thathiah, A. (2015). Regulation of neuronal communication by G protein-coupled receptors. *Federation of European Biochemical Societies Letters, 589,* 1607-1619.

Hullinger, R., O'Riordan, K., & Burger, C. (2015). Environmental enrichment improves learning and memory and long-term potentiation in young adult rats through a mechanism requiring mGluR5 signaling and sustained activation of p70s6k. *Neurobiology of Learning and Memory, 125,* 126-134.

Hymovitch, B. (1952). The effects of experimental variations on problem solving in the rat. *Journal of Comparative Physiological Psychology, 45*(4), 313-21.

Lee, I., Hunsaker, M.R., & Kesner, R.P. (2005). The role of hippocampal subregions in detecting spatial novelty. *Behavioral Neuroscience 119,* 145–153. doi: 10.1037/0735-7044.119.1.145

Li, S., Jin, M., Zhang, D., Yang, T., Koeglsperger, T., Fu, H., & Selkoe, D. J. (2013). Environmental novelty activates β_2-adrenergic signaling to prevent the impairment of hippocampal LTP by Aβ oligomers. *Neuron, 77,* 929-941.

Lu, C.,-Q. Zhong, L., Yan, C.-H., Tian, Y., Shen, X. (2017). Effects of preweaning environmental enrichment on hippocampus-dependent learning and memory in developing rats. *Neuroscience Letters, 640*, 117-122. doi: 10.1016/j.neulet.2016.12.053

Lynn, D. A., & Brown, G. R. (2009). The ontogeny of exploratory behavior in male and female adolescent rats (*Rattus norvegicus). Developmental Psychobiology, 51*(6), 513-20.

Mármol, F., Sánchez, J., Torres, M. N., & Chamizo, V. D. (2017). Environmental enrichment in the absence of wheel running produces beneficial behavioural and anti-oxidative effects in rats. *Behavioural Processes, 144,* 66-71.

Makara, J. K., Losonczy, A., Wen, Q., & Magee, J. C. (2009). Experience-dependent compartmentalized dendritic plasticity in rat hippocampal CA1 pyramidal neurons. *Nature Neuroscience, 12*(12), 1485-1487.

Mesa-Gresa, P., Pérez-Martinez, A., & Redolat, R. (2013). Environmental enrichment

improves novel object recognition and enhances agonistic behavior in male mice.

Aggressive Behavior, 39, 269-279.

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B.,

… Bogdanovic, N. (2002). Environmental enrichment and the brain. *Progress in

Brain Research*, 138, 109-120.

Mora-Gallegos, A., Rojas-Carvajal, M., Salas, S., Saborío-Arce, A., Fornaguera-Trías, J., &

Brenes, J. C. (2015). Age-dependent effects of environmental enrichment on spatial

memory and neurochemistry. *Neurobiology of Learning and Memory, 118,* 96-104.

Neidl, R., Schneider, A., Bousiges, O., Majchrzak, M., Barbelivien, A., Pereira de

Vasconcelos, A., … Boutillier, A.-L. (2016). Late-life environmental enrichment

induces acetylation events and nuclear factor κB-dependent regulations in the

hippocampus of aged rats showing improved plasticity and learning. *The Journal of

Neuroscience, 36*(15): 4351-4361.

Nitz, D. & McNaugton, B. (2004). Differential modulation of CA1 and dentate gyrus

interneurons during exploration of novel environments. *Journal of Neurophysiology,

91*, 863-72. doi: 10.1152/jn.00614.2003

Novokovic, T. Heumann, R., & Manahan-Vaughan, D. (2015). Ras does not contribute to the

facilitation of hippocampal synaptic plasticity enabled by environmental enrichment.

Neuroscience, 309, 214-223.

Paxinos, G., & Watson, C. (1998). *The rat brain: In stereotaxic coordinates*(4th ed.). San

Diego: Academic Pr.

Pellegrino, L. J., Pellegrino, A. S., & Cushman, A. J. (1979). *A stereotaxic atlas of the rat brain* (2nd ed.).New York, NY: Plenum Publishing.

Price, J. L. (2016). Olfactory higher centers anatomy. In *Encyclopedia of Neuroscience*. (Vol. 1, pp. 129-136). Springer Berlin Heidelberg.

Ramírez-Rodríguez, G., Ocaña-Fernández, M. A., Vega-Rivera, N. M., Torres-Pérez, O. M., Gómez-Sánchez, A., Estrada-Camarena, E., & Ortiz-López, L. (2013). Environmental enrichment induces neuroplastic changes in middle age female BalbC mice and increases hippocampal levels of BDNF, P-Akt, and P-MAPK1/2. *Neuroscience, 260*, 158-170.

Riedel, G., & Micheau, J. (2001). Function of the hippocampus in memory formation: Desperately seeking resolution. *Progress Neuro-Psychopharamacology & Biological Psychiatry,* 25, 835-853.

Sagar, S. M., Sharp, F. R., & Curran, T. (1988). Expression of c-fos protein in brain: Metabolic mapping at the cellular level. *Science*, 240(4857), 1328-1331.

Simpson, J., & Kelly, J. P. (2011). The impact of environmental enrichment in laboratory rats – Behavioural and neurochemical aspects. *Behavioural Brain Research, 222*, 246-264.

Soares, R. O., Horiquini-Barbosa, E., Almeida, S. S., Lachat, J. J. (2017). Environmental enrichment protects spatial learning and hippocampal neurons from the long-lasting effects of protein malnutrition early in life. *Behavioural Brain Research, 335,* 55-62.

Stein, L. R., O'Dell, K. A., Funatsu, M., Zorumski, C. F., & Izumi, Y. (2016). Short-term environmental enrichment enhances synaptic plasticity in hippocampal slices from aged rats. *Neuroscience, 329,* 294-305.

Steinberg, L. (2007). Risk taking in adolescence: New perspectives from brain and

    behavioral science. *Current Directions in Psychological Science*, 16(2), 55-59.

Valero-Aracama, M. J., Sauvage, M. M., & Yoshida, M. (2015). Environmental enrichment

    modulates intrinsic cellular excitability of hippocampal CA1 pyramidal cells in a

    housing duration and anatomical location-dependent manner. *Behavioural Brain*

    Research, 292, 209-218.

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of

    environmental enrichment. *Nature Reviews: Neuroscience, 1*, 191-198.

VanElzakker, M., Fevurly, R. D., Breindel, T., & Spencer, R. L. (2008). Environmental

    novelty is associated with a selective increase in Fos expression in the output

    elements of the hippocampal formation and the perirhinal cortex. *Learning and*

    Memory, 15(12), 899-908. doi: 10.1101/lm.1196508

Vivinetto, A. L., Suárez, M. M., & Rivarola, M. A. (2013). Neurobiological effects of

    maternal separation and post-weaning environmental enrichment. *Behavioural Brain*

    Research, 240, 110-118.

White, W. C. (2013). *Effects of Periodic and/or a Single Exposure to an Enriched*

    Environment on Neural C-fos Expression in Adolescent Rats. (Master's Thesis).

    Retrieved from Appalachian State University Library Database. LD175 .A40K Th

    2131.

Wilson, I. A., Gallagher, M., Eichenbaum, H., & Tanila, H. (2006). Neurocognitive aging:

    prior memories hinder new hippocampal encoding. *TRENDS in Neurosciences,*

    29(12), 662-670.

Table 1

Descriptive Statistics for c-FOS+ Neural Densities (in a 200 X 200 X 50 μm Tissue Sample)

in Each Hippocampal Formation Structure of Interest Across All Experimental Groups,

Counted Independently by Two Raters

HF Structure	Rater	*N*	*M*	*SD*
DG	1	72	4.75	2.27
	2	72	4.30	2.12
CA1	1	72	12.15	9.70
	2	72	11.32	9.08
CA2	1	72	6.51	3.99
	2	72	6.04	3.17
CA3	1	72	5.42	3.30
	2	72	5.04	2.86
Sub	1	72	10.08	6.26
	2	72	10.04	6.42

Table 2

Descriptive Statistics for c-FOS+ Neural Densities (in a 200 X 200 X 50 μm Tissue Sample)

in Hippocampal Formation Structures of Interest for Each Experimental Group

		EE-EE		EE-No		No-EE		No-No
	n	*M(SD)*	*n*	*M(SD)*	*n*	*M(SD)*	*n*	*M(SD)*
DG	5	5.2(1.4)	5	3.3(1.5)	4	6.3(2.1)	4	3.1(1.6)
CA3	5	5.1(1.5)	5	3.5(1.8)	4	9.0(2.0)*	4	3.3(2.4)
CA2	5	5.5(2.0)	5	4.6(2.4)	4	10.4(3.5)*	4	4.4(2.5)
CA1	5	8.4(2.8)	5	5.8(3.6)	4	23.5(8.2)*	4	8.3(5.7)
Sub	5	10.2(2.8)	5	4.5(2.6)	4	17.4(4.2)*	4	6.6(4.3)

Note. The significant difference between No-EE and No-No groups is indicated ($p<.03$).

Abbreviations: dentate gyrus, DG; *Cornu Ammonis* 3, CA3; *Cornu Ammonis* 2, CA2; *Cornu*

Ammonis 1, CA1; subiculum, Sub; history of periodic enrichment and an acute enrichment

exposure, EE-EE; history of periodic enrichment without an acute exposure, EE-No; acute

enrichment exposure only, No-EE; non-enriched controls, No-No

Figure 1. Experimental Design with Groups Characterized by History of EE (EE-no and EE-

EE) and/or Presence of Acute EE Exposure Prior to Sacrifice (no-EE and EE-EE)

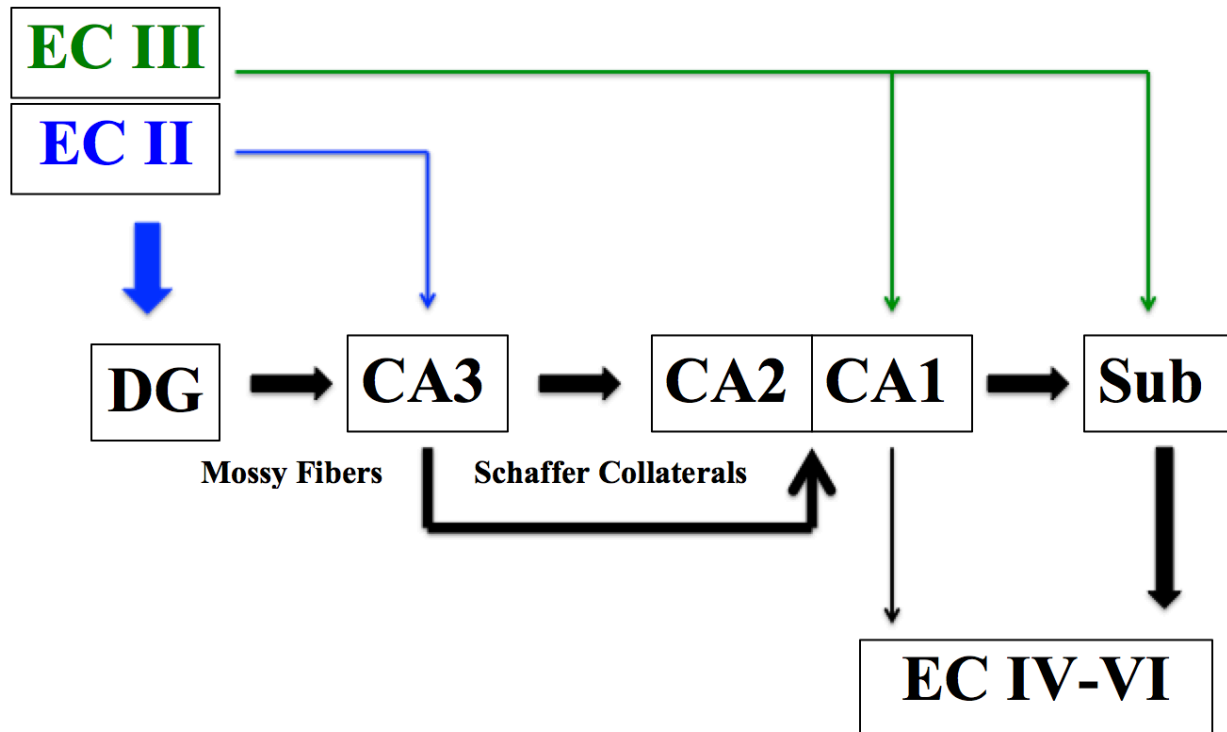|	Acute EE Exposure (Prior to Sacrifice)	
History of Periodic EE During Adolescence	No-No (*n*=4)	No-EE (*n*=4)
	EE-No (*n*=5)	EE-EE (*n*=5)

Figure 2. Schematic diagram, adapted from Wilson et al. (2006), of the sequence in which major regions of the hippocampal formation (HF) are activated by input from the entorhinal cortex (EC). Layer III of EC (EC III) provides input to *Cornu Ammonis* 1 (CA1) and subiculum (Sub), which serve as output structures of the HF and eventually feed back to EC layers IV-VI (EC IV-VI). EC layer II (EC II) provides input to dentate gyrus (DG) and *Cornu Ammonis 3* (CA3). DG feeds into CA3 via mossy fibers, and CA3 feeds into *Cornu Ammonis* 2 (CA2) and CA1 via Schaffer collaterals. Bolded arrows indicate major input/output regions, and smaller arrows represent areas that receive minor input/output. Pathways not summarized in this figure include: lateral versus medial EC projection systems, feedback inputs to/from CA3 via auto-associative fibers, and hilar neurons, which are reciprocally connected to DG.

Figure 3. Example enrichment cage with objects of varying size, shape, color, and texture.

Objects featured in this particular set-up include a mug, foam football, gardening glove, and

others. The male enrichment cage (not shown in this figure) was designed as a mirror image

of the female enrichment cage, featuring an identical arrangement of objects. Objects such as

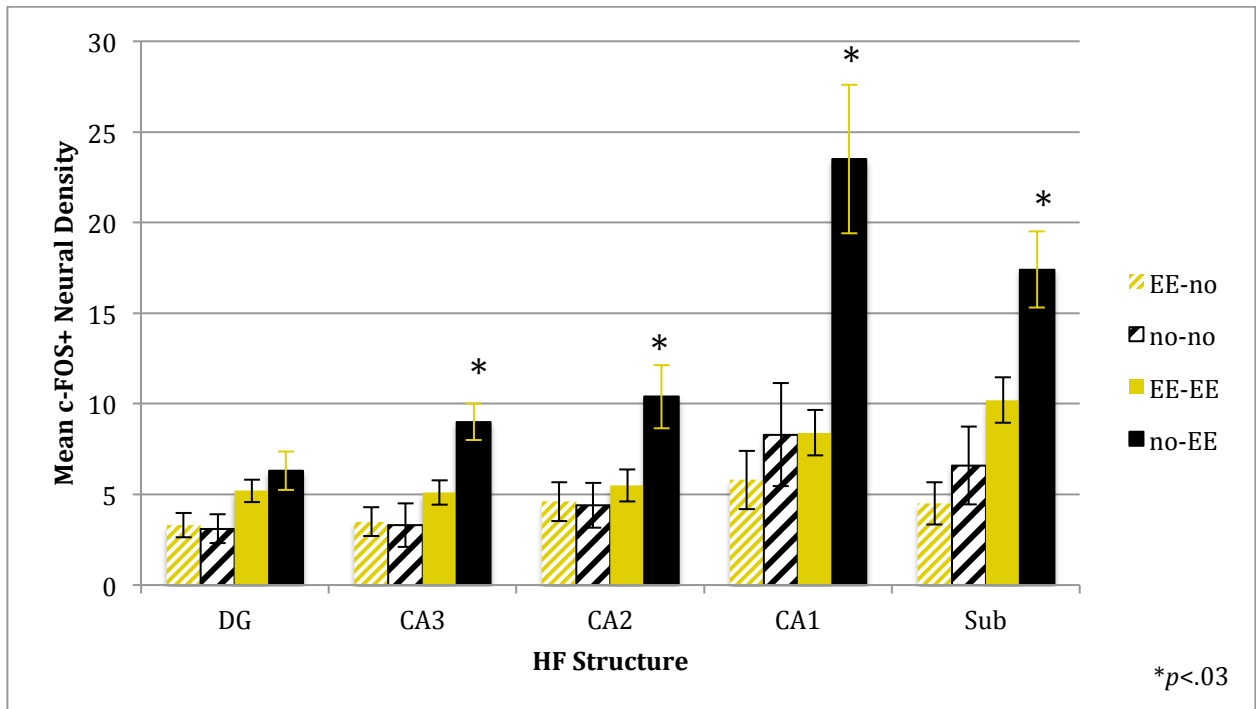food and running wheels were excluded from enrichment cages.

Figure 4. Mean neural densities of c-FOS+ neurons (per 200 X 200 X 50 μm sample) in dentate gyrus (DG), *cornu ammonis* 3 (CA3), *cornu ammonis* 2 (CA2), *cornu ammonis* 1 (CA1), and subiculum (Sub) for each experimental condition. Error bars represent standard error of mean (SEM) for each experimental condition and HF structure. Across all HF strucutres, an acute enrichment exposure prior to sacrifice yielded greater c-FOS+ neural densities. Periodic enrichment exposures yielded slightly suppressed c-FOS activation compared to No-No controls in CA1 and Sub output structures. Less robust group differences were observed in DG and CA3 input structures.
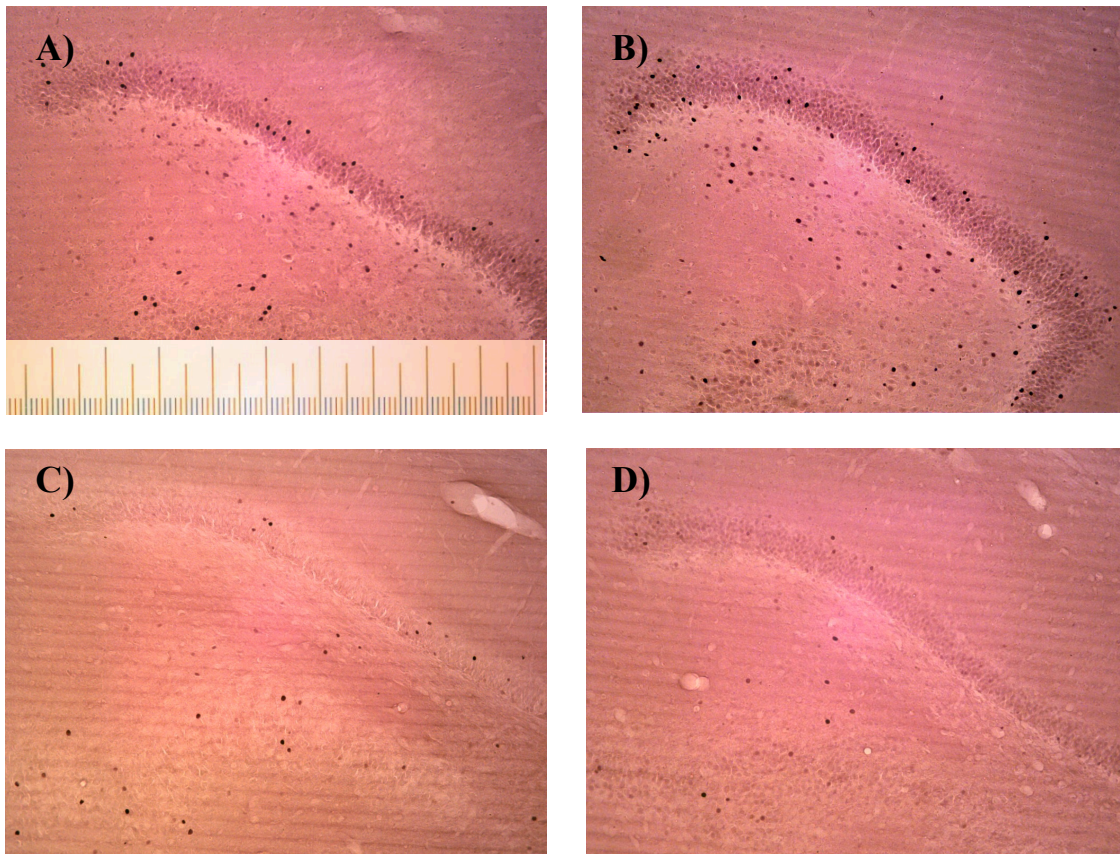
Figure 5. Digital microscopy images of dentate gyrus (DG) granule cells in tissue sections (200 X 200 X 50 µm), representative of each experimental group. A) DG region of hippocampus in control (No-No) rats. B) DG region in No-EE rats. Relative to No-No rats, the DG region of No-EE rats exhibited an average of 105% more c-FOS+ neurons (*p*=.053). Relative to the EE-EE rats (D), the DG region of No-EE rats exhibited an average of 17% fewer c-FOS+ neurons (*p*=.20). C) DG region in EE-No rats. D) DG region in EE-EE rats. Each image is 1000 µm, indicated by the scale (A), with larger divisions representing 100 µm and smaller divisions representing 10 µm.
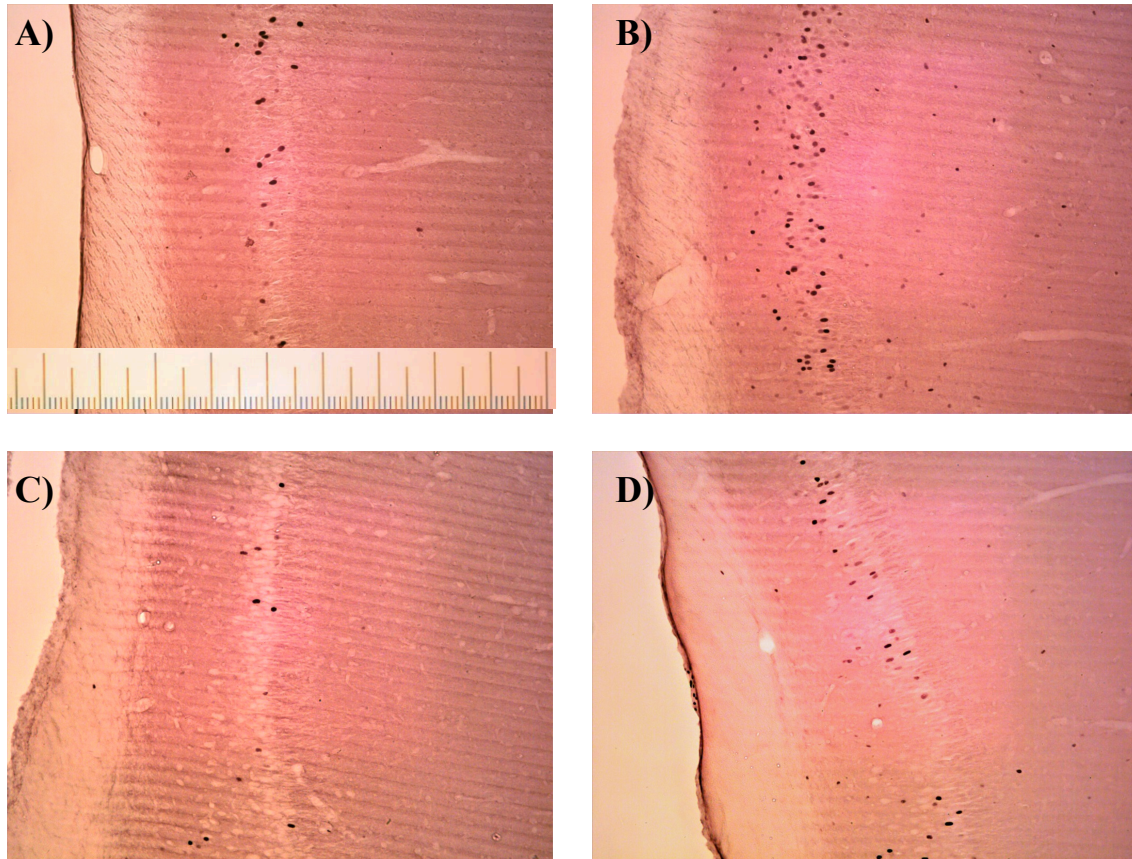
Figure 6. Digital microscopy images of *Cornu Ammonis* 3 (CA3) pyramidal cells in tissue sections (200 X 200 X 50 µm), representative of each experimental group. A) CA3 region of hippocampus in control (No-No) rats. B) CA3 region in No-EE rats. Relative to the No-No rats, the CA3 region of No-EE rats exhibited an average of 170% more c-FOS+ neurons (*p*<.03). Relative to the EE-EE rats, the CA3 region of No-EE rats exhibited an average of 76% more c-FOS+ neurons (*p*<.02). C) CA3 region in EE-No rats. D) CA3 region in EE-EE rats. Each image is 1000 µm, indicated by the scale (A), with larger divisions representing 100 µm and smaller divisions representing 10 µm.
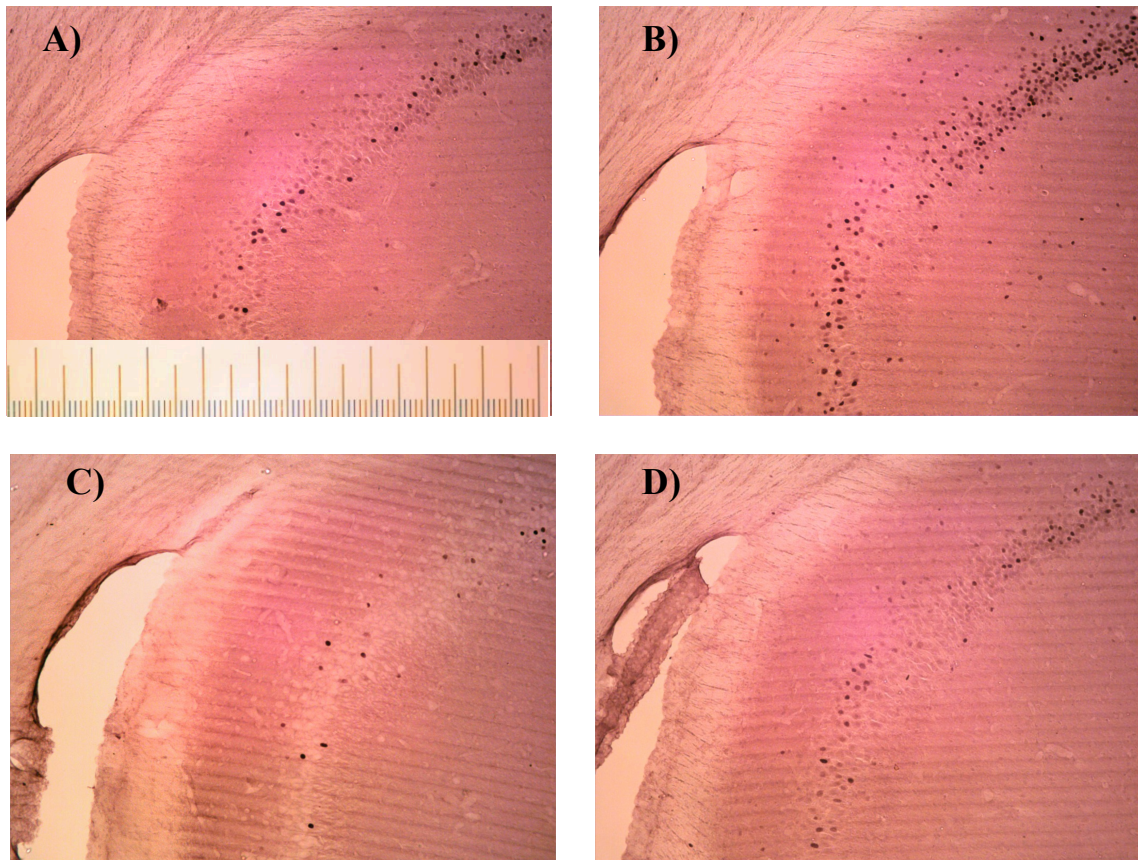
Figure 7. Digital microscopy images of *Cornu Ammonis* 2 (CA2) pyramidal cells in tissue sections (200 X 200 X 50 μm), representative of each experimental group. A) CA2 region of hippocampus in control (No-No) rats. B) CA2 region in No-EE rats. Relative to the No-No rats, the CA2 region of No-EE rats exhibited an average of 135% more c-FOS+ neurons (*p*<.02). Relative to the EE-EE rats, the CA2 region of No-EE rats exhibited an average of 88% more c-FOS+ neurons (*p*<.03). C) CA2 region in EE-No rats. D) CA2 region in EE-EE rats.
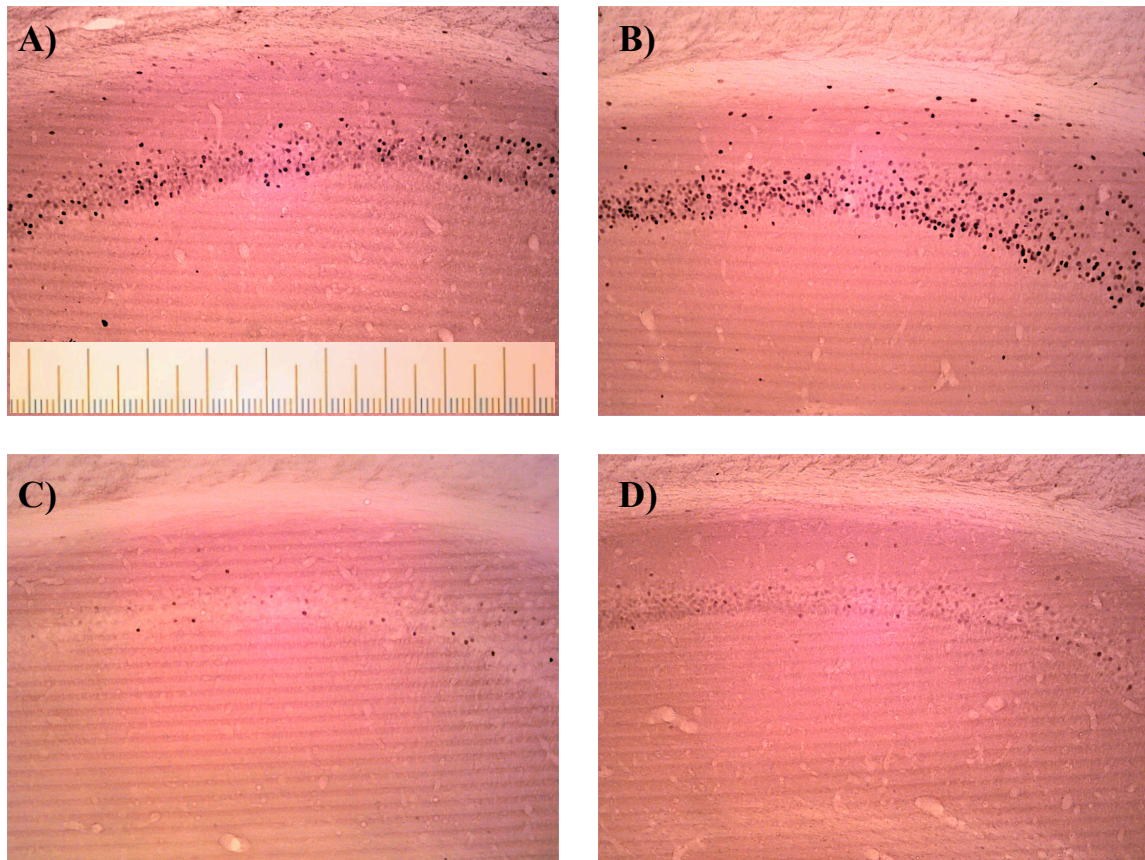
Figure 8. Digital microscopy images of *Cornu Ammonis* 1 (CA1) pyramidal cells in tissue sections (200 X 200 X 50 µm), representative of each experimental group. A) CA1 region of hippocampus in control (No-No) rats. B) CA1 region in No-EE rats. Relative to the No-No rats, the CA1 region of No-EE rats exhibited an average of 185% more c-FOS+ neurons (*p*<.03). Relative to the EE-EE rats, the CA1 region of No-EE rats exhibited an average of 182% more c-FOS+ neurons (*p*<.03). C) CA1 region in EE-No rats. D) CA1 region in EE-EE rats. Each image is 1000 µm, indicated by the scale (A), with larger divisions representing 100 µm and smaller divisions representing 10 µm.
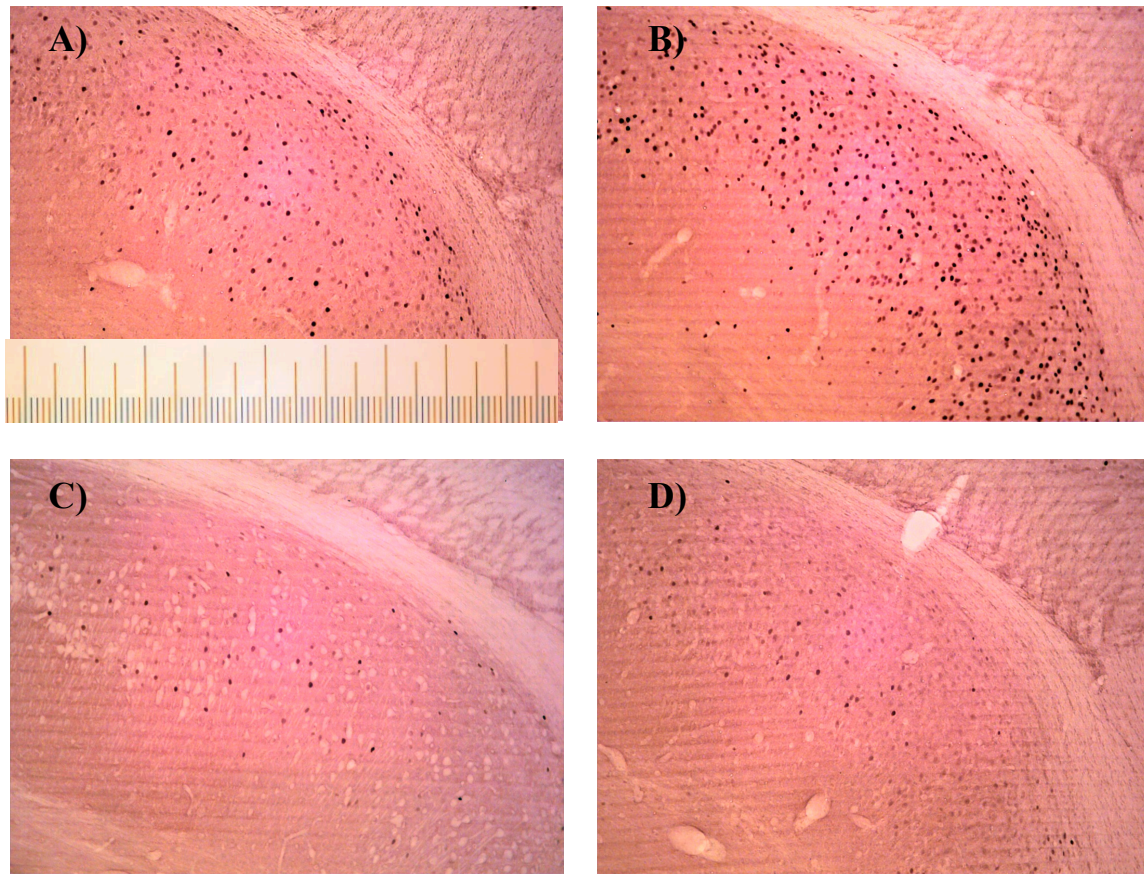
Figure 9. Digital microscopy images of Subiculum (Sub) in tissue sections (200 X 200 X 50 µm), representative of each experimental group. A) Sub region of hippocampus in control (No-No) rats. B) Sub region in No-EE rats. Relative to the No-No rats, the Sub region of No-EE rats exhibited an average of 163% more c-FOS+ neurons (*p*<.02). Relative to the EE-EE rats, the Sub region of No-EE rats exhibited an average of 72% more c-FOS+ neurons (*p*<.03). C) Sub region in EE-No rats. D) Sub region in EE-EE rats.

Appendix

TO: Dr. Mark Zrull
                       Department of Psychology

FROM: Dr. Ted Zerucha, Chair
                       Institutional Animal Care and Use Committee

DATE: August 14, 2014

SUBJECT: Institutional Animal Care and Use Committee
                       Request for Animal Subjects Research

REFERENCE: *Environmental enrichment, object placement preference,
                       social preference, and associated evoked neural activity in
                       adolescent rats*

                       IACUC Reference #15-02

                       Initial Approval Date – August 14, 2014
                       End of Approval Period – August 13, 2017

The above referenced protocol has been approved by the IACUC for a period of three years.
A list the individuals cleared for research activities with live, vertebrate animals will be sent in a
separate email.

Best wishes with your research.

TZ/rst