EFFECTS OF PERIODIC AND/OR A SINGLE EXPOSURE TO AN ENRICHED ENVIRONMENT ON NEURAL C-FOS EXPRESSION IN ADOLESCENT RATS

A Thesis
by
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Abstract

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Environmental enrichment of laboratory animals consists of cognitive, social and physical enhancement of the subjects' life experiences. This is typically accomplished by providing a wide array of objects and spatial regions for the animals to explore and increasing the number of cage-mates. Previous research with rats has shown that enrichment can expedite recovery from traumatic brain injury or lesion and promote the generation and proliferation of stem cells. In the present study, 16 Long-Evans rats (8 male and 8 female) were randomly assigned to one of four experimental groups on postnatal day 34 (n = 4 for each group). One group received twenty 90 min exposures to an enriched environment from postnatal day 34-64 while a different group received this same periodic enrichment from postnatal day 34-64 as well as an acute 90 min exposure before perfusion. A third group was housed in standard home cages throughout life before receiving a single 90 min exposure just before perfusion. Control animals were held in standard home cages throughout life. Rats were sacrificed on post-natal day 77-79, and 72 sagittal sections (50 μm) were taken from

each brain, then processed for visualization of the c-fos protein. C-fos is an immediate-early gene and its translated protein is commonly used as a marker for recent neuronal activation. There were more c-fos positive neurons in the CA1 region, entorhinal cortex and subiculum of rats that received an acute enriching experience only, compared to controls. Additionally, more c-fos positive neurons were observed within these three structures in rats that only received an acute enriching experience compared to rats that received periodic enrichment and acute enrichment. These findings suggest that, in the mammalian brain, diversity of experience during adolescence produces changes in neural circuitry that may foster improved mental acuity in adulthood.

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Foreword

This thesis is written in accordance with the style of the Publication Manual of the American Psychological Association (6th Edition) as required by the Department of Psychology at Appalachian State University.

Effects of Periodic and/or a Single Exposure to an Enriched Environment on Neural C-fos Expression in Adolescent Rats

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Abstract

Environmental enrichment of laboratory animals consists of cognitive, social and physical enhancement of the subjects' life experiences. This is typically accomplished by providing a wide array of objects and spatial regions for the animals to explore and increasing the number of cage-mates. Previous research with rats has shown that enrichment can expedite recovery from traumatic brain injury or lesion and promote the generation and proliferation of stem cells. In the present study, 16 Long-Evans rats (8 male and 8 female) were randomly assigned to one of four experimental groups on postnatal day 34 (n = 4 for each group). One group received twenty 90 min exposures to an enriched environment from postnatal day 34-64 while a different group received this same periodic enrichment from postnatal day 34-64 as well as an acute 90 min exposure before perfusion. A third group was housed in standard home cages throughout life before receiving a single 90 min exposure just before perfusion. Control animals were held in standard home cages throughout life. Rats were sacrificed on post-natal day 77-79, and 72 sagittal sections (50 μm) were taken from each brain, then processed for visualization of the c-fos protein. C-fos is an immediate-early gene and its translated protein is commonly used as a marker for recent neuronal activation. There were more c-fos positive neurons in the CA1 region, entorhinal cortex and subiculum of rats that received an acute enriching experience only, compared to controls. Additionally, more c-fos positive neurons were observed within these three structures in rats that only received an acute enriching experience compared to rats that received periodic enrichment and acute enrichment. These findings suggest that, in the mammalian brain, diversity of experience during adolescence produces changes in neural circuitry that may foster improved mental acuity in adulthood.

Effects of Periodic and/or a Single Exposure to an Enriched Environment on Neural C-fos Expression in Adolescent Rats

Environmental enrichment (EE) consists of increased cognitive, social and physical stimulation relative to standard housing conditions. Experimental studies of enrichment often use smaller mammals such as laboratory rats and mice due to the simplicity of maintaining and manipulating the environments and the genetic consistency of these species. EE is functionally defined as allowing same-sex litter-mates free access to a large cage with a wide array of objects and spatial regions to explore. Objects are usually rearranged or replaced with new objects periodically during the enrichment period. Some EE paradigms also include a running wheel for voluntary exercise (Fabel et al., 2009). Rats and mice held in standard housing conditions are grouped with fewer litter-mates in smaller cages, with little (if any) stimuli available for exploration, or to use as landmarks for spatial reasoning and navigation of the environment (Van Praag, Kemperman, & Gage, 2000). The housing conditions and/or experiences provided for subjects are generally referred to as “enriched” only when compared to another group of animals held in measurably less expansive and/or diverse environments, or exposed to fewer novel objects/locations.

Complex environments that require active cognitive processing and facilitate the execution of intricate and varied behaviors produce changes in neural anatomy and physiology. These changes are apparent in the rodent brain when animals are raised in more diverse or elaborate environments, compared to those reared in standard laboratory housing conditions (Ferchmin, Bennett, & Rosenzweig, 1975; Simpson & Kelly, 2011). Findings from animal models of enrichment serve to inform human theories of cognitive and neurological development, providing support for the mental-exercise and use-it-or-lose-it

hypotheses (Hertzog, Kramer, Wilson, & Lindenberger, 2009; Kumar, Rani, Tchigranova, Lee, & Foster, 2012; Salthouse, 2006).

There is a long history of human research aimed at identifying the environmental factors that best predict or contribute to adaptive behavioral and social outcomes, as well as cognitive ability and performance throughout life (Hebb, 1947; Hertzog et al., 2009; Salthouse, 2006). Many studies use animal models of EE to make inferences about how environmental stimulation may influence human development and performance. In the current study, the age of the subjects is of particular relevance to this line of research and its human implications. Periodic enrichment exposures were provided (for half of the subjects) from post-natal day (PND) 34-64, beginning about two weeks after weaning. During the first three weeks of a rat's life rapid development of motor skills and cognitive abilities occurs, just as human infants acquire mobility and language skills at an accelerated rate during infancy. Importantly, the mossy fibers of the dentate gyrus seem to reach maturity around PND 18, with acquisition of spatial abilities similar to that of adult rats occurring around PND 19 (Wishaw & Kolb, 2005).

This study focused on how exposure to EE throughout life, an acute enrichment experience, and the interaction of the two recruit processing of brain structures analogous to the human medial temporal lobe memory system (see Figure 1); thus, activation of the entorhinal cortex (EC), dentate gyrus (DG), amygdalo-hippocampal transition area (AHA), subiculum (SUB) and CA1 region of the hippocampus was evaluated in all subjects. In the present study, neural activation in adolescent rats was assessed after: twenty 90 min exposures to EE over 30 days; an acute 90 min exposure just preceding sacrifice; exposure to both periodic and acute EE as described; or no exposure to EE at all. Controls were handled

each time rats in the other groups were moved to or from the EE cage and were housed in standard home cages throughout life. Neural activation was assessed by immunohistochemical visualization and quantification of the immediate-early gene (IEG) c-fos in neuronal nuclei. Visualization of c-fos is commonly used to index recent activation of specific neurons (Sagar, Sharpe, & Curran, 1988).

Studies of enrichment using rats and mice vary greatly in the gender/strain of the animals, the age of subjects, the schedule and duration of EE, the types of objects provided, the treatment of controls, how objects were rearranged, the number of cage-mates, as well as the size and layout of the environment. This variation is a product of the lack of a standard operational definition for EE and the often disparate goals of researchers (Van Praag et al., 2000). Giving rodents free access to an enriched environment increases their opportunities for (and the necessity of) socialization with others, spatio-temporal processing and physical exercise. Some studies have attempted to identify the differential effects of social enrichment (Ferchmin et al., 1975; Lehmann & Herkenheim, 2011), cognitive enrichment (Mandolesi et al., 2008), and physical enrichment (Fabel et al., 2009; Simpson & Kelly, 2011). The findings from this line of research suggest that the biological and behavioral effects of EE may be a function of the combination and interactions of stimuli, more so than discrete measureable effects from each individual source of stimulation (Mora, Segovia, & Del Arco, 2007; Van Praag et al., 2000). Despite decades of research aiming to separate and quantify the respective aspects of EE that produce behavioral and neurological change many questions and discrepancies still exist in the literature; specifically, where in the brain does EE-evoked activity occur and why?

EE can change the post-exposure behavior of non-lesioned animals and expedite recovery from brain injury or lesion (Buchold et al., 2007; Mandolesi et al., 2008; Varty, Paulus, Braff, & Geyer, 2000). In light of these findings, a large amount of research has been devoted to the promotion of EE-rearing for all laboratory rats and mice, in order to improve the general health of the strains. Some researchers also infer that the EE paradigm is more akin to the natural environment of the animals and therefore, is a more suitable standard home cage for laboratory animals used in research (Simpson & Kelly, 2011; Van Praag et al., 2000). Nevertheless, early findings suggest that rats given relatively brief exposures to EE after brain lesion will display recovery of spatial skills comparable to rats housed continuously in an enriched environment (Will, Rosenzweig, Bennett, Hebert, & Morimoto, 1977). More recent investigations of enrichment and behavioral change in non-lesioned rats report increased retention of open-field habituation and improved performance on spatial and working memory tasks in subjects with more exposure to EE (Amaral, Vargas, Hansel, Izquierdo, & Souza, 2008; Birch, McGarry, & Kelly, 2013). Additionally, EE exposure prior to brain lesion produces recovery and maintenance of function effects similar to those observed after post-operative enrichment (Mandolesi et al., 2008). Results regarding how the behavioral and neurochemical effects of enrichment manifest as a function of the time and duration of exposure are mixed and more research in this area is needed.

Early findings from research with normal and lesioned animals (Will et al., 1977; Will et al., 1986), coupled with emerging theories of synaptic plasticity, suggested that EE alone produced changes in neural physiology and development (Mora et al., 2007). These observations led to more precise examinations of EE induced neurological changes, as technical progress allowed. Recent examinations of enrichment demonstrate EE-induced

changes in electrophysiology (Sharp, Barnes, & McNaughton, 1987), expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF; Angelucci et al., 2009), and gene expression (Ali, Wilson, & Murphy, 2009; Pinaud, Penner, Robertson, & Currie, 2001; Rampon et al., 2000). These findings suggest that the neural networks responsible for processing sensory input and spatial information, as well as systems involved in facilitating socialization are optimized by exposure to EE, which produces more efficient perception of (and responding to) stimuli, improved spatial navigation and more adaptive social behavior (Amaral et al., 2008; Simpson & Kelly, 2011; Varty et al., 2000).

A multitude of findings indicate that EE can modulate the expression of genes and hormones related to synaptic plasticity (Ali et al., 2009; Birch et al., 2013; Davis, Bozon, & Laroche, 2003; Pinaud, 2004; Pinaud et al., 2001; Rampon et al., 2000). Specifically, the pattern of IEG transcription regulates the basic metabolic functions of the neuron and influences how secondary response genes are expressed, effectively dictating how the neuron grows and functions (Gass, Herdegen, Bravo, & Kiessling, 1993). Expression of IEGs (such as c-fos) is induced rapidly by changes in neural membrane potentials due to neurotransmitter, neuromodulator or growth factor binding, then quickly returns to baseline expression levels (see Figure 2 for induction pathway). Therefore, c-fos and other IEGs serve to index neural activity and provide a specific transcriptional blueprint that guides neural development and apoptotic mechanisms based on experience. Some findings also suggest that transcriptional modification of IEGs and their translated proteins is necessary for conditioning related synaptic plasticity (Loebrich & Nedivi, 2009). As its induction is dependent upon depolarization or growth factor binding, c-fos has been used as a marker for recent neuronal activation in a variety of studies since the late 1980s (Sagar et al., 1988).

As mentioned, a great deal of research on the neurobiological effects of EE has been focused on long-term changes in neuronal structure and function. Therefore, many of the documented effects of EE have been derived from animals housed continuously in an enriched environment for weeks or months before any biological data was collected. Clearly, there is a large discrepancy in the EE exposure schedule and total exposure time for subjects reared full-time in an enriched environment, compared to the 90 min periodic and/or acute exposures provided in the current study. Pinaud et al. (2001) assessed recent neural activation and imposed an enrichment schedule that was nearly identical to the periodic enrichment treatment in the present study. In this study, rats were enriched for 1 hr every day for three weeks and neural tissue sections were processed for visualization of the IEG arc, which is expressed slightly later after activation and under circumstances similar to those which evoke c-fos expression. Increased arc expression was observed in the striatum, layers III and V of the cerebral cortex, and in the CA1, CA2 and CA3 hippocampal subareas, when compared to controls housed in standard home cages throughout life. These results are in accordance with findings from studies of rats reared full time in an enriched environment, especially regarding the hippocampal effects (Simpson & Kelly, 2011; Van Praag et al., 2000); as indicated by the expression of NGF and BDNF (Angelucci et al., 2009; Kuzumaki et al., 2011) as well as electrophysiological recordings (Sharp et al., 1987), but not c-fos expression. Importantly, measureable changes in the expression of growth factors and long-term potentiation/depression are indicative of later neuronal accommodations to environmental stimulation, while c-fos expression is induced acutely and transiently in neurons after activation (Loebrich & Nedivi, 2009; Sagar et al., 1988). Some findings suggest that prior

exposure to EE may attenuate the activation of brain structures involved in learning and memory evoked by an acute experience (Leger, et al., 2012)

Ali et al. (2009) implemented an acute enrichment condition very similar to the acute EE condition in the present study. These researchers demonstrated EE-evoked activity in the sensory and prefrontal cortices, as well as the DG and CA1 regions of the hippocampus, claustrum, amygdala and hypothalamus of fos-tau-lacZ transgenic mice after a single, 1 hr enriching experience (compared to controls). Notably these subjects were mice, not rats, and were bred specifically to over-express the c-fos protein; however, a very limited number of studies examining EE-evoked neural activation in rats or mice without a history of enrichment were available. Therefore, results from the Ali et al. experiment contributed considerably to expected outcomes pertaining to evoked c-fos expression in subjects that received the acute enrichment experience in the present study. Because of the swift induction and transient nature of IEGs like c-fos (Sagar et al., 1988), the acute enrichment condition in the current study may produce systematically different brain regions with observable changes in c-fos expression. This presumption, the findings of Ali et al. and those of Pinaud et al. (2001) heavily influenced the rationale for which brain regions to include in the counting protocol for the present study.

During EE, subjects are exposed to a marked increase in the complexity of environmental stimuli, as well as an increase in the complexity of the spatial relationships between stimuli. This inherently leads to the formation and retrieval of memories for these objects, and for the cage as a whole, via modulation of hippocampal pyramidal cell activation. Across laboratories and EE paradigms, the brain region most consistently displaying EE-induced changes in neural morphology, activation, and gene expression is the

hippocampal formation (Ali et al., 2009; Angelucci et al., 2009; Birch et al., 2013; Kuzumaki et al., 2011; Pinaud et al., 2001; Sharp et al., 1987; Simpson & Kelly, 2011). Subjects also make more errors on spatial memory tasks after lesioning of the hippocampus and exposure to an enriched environment can mitigate these lesion-induced memory deficits (Will, Deluzarche, & Kelche, 1983; Will et al., 1986). These findings strongly imply that neural circuits in rats, which are analogous to the medial temporal lobe memory circuits in humans, are activated by exposure to an enriched environment and that increased activation of this system can improve performance on a variety of spatial and learning-dependent tasks. Thus, c-fos expression in the lateral AHA and EC, dorso- and ventrolateral DG, ventrolateral SUB and dorsolateral CA1 region of the hippocampus will be analyzed in the current study (see Figure 1).

The EC integrates polymodal and unimodal inputs from cortical association areas and is the primary input pathway to the hippocampus, providing information that is vital for spatial processing and declarative memory formation (Ferreira, Da Silva, Medina, & Izquierdo, 1992; Kajiwara, Takashima, Mimura, Witter, & Iijima, 2002). Therefore, the EC may exhibit measureable changes in c-fos expression before these changes can be observed in hippocampal neurons. The AHA receives input from the amygdaloid nuclei and has afferent and efferent connections with the ventral CA1 hippocampal subfield. This structure has been implicated in memory processing related to the integration of temporal context, and environmental stimuli. Specifically, lesioning of the AHA produces interruptions in contextual conditioning and eliminates the primary locus of afferent and efferent fibers connecting the amygdala and the hippocampus. Therefore, the AHA is vital for emotional modulation of memory (Fujisaki, Hashimoto, Iyo, & Chiba, 2004).

In the present study three hypotheses were tested. The first was that the number of c-fos positive neurons in these five brain structures of rats that received periodic EE only would be not equal to the number of c-fos positive cells in these structures for control animals. The next hypothesis tested was that the number of c-fos positive cells for each of these five brain structures in rats that received an acute enriching experience only, with no history of EE, would be greater than the number of c-fos positive neurons in controls. The third hypothesis was that the number of c-fos positive cells in these five brain structures of rats that received periodic EE and an acute enrichment experience just preceding sacrifice would be greater than the number of c-fos positive cells in animals that were exposed to periodic EE, but did not receive an acute enrichment experience.

Methods and Materials

Brain tissue for this study is archival. The tissue came from normal control rats used in the last of a series of studies conducted between September 2008, and July 2011, in which the effect of periodic enrichment on behavior altered by a history of seizures was examined. All care and use of the rats was approved initially on August 29, 2008, by the Institutional Animal Care and Use Committee at Appalachian State University with annual reviews in August 2009 and 2010 (see Appendix, Protocol #09-02). In addition to the specific methods used to analyze neural activity in this study, a description of the methodology used to produce the neural tissue is included.

Subjects/Experimental Groups

Sixteen Long-Evans rats (8 males, 8 females) were obtained from the animal breeding colony at Appalachian State University in Boone, North Carolina. After weaning, rats were housed in standard shoebox cages with one or two same-sex littermates and exposed to a 12

hr/12 hr light/dark cycle with food and water available ad libitum throughout the study. The vivarium also provided a temperature and humidity controlled environment. On postnatal day (PND) 34 experimental procedures began. Rats were randomly assigned to one of four groups described below (*n*=4, 2 males, 2 females for each group):

1) *Periodic Enrichment (EENO)*: Rats were enriched for one 90 min period for 2 consecutive days with no enrichment every third day, for a total period of 30 days. Subjects in this group received a total of twenty 90 min exposures to EE from PND 34 to PND 64 and were held in standard home cages for the remainder of the study after enrichment exposures. These rats were sacrificed on PND 77-79 after spending 90 min in a quiet, dark setting.

2) *Periodic Enrichment and Single Exposure (EEEE)*: These subjects were exposed to the same enrichment schedule described for the periodic enrichment group. In addition, subjects received a single 90 min enrichment exposure between PND 77-79, just preceding sacrifice.

3) *Single Exposure (NOEE)*: Subjects were housed in standard cages until PND 77-79 when they experienced a single 90 min enrichment exposure preceding sacrifice. These subjects were picked up twice for about 15 sec each time rats in the periodic enrichment conditions were moved to or from the enrichment cage to control for handling effects.

4) *Controls (NONO)*: Rats in the control group were held in standard home cages throughout life and were sacrificed on PND 77-79 after spending 90 min in a quiet, dark setting. These subjects were picked up twice for about 15 sec each time rats in the periodic enrichment conditions were moved to or from the enrichment cage to

control for handling effects. This group will provide baseline neural activation levels, to which the experimental groups will be compared.

Environmental Enrichment Procedures

The enriched environment was the same for all experimental groups. EE was conducted in a four-level wood frame and ½ in. hardware cloth cage (46 x 46 x 61 cm) with aspen bedding on the bottom level of the enclosure and 8 objects dispersed throughout during any particular session. Male and female EE cages were separate mirror images of one another. Eight same-sex rats were exposed to this environment simultaneously during all periodic enrichment sessions. During any particular EE session for any specific rat, some of the other rats in the enrichment cage were familiar and some were not. For subjects exposed to periodic enrichment, objects were rearranged every three days, such that objects were in a new location in each session following a day without enrichment. The objects were rearranged prior to the single exposure condition and remained unchanged during these 90 min sessions, which were conducted with four rats in the enrichment enclosure. A running wheel was not employed as an environmental stimulus because of the possibility of differential exposure to exercise, or dominance issues. A white tube, tennis ball, blue shower curtain rings, cardboard toilet paper roll, water dish and other small objects were used as environmental stimuli.

Perfusion/Immunohistochemical Procedures

After the final 90 min enrichment exposure or 90 min in the quiet dark environment, rats were given a lethal overdose of sodium pentobarbital (100 mg/kg body weight, intraperitonially). Upon the absence of corneal reflex, subjects were perfused intracardially with 0.9% saline 10 mM phosphate buffer in deionized water (PBS) to clear blood vessels,

followed by 4% paraformaldehyde in 10mM PBS to begin fixation. Brains were then removed from the skulls and floated in 4% paraformaldehyde and 10% sucrose solution at 4°C for one week. The storage solution was then changed to 10mM phosphate buffer (PB) and the brains were refrigerated until sections were cut. Seventy-two 50 µm sagittal sections were taken from one hemisphere of each brain using a Vibratome® tissue slicer. Sections were then floated in PB and held in tissue wells stored in the refrigerator until processed to visualize neurons containing the c-fos protein or for Nissl staining with thionin.

Twelve sections from each brain were selected at approximately 150-200 µm intervals for c-fos immunohistochemistry. The remaining sections were reserved for Nissl staining. Day 1 of the immunohistochemical procedure began with two 5 min rinses in PBS followed by 15 min in 1% hydrogen peroxide to quench endogenous peroxide. Sections were then rinsed two more times (5 min each) in PBS before floating for 60 min in 15% goat serum and 0.25% Triton-X in PBS to block non-specific binding sites. After this step, sections were floated in the primary antibody to the c-fos protein (Calbiochem PC-38, made in rabbit and used at 1:1500 in PBS with 2% goat serum and 0.25% Triton-X) for about 40 hr at 4°C. Section trays were placed on a rotator to ensure even coverage of the tissue by each solution. Day 2 of immunohistochemistry began with six 10 min rinses with PBS to remove as much of the excess primary antibody as possible and to minimize background staining. Sections were then floated for 90 min in biotinylated secondary antibody (Vector Labs goat anti-rabbit used at 1:300 in PBS with 0.2% goat serum and 0.25% Triton-X) before three more 10 min rinses in PBS. After these rinses, the tissue was floated for 1 hr in avidin-biotin complex (Vector Labs), followed by two 10 min rinses in PBS. The tissue was then exposed to the VIP enzyme substrate (Vector Labs) to react the peroxide-labeled avidin-biotin

complex and stain c-fos protein in the nuclei of neurons. Reactions were ended by moving the sections from the VIP solution to cold PBS. These reactions took about 2 min per section. Sections were mounted onto gelatin-coated slides, allowed to dry, dehydrated in graded ethanols, cleared with toluene, and coverslipped using Permount (Fisher).

Sections not used for c-fos immunohistochemistry were used for cell body or Nissl staining, which produced sections that could be used to identify brain areas and aid in the correct location of anatomical regions for microscopy. After selecting sections for c-fos staining, the remaining sections were mounted on gelatin coated slides, air dried, then dehydrated in a graded series of alcohols. Mounted sections were then rehydrated, stained with thionin, differentiated with a 95% ethanol and acetic acid solution, dehydrated again, cleared in toluene, and coverslipped using Permount. All processed slides were cleaned and cleared of excess glue with xylenes.

Counting Procedures

Quantification of c-fos expression in neurons was accomplished using a Nikon® Eclipse microscope and PixeLink® digital camera. Structures of interest were identified initially using the Plan 4 objective; then cell counts were obtained using the Plan 10 objective. When displayed on the monitor, 1 mm in section was 320 mm on the 800 X 600 pixel digital image. Data were recorded by marking activated neurons on an 8.5 X 11 in transparency mounted on top of six transparent counting frames (200 X 200 μm), which were fixed onto the computer monitor where tissue images were displayed. Neurons located on the left and lower boundaries of the counting frame were excluded from the sample, while cells on the right and upper boundaries were included. Neurons in the initial (i.e., upper surface) plane of focus were excluded from cell counts in sections with more than one visible plane.

From each rat, three tissue sections processed for visualization of c-fos in which the lateral AHA and EC, dorso- and ventrolateral DG, ventrolateral SUB and dorsolateral CA1 region were present were selected for analysis. Structures were identified in section using adjacent Nissl stained sections and two rat brain atlases (Kruger, Saporta, & Swanson, 1995; Pellegrino, Pellegrino, & Cushman, 1981). Two observations composed of three independent samples were obtained from each of the three sections for each structure of interest. Each observation was obtained from a specified region of each structure in order to minimize variability between sections and raters. In sections processed for visualization of c-fos, darker cells indicate a greater presence of the c-fos protein. Therefore, activated cells were categorized into three groups based on the darkness of nuclei. These orthogonal categories identified cells that were slightly, moderately or highly activated, respectively. After visual inspection of the range of nuclei staining throughout each structure, the coloration of the slightly activated, moderately activated and highly activated cells was observed, photographed, indicated and provided to all raters. Any coloration that could be clearly distinguished from the background and identified as a neuron was indicated as slightly activated. Neurons that were clearly darker and more distinguishable from the background than slightly activated cells, but also not as dark as the darkest of cells in the structure, were categorized as moderately activated. The darkest cells in the structure were deemed highly activated and were typically dark purple or black.

The possibility of systematic variation in the visualization of neurons resulting from differential exposure to one or more of the chemicals used in the immunohistochemical procedures was taken into consideration when classifying neurons in terms of activation. Because the degree of activation was indicated by the relative visualization of nuclei, general

differences in the nature of the staining between immunohistochemical procedures could increase the variance within groups. Upon visual inspection, differences in staining between sections processed simultaneously for visualization of c-fos and those processed at different times were minimal. To ensure that any observed differences in the degree or coloration of c-fos visualization between immunohistochemical procedures were not actually differences between experimental groups, sections from rats in the same experimental groups were processed on different occasions for comparison. These comparisons revealed relative homogeneity of staining, independent of the time at which the tissue was processed.

Design

This experiment was a completely randomized hierarchical design, such that three samples from a specified region of a structure were averaged to form each observation, with the two observations for each structure occurring in a single brain section. Section was nested in case (i.e., the particular rat or brain) and case, in turn, was nested in group. For purposes of analysis, experimental group was treated as a fixed variable with four levels of enrichment, and case was treated as a random variable as cases (i.e., subjects) were randomly assigned to the experimental groups (Kirk, 1995). Linear contrasts were formed and tested to assess the following hypotheses for all brain structures: that the number of c-fos positive neurons in rats with a history of enrichment only (i.e., EENO), would be not equal to the number of c-fos positive neurons in rats that were never exposed to the enriched environment (i.e., NONO); that the number of c-fos positive neurons in rats that received an acute exposure to the enriched environment only (i.e., NOEE), would be greater than that of NONO animals; and that the mean number of c-fos positive neurons in all brain structures would be greater in rats

that received periodic EE throughout life and an acute exposure before sacrifice (i.e., EEEE), compared to EENO animals.

Results

Data and Reliability

All of the descriptive and inferential statistics produced to test each of the three hypotheses were calculated using the sums of the highly and moderately activated (i.e., c-fos positive) cells in each sample, counted by an initial rater. Neurons indicated as slightly activated were excluded from all analyses. The sums of the highly and moderately activated neurons in six samples from each section of each brain (for each structure) were averaged to produce two observations from each section. This yielded six observations per brain (two from each section) which were derived from 18 samples (three per observation). These observations, as they will be referred to in the following sections of this thesis, served as the unit of analysis. Only the observations produced by the initial rater were used for analyses of primary hypotheses.

To assess the reliability of the initial raters' categorization of c-fos positive neurons, a second rater produced six samples which yielded two observations from the middle section (i.e., the section taken from the area of tissue between the two other sections chosen for analysis) of each brain. Again, only the neurons deemed moderately and highly activated were used for reliability analyses. The counts of the second raters were used, along with the counts of the initial rater, to evaluate the reliability of the initial raters' quantification of c-fos positive neurons only. Thus, observations produced by second raters were not used to assess any hypotheses in this study. Samples were matched by observation, such that values produced by each rater derived from the same general region of the structure. Pearson's

correlation coefficients were calculated using the observations produced by each rater for each of the five structures of interest. Thus, observations from all brains in all 4 experimental groups were included in the reliability estimates for each structure.

The results of reliability analyses were as follows: AHA, $r(39) = .39$, $p = .013$; CA1, $r(37) = .91$, $p < .001$; DG, $r(35) = .62$, $p < .001$; EC, $r(37) = .88$, $p < .001$; SUB, $r(37) = .70$, $p < .001$, (see Table 1 for descriptive statistics for each rater). These correlation coefficients indicate agreement between raters for counts of CA1, EC and SUB. The reliability estimates for counts of the DG and AHA were likely smaller because the manipulation (i.e., EE) did not have a pronounced effect on the recruitment of activation from these brain structures. Therefore, the numbers of c-fos positive neurons in the AHA and DG were highly similar between and within experimental groups for each rater. This similarity produced greater inconsistency in the relative magnitude of the counts, thereby reducing the reliability estimates for counts of activated cells in these structures.

Primary Hypotheses

Linear contrasts were created to reflect the three hypotheses for each of the five structures of interest. All of these contrasts were actually pairwise comparisons because each hypothesis referenced only two groups. The first hypothesis was that the number of c-fos positive neurons in rats with a history of enrichment only (EENO) would be not equal to the number of c-fos positive neurons in rats that were never exposed to the enriched environment (NONO) in all five brain structures. Statistical evaluations of this hypothesis revealed no significant differences between groups for any of the structures of interest, $p = .108$ to $.639$, (see Tables 2 and 3; see Figure 1). The mean values were higher in the AHA (+18%), DG (+57%) and CA1 region (+41%) of control animals (NONO), compared to cell counts in rats

that received periodic exposure to the enriched environment (EENO). For the EC (-19%) and SUB (-57%), mean cell counts were lower for rats in the NONO group compared to the EENO group.

Linear contrasts were also created to test the second hypothesis, that the number of c-fos positive neurons in all five brain areas of NOEE rats, would be greater than that of control animals. Cell counts derived from the CA1 region were significantly higher (+292%) for rats in the NOEE group, compared to the NONO group, $t(15) = 3.92$, $p < .001$ (see Tables 2 and 3; see Figure 1). This hypothesis was also supported with respect to the activation of the EC (+129%), $t(15) = 3.02$, $p = .004$, as well as the SUB (+363%), $t(15) = 4.31$, $p < .001$ (see Tables 2 and 3; see Figure 1). The mean cell counts for the DG (+19%, p=.284) and the AHA (+46%, p=.106) were also larger for rats in the NOEE group compared to the counts from control animals (NONO), though these differences did not reach statistical significance.

The third hypothesis tested in this study was that the mean number of c-fos positive neurons in all brain structures would be greater in EEEE rats, compared to EENO animals. Pairwise comparisons revealed no significant differences in the number of c-fos positive neurons for any of the structures of interest ($p = .119$ to .478); however, the mean cell counts of all brain structures in EEEE rats were higher (+6% for CA1 and EC, +23% for SUB, +47% for AHA and +56% for DG) than the counts from EENO rats (see Tables 2 and 3; see Figure 1).

Additionally, linear contrasts were created and analyses were conducted in order to compare counts of c-fos positive neurons from EEEE rats and NOEE animals, for each of the five structures of interest. These pairwise comparisons were deemed appropriate during the interpretation of results from this study and no a priori hypothesis regarding the relative

magnitude of neural activation between these two groups was made. Therefore, *t*-values were evaluated using a two-tailed distribution. These analyses were conducted to examine the effects of periodic exposure to EE throughout life on EE-evoked activity. Rats in the NOEE group displayed significantly more c-fos positive neurons in the CA1 region, $t(15) = 5.34$, $p < .001$; EC, $t(15) = 2.62$, $p = .019$, and SUB, $t(15) = 3.46$, $p = .003$, compared to EEEE rats (see Table 2 and Figure 1 for descriptive statistics). Counts from the AHA, $t(16) = 0.76$, $p = .457$, and DG, $t(14) = 1.82$, $p = .090$, were not significantly different between groups, though counts from NOEE rats were consistently larger than those from EEEE animals across all structures (+20% to +531%).

Summary

Considering the observed increases in activation found in the EC, CA1 and SUB of NOEE rats compared to NONO rats, acute exposure to EE tends to recruit activation of structures in the perforant pathway. In addition, the increased activation of CA1, EC and SUB in NOEE rats relative to EEEE rats suggests that periodic enrichment during adolescence attenuates the evoked response of these structures to an acute EE experience. A history of EE without an acute exposure may also suppress the activity of some of the structures in the perforant pathway when compared to animals exposed to EE directly preceding perfusion; however, the observed cell counts for all five structures of interest in EENO animals were not statistically different from controls.

Discussion

Primary Hypotheses

The first hypothesis evaluated in the current study was that the activation of the AHA, CA1, DG, EC and SUB of EENO rats would be not equal to that of animals in the NONO

group. No significant differences in the number of c-fos positive neurons were observed in any brain structures of EENO rats, compared to control animals. Notably, the experience of rats in each of these groups was identical for the two weeks preceding sacrifice. The lack of differences between groups may be attributed to the rapid onset and relatively short duration of increased c-fos expression following neurotransmitter, neuromodulator, or growth factor binding (Loebrich & Nedivi, 2009; Sagar et al., 1988). Therefore, some long-term changes in the functioning of circuits or the efficacy of synapses may not have been revealed or accurately represented by the number of c-fos positive neurons in these structures at the time of sacrifice (Buschler & Manahan-Vaughan, 2012). In general, the mean counts were higher for control rats, though not significantly larger than counts from rats in the EENO group. This suggests that a history of EE may indirectly produce long-term changes in the recruitment of activity from structures in the perforant pathway, which might be portrayed more faithfully by dendritic branching, electrophysiological recordings, or by the presence of delayed-early-genes such as arc in neurons (Davis et al., 2003; Pinaud et al., 2001; Van Praag, 2000).

The wealth of research demonstrating the cognitive benefits of chronic and periodic EE (Amaral et al., 2008; Birch et al., 2013; Hebb, 1947; Hymovitch, 1952; Leger et al., 2012; Simpson & Kelly, 2011) strongly suggests that the efficacy of circuits responsible for processing the environment is increased by exposure to EE. The findings from the present study regarding EENO and NONO rats indicate that any improvement in spatial and working memory produced by periodic EE may not be reflected by the number of c-fos positive neurons in these brain structures, after animals are deprived of EE for two weeks before perfusion. In other words, more activated neurons within a brain structure does not directly

reflect the potential depth or efficiency of processing for that structure (Buschler & Manahan-Vaughan, 2012). Therefore, periodic EE may alter the function of structures in the perforant pathway by increasing the efficiency of neurotransmission; such that fewer neurons in a given structure must become active, ultimately, in order to produce the same cognitive or behavioral output.

The second hypothesis assessed in this study was that the number of c-fos positive neurons in all five brain structures of NOEE animals would be greater than the cell counts from NONO rats. There were significantly more c-fos positive neurons in the CA1 region, EC and SUB of rats in the NOEE group compared to NONO animals. The DG only receives input from the EC, while CA1, SUB and EC all receive input from at least two structures within the perforant pathway (see Figure 1). This may be why group differences in the activation of the DG were less robust than differences in other structures. The activation of the AHA is dependent upon input from the amygdala (Fujisaki et al., 2004). The amygdala becomes active when an animal encounters an unfamiliar or fear-inducing circumstance (Kolb & Wishaw, 2008). The enrichment experience certainly meets these criteria for activating the amygdala, thereby evoking c-fos expression in the AHA; however, the experience of being alone in the novel quiet and dark environment just preceding sacrifice for control animals also meets these criteria. This step was implemented for animals that did not receive an acute EE exposure in order to standardize their experiences before perfusion, yet may have inadvertently evoked some activity in the amygdala, producing input to the AHA. Additionally, the activation of the amygdala may play a greater role in the processing of, and behavior during the enrichment experience, for smaller or less dominant animals. Thus,

individual differences in the activation of the AHA via input from the amygdala may have also contributed to the lack of group differences in activation for NOEE and NONO rats.

The third and final hypothesis tested in the present study was that the number of c-fos positive neurons in the AHA, CA1, DG, EC, and SUB of rats in the EEEE group would be greater, compared to cell counts of rats in the EENO group. There were no significant differences in the number of c-fos positive neurons within any of the five brain structures of EEEE rats, compared to rats in the EENO group. Though none of the group differences were statistically significant, the mean cell counts for EEEE rats were higher than those of EENO animals across all brain structures (see Table 2). Considered together, these small mean differences, along with the modest to large increases in cell counts observed in NOEE rats compared to EEEE rats (+20% to +531%) suggest that periodic EE attenuates the activation of these brain structures in rats, regardless of whether they receive an acute exposure to EE or not. Additionally, the fact that mean cell counts of EEEE animals were slightly larger than counts of EENO rats for all five brain structures implies that the acute enriching experience for rats with a history of EE may still evoke activity of these structures over and above being held in a quiet, dark environment. The EE induced activity of these brain structures in non-EE-naïve rats would likely be larger if steps were taken to make the acute experience more novel for these animals. For example, providing less prior exposure to the enriched environment, implementing a longer period of abstinence from EE between the final periodic exposure and the acute EE experience, or using a completely different environment for the acute exposures, would likely increase the EE-evoked activity of these structures in animals with a history of enrichment.

The following sections of this thesis will discuss the effects of acute EE without prior exposure, periodic exposure to EE followed by no EE for several days before assay, and prior exposure to EE along with an acute exposure before sacrifice, on the number of recently activated neurons of brain structures in the perforant pathway. The present findings will be interpreted in the context of previous research in the field and recommendations for future studies will be provided.

Effects of Acute EE

The present study found that an acute exposure to EE significantly increases evoked activity in the EC, CA1, and SUB of EE-naïve rats, compared to rats that were held in a quiet and dark environment before sacrifice. These findings support previous reports of the acute effects of EE (Ali et al., 2009), with some variation. Notably, Ali et al.'s subjects were mice that were genetically modified to over-express c-fos. These researchers also reported large increases in activation after acute EE for the DG and only moderate increases in the activation of the CA1 region. The current study found that acute EE drastically increased activation of CA1 (+292%), yet increases in the activity of DG were small and not significant. While the relative magnitude of increased activation induced by acute exposure to EE for each separate region comprising the perforant pathway remains to be elucidated, it seems that an acute exposure to EE does evoke activity in many of these structures. The EE-evoked activity in specific areas of tissue within each of these brain structures should be selected and sampled from with great precision in future studies in order to pinpoint functional circuits.

Effects of Periodic EE

A large number of previous findings, from a variety of researchers utilizing a variety of EE paradigms indicates: enhanced working memory and spatial skills (Amaral et al., 2008; Birch, 2013), altered NGF and BDNF expression (Angelucci et al., 2009), expedited recovery of function after brain injury (Buchold et al., 2007; Will et al., 1977) and enhanced early long-term-potentiation at CA1 synapses (Buschler & Manahan-Vaughn, 2012) after periodic or continuous enrichment throughout life. All of these findings reflect the long-term cognitive and neurological changes produced by chronic exposure to EE, and are not dependent upon the number of activated neurons in certain brain regions during the hours preceding sacrifice. Pinaud et al., (2001) found increased expression of arc in the hippocampus of rats that were enriched periodically through late adolescence, without an acute enriching experience, compared to rats housed in standard home cages throughout life. Upregulation of arc occurs in circumstances similar to those which evoke c-fos expression, however arc is a delayed-response-gene, therefore c-fos expression must be induced before arc can be expressed (Loebrich & Nedivi, 2009). Thus, increased presence of arc implies an earlier increase in c-fos expression (see Figure 2 for induction pathway). Though no statistically significant findings from the present study directly oppose the findings of Pinaud et al., the current data imply that periodic exposure to EE throughout adolescence may reduce EE-evoked IEG expression (i.e., activation) by neurons of brain structures involved in the initial processing of the experience, when animals are deprived of EE for several days before assay. In future studies, the performance of enriched and non-enriched animals on spatial reasoning or object recognition tasks should be quantified and analyzed along with the

activation of these brain structures to better understand the effects of periodic EE on processing capability.

Effects of Periodic EE on EE-Evoked Activity

A history of exposure to EE seems to attenuate the evoked response of brain structures responsible for processing novel environments. Leger et al., (2012) housed adult mice in enriched or standard housing conditions for 3 weeks, then administered a novel object preference test to all subjects just before perfusing and processing tissue for visualization of c-fos protein. The processing demanded by this novel object preference task was similar to the processing required to negotiate the enriched environment. These researchers found more c-fos positive neurons in the hippocampus and perirhinal cortex of non-enriched animals compared to enriched animals. Additionally, there were significantly fewer c-fos positive cells in the basolateral amygdala of enriched mice. These findings, along with those of Pinaud et al., (2001), Ali et al., (2009) and the results of the present study regarding NOEE and EEEE animals, suggest a negative relationship between prior EE exposure and the EE-evoked activity of brain structures involved in learning and memory.

As the amount of prior experience in a given enriched environment increases, the novelty of that environment decreases. Additionally, frequent exposure to virtually any stimuli or circumstance leads to improved performance when negotiating that situation in the future (i.e., the act of learning). Thus, when animals are enriched periodically over time in a similar manner, the activity of circuits responsible for processing the event may become optimized for directing behavior during future instances of that event. Based on the present findings, this optimization seems to result in (or be a result of) fewer neurons of structures in the perforant pathway becoming active during an EE exposure, when animals have a history

of EE. Future examinations of EE-evoked activity in animals with a history of EE should vary the degree of periodic EE and the period of abstinence from EE before the acute exposure to EE and assay. This would provide a greater understanding of how the evoked activity of structures in the perforant pathway is modified by exposure to EE over time, and how temporal distance from the last periodic exposure to the acute EE experience alters the evoked response of these structures.

Summary and Conclusions

Acute exposure to EE evokes activity in neurons of the EC, CA1 region and SUB of rats, relative to being held in a quiet and dark environment preceding perfusion. Therefore, these brain structures are integral for processing and negotiating the enriched environment. Periodic exposure to EE during adolescence may decrease basal activation levels of the EC, CA1 region and SUB in animals that have not been exposed to EE for several days before assay. We postulate that this small yet consistent reduction in activity after periodic EE is due to the impoverished nature of the home cage and the quiet, dark environment relative to the enriched environment. Additionally, periodic EE attenuates the EE-evoked activity of the EC, CA1 region and SUB; which may result from increased processing efficiency of these structures.

EE produces an immediate effect on the activation of brain structures involved in learning and memory processes, as well as a lasting effect on the functioning of these circuits. These findings suggest that, in mammals, diversity of experience during childhood and adolescence leads to changes in neural circuits that support improved mental acuity in adulthood. Additionally, the results correspond to the notion of mental exercise preparing the brain for enhanced cognitive processing in the future.

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Table 1

Descriptive Statistics for the Number of c-fos Positive Neurons Produced by Each Rater for Each Structure Across all Experimental Groups

Structure	Rater	*n*	*M*	*SD*
AHA	1	116	6.55	3.83
	2	40	3.17	3.01
CA1	1	114	6.50	8.08
	2	38	9.26	8.34
DG	1	108	2.89	2.40
	2	36	6.91	4.20
EC	1	114	13.41	7.62
	2	38	6.61	7.78
SUB	1	108	3.35	2.98
	2	38	3.85	3.96

Note. Data produced by both raters from the middle section of each brain only were used for reliability estimates. Only matched observations available for both raters were used to compute Pearson's *r*'s.

Table 2

Descriptive Statistics for the Number of c-fos Positive Neurons in Each Structure for Each Experimental Group

	EEEE		EENO		NOEE		NONO	
	n	*M*(*SD*)	*n*	*M*(*SD*)	*n*	*M*(*SD*)	*n*	*M*(*SD*)
AHA	34	7.05(3.92)	30	4.78(2.42)	28	8.49(4.94)	24	5.81(2.39)
CA1	36	2.62(2.74)	30	2.48(1.57)	30	16.54(9.68)	18	4.22(3.24)
DG	30	2.41(2.28)	30	1.54(1.23)	30	4.29(2.82)	18	3.59(1.93)
EC	36	12.09(5.74)	30	11.38(5.01)	24	21.82(9.48)	24	9.53(3.99)
SUB	36	3.05(2.67)	28	2.48(1.71)	20	7.27(2.91)	24	1.57(1.52)

Note: These statistics were derived from the values produced by an initial rater only. Each observation was composed of the mean of three samples, *n* = number of observations.

Table 3

Results of the Pairwise Comparisons Evaluating Each of the Three Alternative Hypotheses in All Five Structures

	EENO ≠ NONO		NOEE > NONO		EEEE > EENO	
	t(df)	*p*	*t*(df)	*p*	*t*(df)	*p*
AHA	0.51(16)	.619	1.30(16)	.106	1.22(16)	.119
CA1	0.55(15)	.587	3.92(15)	<.001	0.05(15)	.478
DG	1.72(14)	.108	0.58(14)	.284	0.84(14)	.208
EC	0.48(15)	.639	3.02(15)	.004	0.21(15)	.420
SUB	0.75(15)	.467	4.31(15)	<.001	0.52(15)	.306

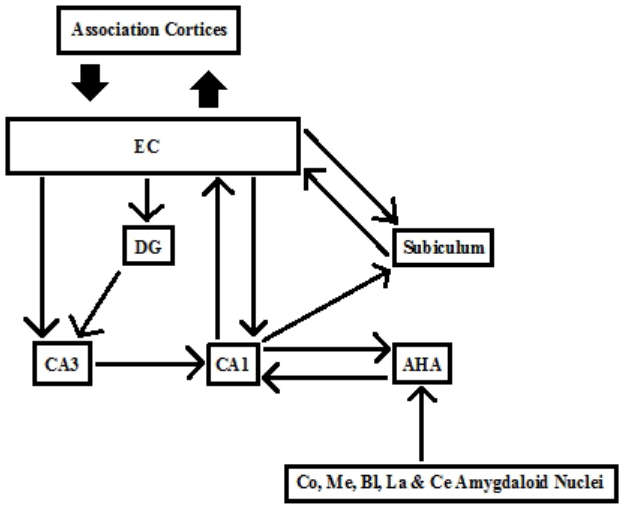

Figure 1. Schematic diagram of afferent and efferent connections among several structures comprising the perforant pathway in rat. EC-entorhinal cortex, DG-dentate gyrus, AHA-amygdalo-hippocampal transition area, CA1 & 3-cornu ammonis (ram's horn) subfields of the hippocampus, Co-cortical, Me-medial, Bl-basolateral, La-lateral, Ce-central.

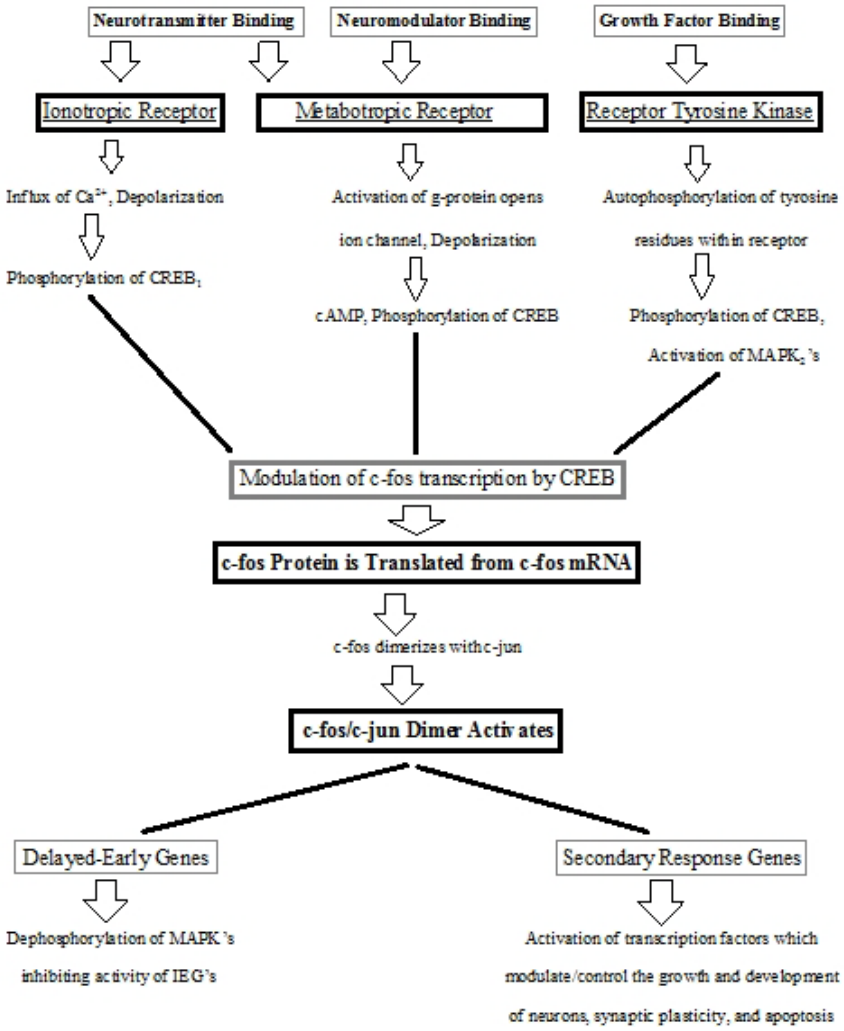

Figure 2. Flowchart mapping signal transduction pathways leading to transcriptional modification of the c-fos gene and subsequent protein translation, ending with expressional modulation of later genes. cAMP-cyclic adenosine monophosphate (2nd messenger), CREB-cAMP response element binding (binds to cAMP response elements on DNA influencing c-fos transcription), MAPK-mitogen activated protein kinase.

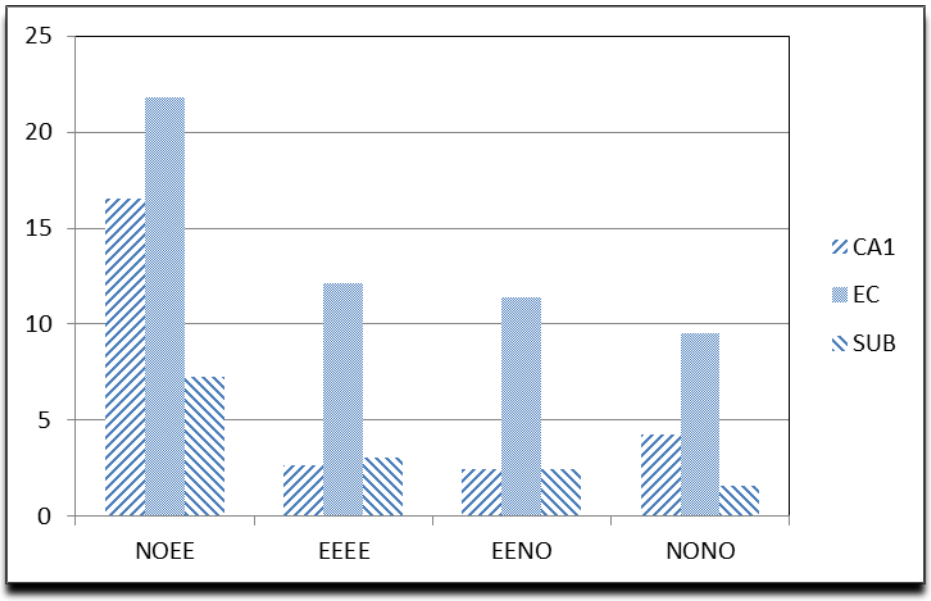

Figure 3. Mean numbers of c-fos positive neurons per 200x200x50µm tissue sample for CA1, EC and SUB.

Appendix

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TO: Dr. Mark Zrull
Department of Biology

FROM: James C. Denniston, Chair
Institutional Animal Care and Use Committee

DATE: August 29, 2008

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

REFERENCE: "Investigating how exposure to an enriched environment may alter how generalized seizures affect various behaviors in young rats"

IACUC Reference #09-2

Initial Approval Date – August 29, 2008
End of Approval Period – August 28, 2011t

The above reverenced protocol has been approved by the IACUC for a period of three (3) years.

Best wishes with your research.

JCD/lab

Vita

Wade Cates White, Jr. was born in Columbus, Georgia to Wade and Lynn White on November 17, 1987. He graduated from Hardaway High School in Georgia in May 2006. The following autumn, Mr. White entered Columbus State University to study Psychology, and in December 2010 he was awarded the Bachelor of Science degree. In the Fall of 2011, he accepted a research assistantship, working in a neuroscience laboratory within the Department of Psychology at Appalachian State University, where he also began study toward a Master of Arts degree. Mr. White then accepted a teaching assistantship, guiding laboratory sections of Research Methods in Psychology during the 2012-2013 school year. He was awarded his M.A. in General Experimental Psychology during the Fall of 2013.

Mr. White currently resides in Columbus, Ga and plans to seek acceptance to a behavioral neuroscience PhD program in the Fall of 2014.