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A Novel Experience of Environmental Enrichment Affects the Basolateral and Lateral Amygdala Differently in Adolescent Rats

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Abstract

Environmental enrichment (EE) provides sensory and motor stimulation with same sex conspecifics in a setting often containing objects, ramps, and platforms. The experience evokes behavioral responses to an emotionally arousing situation, which impacts brain development of adolescents who often make decisions based on emotions and novelty. The lateral amygdala (LA) is the primary source of sensory input to the basolateral amygdala (BLA) which initiates emotional behavior and fear learning and acts as an efferent pathway to other brain structures. The purpose of this study was to examine how enriching experiences might affect activation of LA and BLA neurons. Subjects were groups of five adolescent rats, enriched periodically with or without a last EE exposure and unenriched rats with or without a single EE exposure prior to sacrifice. After sacrifice, brain tissue was processed to visualize the neural activity marker c-FOS. There were 25% more c-FOS positive neurons in LA and 16% in BLA of rats with one EE experience compared to those with a history and last EE experience. Enriched rats showed more activated neurons in LA and BLA than in unenriched rats ($p<.05$). As expected, an EE experience, which includes novelty and evokes an emotional response, enhances amygdala activity as compared to a baseline. The data also indicate that rats may become accustomed to EE with periodic exposure, which explains less neural activation in rats with an enrichment history. Lastly, the greater discrepancy in activation of LA neurons may be due to its role as a primary target for amygdala input in comparison to the integrating and output role of BLA. Additional experiments designed to examine intra-amygdaloid circuitry might help explain the differences in numbers of activated LA and BLA neurons.

Keywords: Environmental Enrichment (EE), Basolateral Amygdala (BLA), Lateral Amygdala (LA)

1. Introduction

Adolescence is a critical period of brain development when impulsive decisions tend to be made based on emotions[14,15]. Novel experiences in adolescence have demonstrated an influence on these risk-taking behaviors. Environmental enrichment (EE) includes interactions with a novel environment which utilizes multiple ramps, objects, and platforms[8,13,17]. EE provides a setting for sensory and motor stimulation as well as social interaction which generates behavioral responses to emotionally arousing stimuli[8,13,17]. Enrichment has been found to induce changes in the biochemistry as well as the morphology of the brain by strengthening connections between synapses, increasing dendritic branching, and increasing overall brain weight and size[5,6]. Ultimately, environmental enrichment can produce a lasting effect on learning, memory, and exploratory behavior by means of environmental novelty.

The amygdala is a region of the brain responsible for producing behavioral responses to novel situations. The two major divisions of the amygdala are the lateral amygdala (LA) and basolateral amygdala (BLA). LA is the primary input source of auditory, somatosensory, and visual stimuli, working to integrate information from multiple brain regions outside of the amygdala[3,5,10]. LA filters these stimuli and provides input for BLA, which relays information to the central nucleus of the amygdala (CeA). The CeA functions largely as an output structure that sends information to

other areas of the brain in order to produce a behavioral response[3,5,10]. The purpose of this investigation was to observe the effects of a history and a final exposure of environmental enrichment upon activation within the two major regions the amygdala. Through methods of quantifying activated neurons in the BLA and the LA, these regions can be further differentiated due to their separate functions.

1.1 Environmental Enrichment

Environmental enrichment (EE) is a term used to describe an environment which is both socially and physically more stimulating than a standard environment[7,8,13,17]. An enriched environment includes objects such as ramps, platforms, toys, and other novel conspecifics to interact with during set schedules. EE has a large impact on exploratory behavior, problem solving, memory, learning, and novelty seeking in adolescent rats[2,7,13]. These effects are primarily due to routine exposure to novel objects put in different spatial arrangements and new same-sex age matched rats. A number of protocols on the duration of EE typically specify between one to two months of enrichment[13]. Repetitive exposure to EE over a period of time is important for long term effects.

Environmental enrichment provides an opportunity for learning to take place through novel experiences. Exposure to EE has been shown to induce modifications in the morphology, as well as synaptic plasticity, of rodent brains[7,13,17]. Neural plasticity allows for stronger connections between and within brain structures for increased neural activity and an enhanced capacity for learning. Novel enrichment has been demonstrated in previous studies to produce particularly profound effects in adolescents compared to adults[14,15]. During adolescence, the brain is developing decision-making habits, as well as cognitive processing and neural circuitry. The brain produces behavior that is more impulsive and exploratory in novel situations during this period, suggesting that decisions are based more off of emotions or sensations[14,15]. A particular brain region that is critical in processing emotionality of responses is the amygdala.

Amygdaloid structures are responsible for processing and producing emotion-based behavior. The main subregions of the amygdala, LA and BLA, have also been associated with processing fear and anxiety[16]. When considering the role of emotion-based decision making and the benefits of enrichment in adolescence, repeated exposure to EE could decrease a fear response through mechanisms of neural plasticity in young rats. An alternate study found that manipulation of the environment resulted in morphological changes in both the synapses and neurons within amygdaloid nucleus of rats[6]. A history of enrichment decreases stress responses in animals that are otherwise not enriched[7]. Furthermore, consistently enriched rats were also noted to display lower levels of neural activity at a 24-hour post-experiment sacrifice, suggesting that the enriched rodents were more capable of recovering from a stressful experience by adaptation[6,13].

Environmental enrichment implements a learning environment that will increase the likelihood of adaptation to novel experiences. Observed benefits of enrichment include increased dendritic branching and cell proliferation, as well as equipping the brain for more complex development of behavior through emotion-based decision making within the amygdala[5,6]. The amygdala is crucial in tasks requiring attention, the development of memories, and regulating how sensory information can be perceived through an emotional lens[9,10]. Reinforcing the amygdala's extrinsic and intrinsic neural connections would also create stronger dendritic branching. This could allow for more calculated decision making in the BLA, which has connections with several important brain regions that produce behavioral responses[5].

1.2 Current Investigation

A central part of the corticolimbic circuit, the amygdala, is the region of interest due to its role in regulating stimulatory input and output[3]. In the current study, neural activation in the BLA, a central component of anxiety-related neuronal circuitry, and the LA, the primary input source for the amygdala, were compared in adolescent rats. The majority of inputs to the amygdala are from cortical and thalamic structures, as well as the hypothalamus or brainstem[12].

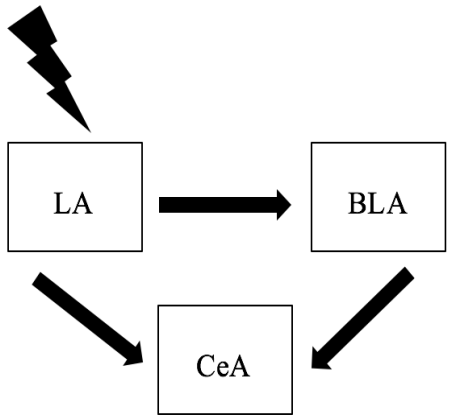

Figure 1. Schematic diagram of the neural circuitry and major input regions of the amygdala.

Figure 1 represents the inputs and outputs of the LA, BLA, and the CeA. The LA receives afferent input from visual, auditory, and somatosensory cortices, as well as sensory nuclei in the hippocampal formation, thalamus, and prefrontal cortices[3]. The neurons of LA largely function to relay this input to the basal nuclei, or in some cases, directly to the CeA[3]. The subcortical nuclei of BLA have many behaviorally important neuronal connections with regions such as the frontal, temporal, and entorhinal cortices[5]. These extrinsic circuitries could allow for integration of various inputs to produce an appropriate behavioral response, although not immediate. In the case of bypassing BLA, and information being relayed directly from the LA to the CeA, a shortcut is created to produce a more rapid behavioral or physical response. The CeA ultimately functions to produce efferent projections as the primary output structure of the amygdala.

The amygdala primarily utilizes these inputs for the processing of emotion to produce a behavior. While the LA has been strongly associated with fear perception, the BLA is more coherent to processing anxiety[16]. Environmental enrichment is likely to produce either one or both of these emotions in a novel experience. Histochemical detection of c-FOS protein from *c-fos* expressing cells is an effective manner to distinguish activated neurons[1]. In the case of the amygdala, these activated cells would indicate emotional processing in the LA and BLA and the efferent projection to produce behavior emitted by the CeA. Previous studies have linked novel enriching exposures to an increase of c-FOS expressing, activated neurons, as well as amygdaloid neuron proliferation[8,4]. As such, the current investigation was posed to examine how novel enriching experiences may activate neurons in the LA and BLA to produce emotion based behavior.

2. Materials and Methods

2.1 Subjects

The subjects in this study were 21 adolescent Long-Evans hooded rats, 10 of which were male and 11 of which were female. They were housed in standard conditions in separate same-sex plastic shoebox cages in groups of 3-4 within a humidity- controlled vivarium on a 12 hour light and 12 hour dark cycle. Food and water were supplied *ad libitum*. The subjects were bred 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, M.C. Zrull, PI).

2.2 Environmental Enrichment

Between postnatal day (PND) 25 and PND 48, a group of rats (n=11) were enriched on a schedule using EE for a duration of 1.5 hours per session and experienced a total of 18 sessions. The subjects included in this enrichment

schedule are only the subjects with a history of enrichment; (EEEE) group and the (EENO) group. In order to control for any extraneous variables in handling, the subjects without a history of enrichment; (NOEE) group, and the non-enriched (NONO) group were stimulated by being picked up and placed back into their home cages twice on the same days that scheduled enrichment took place.

Enrichment took place in a 45.7 X 48.3 X 78.7 cm (w X d X h) wooden frame with a 1/2-in. thick hardware cage covered in aspen bedding that was closed by a latched door. A wide array of different color, shape, and textured objects were placed within the cage on platforms and the bed of the cage as well hung from the mesh ceiling and ramps inside the cage as seen in Figure 2. The platforms were located at 14.0, 24.8, 43.2, and 61.0 cm above the floor of the cage and were made accessible to the subjects via ramps. In each session the subjects experienced, objects were swapped out and placement of objects were changed with a total of four different setups simulating novel environments. In the enrichment cages, the subjects were able to interact with familiar and novel same sex conspecifics that they were not housed with regularly.

Figure 2. Example of one set-up in a female enrichment cage containing various ramps, objects, and platforms.

The picture of the enrichment cage in Figure 2 shows a female enrichment cage displaying one of the various setups used in enrichment trials. Some of the objects included are a mug, foam football, shoestring, dish, and hanging rings amongst others. The male cage is a mirror image of the female cage with the same objects arranged in the same fashion on each enrichment day.

2.3 Final Exposure to Environmental Enrichment

On PND 49, subjects were either exposed to a final enrichment session or not exposed to a final enrichment session. Subjects that were exposed to an enrichment session included the (NOEE), and (EEEE) groups. Subjects that did not experience this final enrichment session were the (EENO), and (NONO) groups. The purpose of this final experience was to make a distinction between subjects that were enriched continuously with a history of enrichment, versus those that had no prior history of enrichment, but experienced a final enrichment session prior to sacrifice.

2.4 Histology

Following the final enrichment experience or not, the subjects were placed in the quiet and dark for approximately 90 minutes then sacrificed by lethal injection of sodium pentobarbital (100 mg/kg b.w., ip). Upon the absence of corneal and tail reflex, each subject was then intracardially perfused using phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB). Following the perfusion, brains were collected and fixed in a 10% sucrose, 4% paraformaldehyde solution at 4°C for a week and then stored in a PB and 0.05% sodium azide solution for storage until the tissue was processed. The brain tissue was then cut into 50 micron sagittal sections using a

Vibratome Series 1000 Sectioning System and were processed using floating section immunohistochemistry (IHC) which was utilized for c-Fos staining as a neural marker of activation.

On the first day of IHC, floating sections were rinsed in PBS (2 x 5 min) and incubated for 15 minutes in 0.5% hydrogen peroxide. Sections were rinsed again in PBS (2 x 5 min) and subsequently incubated in 15% goat serum in 0.25% Triton-X for one hour. The sections were then placed in rat anti-c-Fos made in rabbit (Santa Cruz Biotechnology, SC-52) for a total of 40 hours.

On the second day of IHC, floating sections were rinsed in PBS (6 x 10 min) and incubated for one hour in biotinylated goat anti-rabbit secondary antibody (Vector Labs) and rinsed again in PBS (3 x 10 min). Sections were floated in a peroxidase-labeled avidin- biotin complex for one hour (Vector Labs), and rinsed again in PBS (2 x 10 min). Then sections were exposed to a VIP enzyme substrate (Vector Labs) for two minutes followed by removal of the enzyme and placement into chilled PBS until the sections were mounted onto gel-coated slides. They were cover-slipped with Permount (Fisher) following dehydration using graded ethanols and clearing with toluene.

2.5 Microscopy And Data Analysis

In this study, c-FOS, a phosphoprotein used as a neural marker was transiently expressed after synaptic stimulation occurred[11]. The immediate early gene, *c-fos*, is expressed when extracellular stimulation via neurotransmitters or other trophic substances has taken place[1,11]. When the c-FOS protein is stained for using immunohistochemistry methods, it presents itself in the nuclei of neurons[11]. When quantifying the c-FOS activated nuclei, it is evident due to a darkening in the cells where a change has occurred.

Neuronal activation was defined by cell nuclei that were activated by the c-FOS protein and quantified by counting. Stereological techniques were used to compare levels of activation between enriched and unenriched rats with or without a history and last experience of enrichment. Images were viewed at a Plan 10 objective and 1024 x 768 pixel image size then collected using a Nikon Eclipse microscope and PixeLink digital camera with photo capture software. The structures within the amygdala (BLA & LA), were distinguished using an atlas of the rat brain.

Cells that were found to be activated were darkened as seen in Figure 3. Generally, cells which were darker had higher levels of activation than those which were lighter. Only the cells which were the darkest were included in the counting methods and represented in the final dataset using Microsoft Excel.

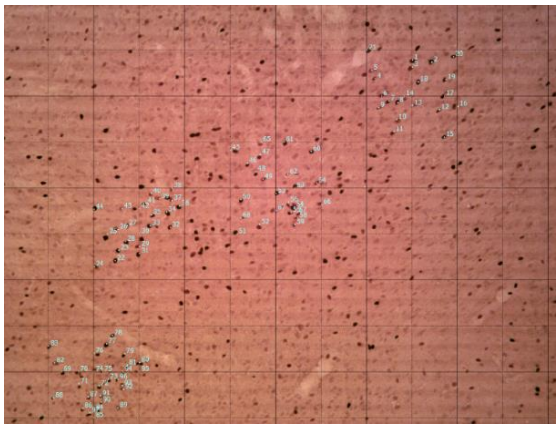

Figure 3. A representation of the method for quantification of the c-FOS expressing activated neurons in the basolateral amygdala.

Represented in Figure 3 is the method of counting that was used. After pictures were collected, brain tissue sections were analyzed. Using Adobe Photoshop CS4, 2x2 inch block grid lines were put in place for counting purposes. Due to regional size differences in the BLA and LA, four samples were counted for the BLA and three samples were counted for the LA. Each samples accounted for included four 2x2 inch blocks in a square arrangement, and each sample was separated by at least one 2x2 block to account for the entirety BLA or LA in the captured image. Individual activated neuronal cells were counted within the samples. Cells which fell on a grid line were not quantified, but those which were inside the grid lines were counted.

3. Results

Table 1. The mean and standard deviations of the expression of c-Fos activated nuclei in the basolateral amygdala and lateral amygdala across conditions with or without a history of enrichment, and with or without a last experience of enrichment.

	Basolateral Amygdala	**Lateral Amygdala**
Condition	*Mean (SD)*	*Mean (SD)*
EEEE	9.8 (3.3)	10.4 (4.7)
EENO	6.4 (2.3)	6.4 (1.7)
NOEE	11.4 (2.3)	13.0 (2.0)
NONO	8.6 (3.3)	7.3 (2.1)

This table displays the quantity of c-FOS expressing, activated nuclei differences between the BLA and the LA. The mean c-FOS expressing nuclei in rats with a last experience of environmental enrichment were higher than those without a last experience in both the LA (9.8 & 11.4) and in the BLA (10.4 & 13.0). The mean c-FOS activation was even slightly higher when it was the subjects first EE experience without a prior enriching experience (NOEE) group (11.4 & 13.0).

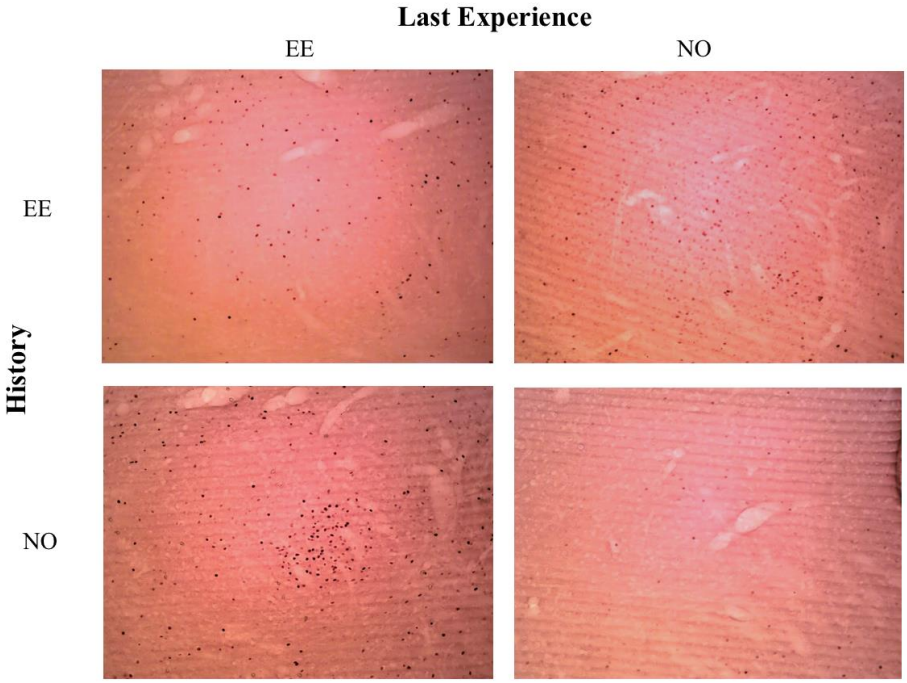

Figure 4. A representation of four LA sections with neuronal activation from each experimental condition with and without a history of enrichment and with or without a last experience of enrichment (EEEE), (NOEE), (NOEE), (NONO).

Indicated in Figure 4, the level of c-FOS expression in the (NOEE) tissue is far greater than that of the expression seen in the (NONO), (EENO), and (EEEE) tissue. The smallest proportion of c-FOS expressing, activated neurons were seen in the (EENO) tissue and second lowest proportion of activation was seen in the (NONO) baseline tissue. These are consistent findings not only in other samples of the LA, but also in samples from the BLA.

From the data in Table 1 and Figure 4, a last experience of EE was determined to have a significant effect in both the LA (p=0.003) and the BLA (p=0.029). History, however, produced no significant differences in the number of c-FOS expressing, activated nuclei. Groups with a final experience of EE differed from those without, regardless of an enrichment history. The effect of a last experience of enrichment was greater in the LA than the BLA because of the ability to account for more variance. Looking at the proportion of variance of the last effect in both cases of LA was 39% and BLA was 23%. This indicates a stronger effect of last exposure in the LA as compared to the BLA.

4. Discussion

The function of the amygdala is primarily for processing vivid emotional memories. As hypothesized, both of the groups that had a last experience of enrichment, (EEEE & NOEE) still showed greater activation than those groups that did not have a last experience of enrichment. This means that even if there is a history of enrichment, when there is a last experience, adolescent rats still process and experience this in a similar way. The neural circuitry is continuously changing especially in adolescents which affects a subject throughout life[14,15].

The amygdala, as a whole, plays an important role in consolidating emotionally arousing memories as well as learned fear in novel situations[9]. The LA and BLA showed different levels of activation which may be explained by the nature of the LA and the BLA. The LA has been shown to respond to emotion-evoking stimuli, which includes anything that is novel to the rat[3,5,10]. The greater effect seen in LA could be due to its nature as a primary input source, in that it activates no matter what, which could contribute to slightly higher levels of activated c-FOS expressing nuclei. Both of these aspects would present as an increase in the number of activated nuclei. BLA is more closely linked to the consolidation or connection of memories to emotional stimuli[3,5,10]. This could potentially account for a lower activation in the BLA due to its role in primarily sending information to the CeA. Repeated exposure to enrichment may serve to decrease anxiety related behaviors that are processed in the BLA[16].

A null effect of history could be deduced from our study, because with more exposure to an enriched environment, novelty would decrease over time. This indicates a decrease in the number of activated cells because structures involved in memory and learning, such as the hippocampus, would predominate. With periodic exposure to novel experiences, an overall decrease in impulsive decisions and risk taking behaviors in adolescents would be expected. Decreased levels of c-FOS positive nuclei in rats with a history of enrichment may also suggest possible neuroplasticity due to repeated exposure, leading to the development of more efficient neural pathways. The effects of neural plasticity are most profound in adolescence, a critical period of development, although effects of enrichment may continue into adulthood[14]. Continued exposures to novel experiences equip these adolescents with the potential to develop more complex behaviors associated with emotion based decision.

A potential limitation to this study could have been that there was a relatively small number of animals. Increasing the sample size would have increased the level of confidence in the results. Relative neural cell densities of the amygdaloid structures may have attributed to the number of activated neurons. In order to account for this variable, a study that will compare the densities of total activated cell bodies using Nissl staining versus c-FOS positive neurons within neighboring brain sections from the same subjects will be conducted. Through this comparison, differences in cell densities between the LA and BLA can be used to determine the percentage of activation within structures.

Although the extrinsic neuronal pathways of the amygdaloid regions have been studied, the intrinsic circuitry and subdivisions of each amygdaloid region has not been as thoroughly investigated[5,10,12,16]. More recent research has divided both the LA and BLA into anterior and posterior regions[4]. Future work will go towards making a distinction between the anterior and posterior BLA due to potential differences in functioning of the anterior and posterior regions. Our research warrants further studies in evoking amygdala activity with alternate tasks to support the theory of neural pathway efficiency as a result of enrichment.

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