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The Influence of a Novel Experience on Neural Activation in the Adolescent Rat Amygdala

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Abstract

Environmental enrichment (EE) fosters physical and social stimulation by exposing animals to interactive floor plans, unique objects, and conspecifics. EE sessions can enhance neural activity in the amygdala, which provides some basis for emotion and motivation as well as processing fearful and rewarding environmental stimuli. Simplified, the rat amygdala consists of an input region (lateral, LA), an integration area (basolateral, BLA), and an output region (central, CeA). While EE manipulations usually occur over time, the present research was conducted to examine the effect of a single enriching experience on neural activity in the amygdala of male (n=6) and female (n=6) adolescent Long-Evans rats. Prior to sacrifice, half of the rats, in same-sex groups, were placed into a multi- level enclosure and experienced EE with a variety of unique objects, while the other six rats served as non-enriched controls. Immunohistochemistry was used to process brain tissue for the neural activity marker c-FOS and microscopy was used to measure the density of c-FOS positive neurons in amygdala structures. Mean densities of activated neurons were greater in amygdala input and output regions, LA (+164.9%) and CeA (+84.8%), of rats exposed to EE than in controls (all $p<.05$). No significant difference within the BLA (+32.6%), a primary integration region for amygdala, between enriched and control rats was observed ($p>.05$). These results suggest the combination of sensory and contextual input to the BLA lowers its level of neural response, compared to the input and output regions which mainly relay current sensory information. The neural activity observed in the input and output regions implies that one novel experience allows for conversion of a large amount of high-resolution sensory information to emotion. For adolescents, such experiences may modify neural circuits in amygdala and consequently alter social and emotional components of adapting to new conditions.

Keywords: Environmental Enrichment (EE), Adolescence in rats, Amygdala

1. Introduction

Adolescence is a particularly important time of transition within human beings, as it marks an essential time for the development of knowledge, basic needs, rights and health. This stage is typically defined as the transition from childhood to adulthood. During adolescent years, individuals commonly initiate stages of rebellion and disobedient behavior that is based primarily on emotion, as there is a substantial increase in amygdala volume during this time[12]. Aoki and fellow researchers suggest that in humans, adolescence is a critical time of maturation and plays a major role in self-advancement whereas individuals who lack progression during this part of life are at risk for underdevelopment in adulthood[2]. Failure to thrive during this period has not only been shown to enhance the risk of deterioration in an individual's social roles, but also in biological development[9]. An additional study by Hair and his team of researchers focused on the significance of the impact of impoverishment on brain development. They showed that impoverished adolescents exhibit atypical structural development in critical areas of the brain including their frontal and temporal lobes, hippocampus, as well as a decrease in total gray matter[6]. Researchers have found that not

only does environment play a role in physical stimulation, but that exercise does as well[3]. Adolescents who are unable to engage in physical activity at a moderate rate are at risk of under developing critical cognitive functioning such as attention levels, behavioral inhibition and memory[3]. Thus, adolescence may serve as a prominent timeframe for studying the outcomes that experiences have on the developing brain both structurally and behaviorally.

These findings in human beings have been used to support further research on the role of adolescence in rats. Adolescence in rats is defined as the transitional time between weaning, typically postnatal days (PND) 21-59[13]. One study in 2009 reported that social isolation for a prolonged period of time within adolescent rats resulted in increased behavioral inhibition, anxiety-like behaviors and latency for social approach and contact within those affected[10]. Lukkes and his colleagues found no success in their efforts to try and combat the effects of adolescent social isolation due to the consequences of social isolation being so ingrained in the rats' brains, resulting in their anxiogenic profiles remaining static throughout adulthood[10]. The effects of behavior and environment during adolescence were further investigated by Amodeo and his team of researchers. They compared the consumption of alcohol in adolescent vs. adult-aged rats and its impact on neurobehavioral functioning. It was found that rats who consumed alcohol as adolescents were more likely to also consume alcohol as adults compared to controls[1]. Furthermore, they suggested that alcohol consumption ultimately led to less inhibited behavior, meaning rats with a history of alcohol consumption during adolescence were more likely to involve themselves in risky behavior as both adolescents and adults[1]. Ultimately, this suggests that behaviors developed in adolescence are likely to progress into adulthood.

1.1 Environmental Enrichment

Environmental enrichment (EE) fosters physical and social stimulation by exposing laboratory animals to interactive floor plans, unique objects and conspecifics[16]. This type of housing cultivates greater levels of stimulation than the laboratory animals would experience under standard housing conditions. EE can be divided into two components: social and physical. Social enrichment involves purposefully socializing subjects with one another. Animals in these conditions are typically placed into large groups rather than with basic littermates or in isolation[16]. The physical aspect of EE involves altering housing conditions, such as manipulating floor plans and toy exposure to allow for more activity[16].

One significant effect of EE that has been observed in rats is that of neurogenesis. Neurogenesis refers to the growth, proliferation and survival of nervous tissue[5]. This is a structural change observed in the brain that can have profound effects on the physical and social aspects of one's life. Although researchers have not been successful in reversing the consequences of social isolation, one study that focused primarily on physical EE found that exercise has the ability to revert disrupted neurogenesis and recover long term potentiation (LTP) in rats[13]. Specifically, Sakalem discovered that the combination of exercise and enriched housing over multiple sessions stimulate cell division and survival, which has the ability to restore overall cognitive ability in rats[13].

In addition to this, Hirase and Shinohara investigated the effects of EE on the neural changes in the cerebral cortex and hippocampus of rats, focusing on anatomical, electrophysiological and molecular differences. They discovered that prolonged EE during adolescence does in fact heighten brain volume, enhance neurogenesis and increase neuropil complexity. Continuous exposure to EE throughout late adolescence and adulthood was found to promote these effects to a greater extent, associated with increased brain weight and cell proliferation[7]. This suggests EE-induced changes in the brain are global with the inclusion of experience-dependent plasticity of pre-existing circuits[7].

EE creates a learning environment that has the ability to increase the likelihood of adaptation to novel experiences if repeated exposure is involved, especially during adolescence. This adaptation increases dendritic branching and cell proliferation in rats, as well as preparing the brain for similar future experiences[17]. Additionally, becoming familiar to these experiences increases complex thought processes and development of emotion-based decision making of rats through the amygdala[17]. The bridge between the hippocampus and the amygdala links emotion to memories and vice versa, reinforcing this increase in dendritic branching. Most of the current literature on EE is focused on prolonged exposure which is what allows for these long-term impacts on the brain to be recorded. The current study focuses on the effect of a single enriching experience in adolescent rats and how this contributes to behavior and neural activity with the amygdala as the prime area of investigation.

1.2 c-*fos* and c-FOS

The current experiment investigates the effect of one enriching experience in rats using a neural protein marker known as c-FOS. This protein marker allows for mapping of functional activity in amygdala subsections, exposing areas with neural activity. Active neurons are associated with a dark color due to c-FOS accumulation. Inactive neurons are

associated with a lighter color due to lack of c-FOS. This protein marker is derived from the immediate early gene (IEG), c-*fos*[4]. Chaudhuri describes the signal transduction sequence that affirms the production of the c-FOS protein. This sequence is most commonly initiated by a calcium (Ca^{2+}) influx into the cell, which commonly occurs through NMDA receptors[4]. From here, different enzymes are turned on which then activates proceeding transcription factors[4]. The IEG is transcribed into c-*fos* mRNA, which is translated into the c-FOS protein[4]. This neural protein marker has been found effective in a number of studies identifying neural activity, and in turn is what was also used in the current investigation[8,14].

1.3 Current Investigation

EE sessions can enhance neural activity in the amygdala, which provides some basis for emotion and motivation as well as processing fearful and rewarding environmental stimuli in rats. The amygdala plays a crucial role in the corticolimbic circuit, regulating stimulatory input and output. Simplified, the rat amygdala consists of lateral amygdala (LA), the primary input region; basolateral amygdala (BLA), the primary integration area; and central amygdala (CeA), the primary output region[15]. While input to LA can essentially be from any region, the majority of information is driven from cortical and thalamic structures, as well as the hippocampus or brainstem[15].

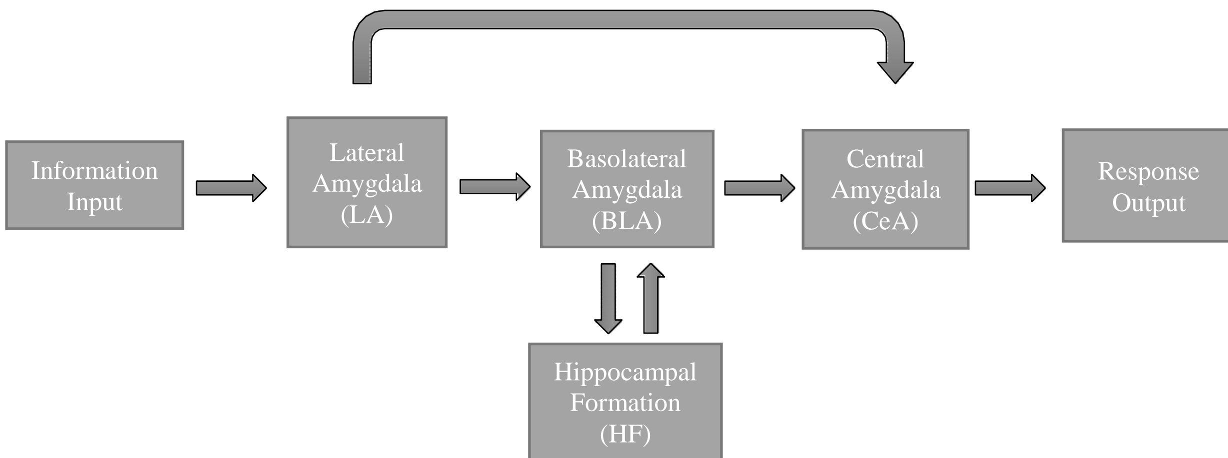

Figure 1. Circuitry of amygdala with hippocampal formation in a rat.

Figure 1 represents the input and output of LA, BLA, and CeA in a rat. LA is the input region, receiving afferent input from visual, auditory and somatosensory cortices, as well as sensory nuclei in the hippocampal formation, thalamus and prefrontal cortices. LA directs its information straight to BLA, or in some cases, to CeA through a bypass channel[15]. This allows raw stimuli to be directed out of the amygdala without having been integrated by BLA, producing a more rapid behavioral or physical response[15]. The nuclei of BLA have many important neural connections with the brain's cortices, allowing for integration of various inputs to produce an appropriate behavioral response, although not immediate[11]. BLA is also able to integrate with the hippocampal formation (HF), meaning it receives both sensory and contextual information. CeA ultimately functions to produce efferent projections as the primary output structure of the amygdala, receiving information from both LA and BLA[15].

The present research was conducted to examine the effect of a single enriching experience on neural activity in all subregions of the amygdala of male and female adolescent Long-Evans rats. It was hypothesized that compared to control subjects, or those not enriched, the experimental group would produce a greater number of activated neurons in all subregions of the amygdala. It is suspected this is most likely due to the short, single exposure that rats have with the novel environment. This is of interest because EE manipulations usually occur over time, however, it is predicted the same EE-induced changes to the brain and developments in behavior will be observed from a single enriching experience.

2. Materials and Methods

2.1 Subjects and Setting

The subjects in this study were twelve (six male, six female) adolescent Long-Evans hooded rats which were cared for in the Arts and Sciences Animal Facility at Appalachian State University. Subjects were initially ran as adolescents, PND 36. All subjects were housed and fed in standard shoebox housing in same-sex groups of three and placed on a 12-hour light-dark schedule. Subjects in the experimental group were separated for enrichment prior to sacrifice in an EE cage containing numerous play objects, while subjects in the control group were exposed to minimal human contact and no play objects. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University.

Figure 2. Multi-level female enrichment cage containing various objects for play.

Figure 2 depicts an enrichment cage used for the female subjects in the experimental group during enrichment sessions. Enrichment cages used for male subjects mirror that of the one pictured in Figure 1. The enrichment cages contained objects that include the following: a foam football, hanging rings, a mug, a tennis ball, plastic fruit and a squish toy.

2.2 Experimental Conditions

2.2.1 experimental group

Six randomly selected males and six randomly selected females were placed into enrichment cages for a 90-minute session. Following the 90-minute enrichment session, the subjects were placed into quiet and dark containment for 90 additional minutes. The quiet and dark containment enabled the subjects time to fully express the neural protein marker, c-FOS, prior to sacrifice.

2.2.2 control group

The remaining subjects remained in regular housing conditions for the duration of their lives leading up to sacrifice. Nevertheless, prior to sacrifice, the subjects in the control group were placed into quiet and dark containment to enable full expression of the neural protein marker, c-FOS. Moreover, subjects in this condition were handled regularly

throughout their lives through being picked up and placed back down in accordance to the experiment group's enrichment handling.

2.3 Histology

Upon completion of the 90-minute quiet and dark session, all subjects were sacrificed by lethal injection of sodium pentobarbital (100 mg/kg b.w., ip). Upon the absence of corneal and tail reflex, each subject was intracardially perfused using phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB). Following perfusion, brains were collected and fixed in a 10% sucrose, 4% paraformaldehyde solution at 4° C for a week and then kept in a PB and 0.05% sodium azide solution for storage until the tissue was processed. The brain tissue was cut into 50 micron sagittal section 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 PBC (2 x 5 min) and subsequently incubated in 15% goat serum in 0.25% Triton-X for one hour. The sections were placed in a 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 PBC (6 x 10 min) and incubated for one hour in biotinylated goat anti-rabbit secondary antibody (Vector Labs) and rinsed again in PBS (2 x 10 min). The 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.

2.4 Data Collection Procedures

The sagittal cut 50 micron sections were cover-slipped with Permount (Fisher) following dehydration using graded ethanol and clearing with toluene. These tissues were stained with c-FOS which presents in the nuclei of neurons, with the darkness of the nuclei indicating the intensity of activation[14].

After the tissues were stained with c-FOS, the microscope slides were examined using a Nikon Eclipse microscope. Pictures of the tissues were taken with a PixeLink digital camera (plan 10 objective) connected to the microscope and subsequently analyzed. Neurons in BLA, CeA, and LA of each sample were quantified using Adobe Photoshop CS4 with 2x2 inch block grid lines. The samples accounted for included four 2x2 inch blocks forming square arrangements, with each sample having a separation of a minimum of a 2x2 inch block. While cells on the grid line were not quantified, those inside the grid lines were. To ensure accurate quantification, all images were counted and averaged by two observers who were blind to the conditions.

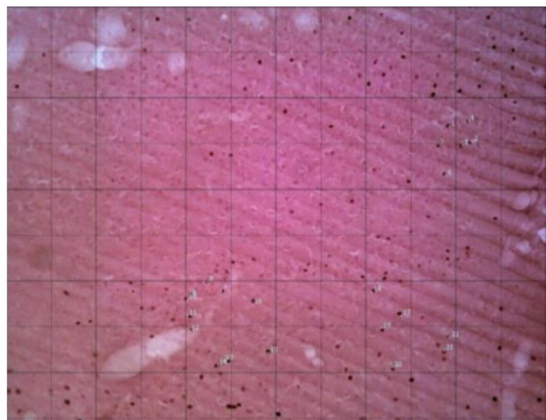

Figure 3. A depiction of the quantification method of neurons in LA expressing c-FOS.
Brain tissue 1711L.

Figure 3 depicts an image of LA taken with a Nikon Eclipse microscope and a PixeLink digital camera (plan 10 objective) being processed in Adobe Photoshop CS4. The blue numbers evident on the image indicate the quantification of c-FOS activated neurons with the Adobe Photoshop counting tool. As mentioned earlier, activated

c-FOS neurons were quantified through counting techniques wherein cells on a grid line were counted, while those inside a grid line were not.

3. Results

Table 1. The mean and standard deviation of c-FOS activated nuclei expressed in BLA, LA and CeA of subjects in the control and experimental conditions.

	Enriched		Control	
Amygdala Structure	*n*	*M(SD)*	*n*	*M(SD)*
LA	5	15.1 (2.5)*	5	5.7 (1.7)
BLA	5	11.4 (2.3)	5	8.6 (3.3)
CeA	5	18.3 (3.0)*	5	9.9 (3.7)

*significant when $p<0.05$

Table 1 displays the mean number of c-FOS activated neurons in BLA, LA and CeA as well as the standard deviations of enriched and control groups. For each structure, variance was pooled across the experimental groups to aid in differentiating the effects of the independent variable (environmental enrichment) from error. Subsequently, the error variance was used in independent sample t-tests that compared the proportions of c-FOS positive nuclei in the enriched vs. control conditions for all three amygdala regions. There was a significant difference in the proportion of c-FOS activated nuclei for LA experimental and control rats, $t(8)=6.95$, $p=.0001$. A similar significant finding was discovered for CeA, $t(8)=3.95$, $p=.0042$. In contrast, there was no significance found in the proportion of c-FOS activated nuclei in BLA between enriched and control groups, $t(8)=1.54$, $p=.1629$.

Additionally, the extent to which EE increased activation of c-FOS positive neurons in the amygdalae of the subjects could be further quantified through percent differences derived from between-subject comparisons. The following percent differences were determined: LA=164.9%, BLA=32.6%, CeA=84.8%. While EE was found to increase the number of c-FOS positive nuclei in all amygdala regions, only LA and CeA were determined to have significant differences. Furthermore, it was observed that the enhancement of activation in LA was nearly twice that of CeA. These findings support the notion that environment enrichment may increase neural activation in the amygdaloid formation.

4. Discussion

It was originally hypothesized that compared to the control group, subjects in the experimental group receiving a single EE experience would have greater neural activity in LA, BLA and CeA. This hypothesis was accepted, as all percent differences and means of the proportions in experimental group were greater than those in the control. In other words, this indicates that similarly to the effect of multiple EE sessions, one single exposure of EE has the ability to generate increased neural activity in all studied subregions of the rat amygdala. Although these increases were observed, not all were statistically significant. The difference in proportion of activated neurons in BLA for experimental rats was not significantly greater than that of control rats. This provokes question as to what might be happening in this integration region and why the proportion of active neurons is substantially less in BLA than in LA or CeA after an EE session.

It can be suggested that the combination of sensory and contextual input to BLA lowers its level of neural response compared to the input and output regions which relay only current sensory information. It has been found that LA primarily responds to emotion-evoking stimuli, which to a lab rat most likely includes all aspects of an environmentally enriched cage[10]. The reasoning behind the significant increase in neural activity observed in the LA lies in the fact that this region is constantly taking in information even when the rat is in standard shoebox housing. The amount of information LA receives substantially increases when the subject is placed into a new, exciting environment. Just like LA, CeA is also always receiving information. Although this information is primarily coming

from other subregions of the amygdala, CeA is still continuously being stimulated. This finding aids in the explanation for the increased neural activity observed in CeA[10]. The percent difference for CeA is less than that of LA most likely because a large portion of the information received by CeA has already been integrated and modified by BLA. In other words, BLA has the ability to elect what information gets sent to CeA, the hippocampus or other cortical regions which lowers the number of active neurons observed in CeA in comparison to LA. As mentioned, BLA is the primary integration region. It is predicted that the reason for a non-statistically significant increase in neural activity is due to the combination of both sensory and contextual stimuli this region receives. The contextual information BLA receives from the HF can be depressing, producing a lower proportion of activated neurons than expected[10]. The neural activity observed in LA input region and CeA output region implies that one novel experience allows for conversion of a large amount of high-resolution sensory information to emotion. For adolescent rats, such experiences may modify neural circuits in amygdala and consequently alter social and emotional components of adapting to new conditions.

Additional reasons for the depressed proportion of active neurons observed in BLA could be due to the relatively small sample size used. In terms of generalizability and validity, a larger sample size with a greater number of trials might have impacted the confidence of the results. In addition to this, the methodology of counting might have accounted for the findings attributed to BLA. To improve upon the certainty of counting, a light-dark contrast system might be employed to determine which dark neurons are counted as active, instead of using subjective reasoning. All suggested improvements should be adapted in future replications.

Although there is a substantial amount of research on the effect of EE over multiple sessions, additional investigations should be promoted to continue with the idea of how one session impacts biology and behavior. One particular study that further explores this idea of a single EE experience was conducted by VanElzakker and researchers. They investigated the impact that EE employs on subregions of the rat hippocampus using c-FOS as their primary neural protein marker[18]. Similarly to the current study, researchers found considerable significant differences in neural activity in two-thirds of the hippocampal regions that were under investigation[18]. These impressive discrepancies found in both the current study as well as the VanElzakker study is a common discovery among previous literature on EE in rats. This further confirms the hypothesis that EE has a substantial positive effect on neural activity in the rat brain. To fully establish understanding of this idea, research should extend outside the amygdala and hippocampus into other regions of the brain that may be significant to this area of study. Overall, these results support the suggestion that what is accomplished over multiple EE sessions can also be achieved by a singular EE session during adolescence. This research further warrants the predictability of behavior during adolescent years, as well as reinforcing its importance.

Additionally, this research can be related back to what we know about the adolescent years of human beings. It is important to realize that although not all aspects of these findings are generalizable to the human population, it should be noted that adolescence is a particularly important stage of aging as it determines our future in terms of behavior, attitudes, ideals and so much more. Without these primitive years of learning and development, individual growth would be minimal and what we as human beings consider unique about ourselves might be nonexistent.

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