The Effect of Early Life Adversity on Basolateral Amygdala Projections Activating Cells in the Prefrontal Cortex

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The basolateral amygdala (BLA) and prefrontal cortex (PFC) pathway is implicated in producing anxiety-like behaviors. In rats, the BLA sends axonal projections to the PFC playing a role in modulating anxiety-like behavioral output. While we know that there are sex differences in anxiety following early life adversity (ELA), research on the connection between the BLA and PFC has primarily been done exclusively on male rats. Honeycutt et al., (2020) reveals that rats who experience ELA develop stronger connections between the BLA and PFC, and these changes occur at an earlier juvenile age in female rats. Interestingly, the changes in the BLA-PFC pathway did not occur until adolescence in male rats. Additionally, the BLA-PFC circuit matures at an earlier age for females than it does for males. However, at an older age, the difference between sexes in degree of maturation is not observed. It is unclear what kind of cells in the PFC are activated by the early BLA projections and how this relates to behavior. We investigated what types of cells are being activated, what the excitatory/inhibitory balance is, whether ELA effects change with age, and what are the sex differences. We performed behavioral analysis, electrical BLA stimulation via stereotaxic surgery, and immunohistochemistry to elucidate answers to our research questions. It is crucial that we elucidate why females are at a higher risk of developing anxiety- and depression- like behavior following ELA so that we can develop individualized treatments.

Honeycutt et al., (2020) was able to determine that females were more vulnerable to the neurological effects of ELA, as they experienced earlier maturation of the BLA to PFC pathway, while males did not. However, that research paper was not able to determine whether the increased connectivity between the BLA and PFC was integrated and/or functional connectivity. There are multiple questions our summer research is investigating. First, what type of cells are being activated by the BLA projections to the PFC and are these innervations integrated? Second, how many of the cells are excitatory/inhibitory? Third, what are the sex differences that exist? Fourth, do the effects of ELA change depending on age?

To investigate these questions, we performed behavioral analysis, BLA stimulation via stereotaxic surgery, and immunohistochemistry. The Honeycutt Lab uses maternal separation to replicate caregiver deprivation as a form of ELA. First, timed-pregnant rats arrive at gestational day 15 and gave birth approximately one week later. Rats were housed under standard laboratory conditions in a 12-hour light/dark cycle (lights on at 0700 and off at 1900) with temperature- and humidity-controlled vivarium access to food and water ad libitum (Lab Diet P500 Prolab RMH 3000). All rats were housed in polycarbonate caging with wire cage tops and heat-treated pine shaving bedding. Following birth (postnatal day [PD]0), litters were assigned as either: CON (control condition) and left undisturbed except for cage changing twice/week, toe clipping (PD5), weighing (PD9 & 21), and handling (2 min/pup on PD11 & 15); or ELA (experimental condition) via maternal and peer separation. On PD1, litters were culled to 10 (+/-2) pups, and an even sex ratio was maintained whenever possible. Rats were considered outliers when they were found to be visibly agitated or otherwise behaving abnormally outside of experimentation and were excluded from subject analysis. ELA pups were separated from dams and littermates in individual cups filled with home-cage bedding placed in a temperature and humidity-controlled incubator (RCom Bird Brooder 60, 35 C) from PD2-10. Cups were also placed within a cardboard box to minimize noise produced by the incubator itself. At PD11, the pups can self-regulate their body temperature, so they were individually placed within smaller cages instead. Pups were separated for 3 ½ hrs a day during PD2-10 and 4 hrs during PD11-20, from 0900 (0930) h -1300h. During this time, they were deprived of maternal and littermate tactile stimulation and nursing, but not from

maternal odor. ELA dams remained in their home cages but were deprived of their entire litters during separation. Pups were weaned at PD21 into same-sex litter pairs and left undisturbed apart from cage changes until surgery/behavioral assessment.

Elevated zero maze performance was evaluated 1 day prior to surgery for all animals. The maze was a circular ring constructed from solid black plastic with four distinct areas: two areas had walls 40cm cm tall on both sides while the other two areas were entirely open with a 100cm diameter. Between each animal, 50% ethanol solution was used to clean the apparatus. Subjects were placed in the closed area while facing the open arm and behavior was analyzed via Ethovision for 5 minutes per subject. Subjects were returned to their cagemate(s) following the analysis. No acclimation process was undergone to evaluate each subjects' response to a novel environment.

Male and female rats from CON and ELA litters at PD28 & 48 underwent stereotaxic stimulation of the right basolateral amygdala (Master-9 pulse stimulator, 0.75mA current for 50x4 pulses with 60 microseconds duration, 0.3 second intervals)**.**

Rats were first anesthetized with Isoflurane in an induction chamber before beginning surgical procedures. After ensuring the rats were anesthetized via a firm toe pinch, Meloxicam (1-2 mg/kg) was injected subcutaneously as a postoperative analgesic. Surgical site was shaved, and the animal was secured via ear bars with top incisors positioned over a bite bar within a nose cone to provide continuous Isoflurane anesthetic during surgery. A skin incision was made along the midline of the skull to visualize Lambda and Bregma. Dorsal-ventral (DV) coordinates for Lambda and Bregma were taken to ensure that the skull was level, and Bregma was used as a landmark to navigate to the position above the BLA, where a small hole was drilled into the right hemisphere of the skull. The electrode was then slowly lowered into the brain until it reached the appropriate DV coordinate (DV was calculated from dura surface to account for differences in skull thickness), and the electrode remained in this position for 5 minutes to give the brain time to accommodate the presence of the electrode. Stimulation then began and lasted for approximately 1 ½ minutes. The electrode remained in the brain for another 60 seconds after the stimulation ended, then the electrode was slowly retracted. The incision was glued (3M Vetbond tissue adhesive), and rats were returned to their cagemates for the 90-minute recovery period before euthanasia.

90-minutes post-surgery, rats were euthanized via CO_2 and transcardially perfused with ice-cold (0.9%) physiological saline, followed by ice-cold 4% paraformaldehyde solution. Brains were extracted and stored in 4% paraformaldehyde solution for 1 week before being transferred to a 30% sucrose solution for 4 days for cryoprotection. All brains were sliced into 40 µm serial sections on a freezing microtome, with serial sections placed in well plates filled with freezing solution for -20°C storage. Sections taken for analysis include PFC, BNST, and BLA. This year, I will continue this project as my Honors and collect more data and conduct analysis to answer my research questions.

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