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Research Plan:

The AVP Sub-populations Involved in Infant-Directed Aggression

Rationale:

The paraventricular nucleus of the hypothalamus (PVN) is a heterogenous nucleus composed of projections to the posterior and anterior pituitary via median eminence (ME) (connects hypothalamus to pituitary) and autonomic centers in the medulla and spinal areas (which are involved in autonomic responses like swallowing and reflexes, respectively). In times of stress, hypothalamic neurons will send signals to the pituitary gland, via the PVN's projections, which will then send hormones to activate the adrenal glands and increase cortisol or corticosterone serum levels (major stress hormones produced by the adrenal gland) (Liu 2010). This means that the PVN plays a role in how we respond to stress. Importantly, a subset of cells in the PVN project to a region of the Hypothalamus called Perifornical Area (PeFA), which is selectively activated during infant-directed aggression and infanticide in virgin male mice. Within the PVN, neurons that express arginine vasopressin (AVP) are the ones that project to PeFA infanticide-activated neurons. Furthermore, there are AVP sub-populations that can be implicated in infant-directed acts of aggression, specifically Prodynorphin (pDyn) and corticotropin releasing hormone (CRH).

Though the results cannot be directly applied to human behavior, they can lead to speculation that human infant-directed behavior might also be affected by changes in these hormone levels. In humans, there is an established link between AVP concentrations in the cerebrospinal fluid (CSF) and acts of aggression. Though all studies do not share the same conclusion, perhaps due to a discrepancy in the populations studied, a positive correlation exists between aggressive behavior and CSF AVP concentrations (Caldwell 2009). Additionally, if mice go through this hormonal transition when first becoming a parent, then perhaps the sam happens in humans. Any hormonal imbalance in this transition could lead to maladaptive

behaviors such as postpartum depression. This study could broaden our understanding of these maladaptive behaviors and, in the future, seeking ways to fix this potential imbalance.

Research Question:

What are the pDyn and CRH AVP sub-populations' role in infant-directed aggression in mice?

Hypothesis:

It is hypothesized that AVP neurons involved in infanticidal behavior or infant-directed aggression will express more CRH and pDyn than those involved in parental behavior; in terms of neuron activation, the AVP cells co-expressing CRH or pDyn are more likely to be active in an infanticidal mouse during pup-exposure.

Procedures:

Role of Mentor:

The mentor will conduct the behavior assays necessary to establish if the mouse was infanticidal or parental, as well as perform the tissue collection.

Behavior Assays:

The mice that will be used for this experiment were 4 virgin males, 4 virgin females, and 4 fathers. Neither the virgin males nor the females will be exposed to pups prior to experimentation and fathers will be separated from their pups within a few days of birth. The pups that will be used will be stranger's pups.

My mentor would conduct a behavioral test that would determine if the mouse was infanticidal, parental, or neglectful as follows: a mouse will be selected and their cage will be placed in a room with red light, so as not to disrupt their reverse light cycle. The cage will be surrounded by three cameras to record behavior. The mouse will be left in the cage for 5 minutes so it can get accustomed to its new environment.

Following these 5 minutes, the mentor will place a pup in the cage; the recording will be started at the same time. The mouse will have 10 minutes to interact with the pup and, unless the

mouse is infanticidal, each ten minute trial will be run to completion. If the mouse is infanticidal, the mentor will stop the trial. If the pup is injured, the pup will be euthanized to prevent further suffering; if not, it will be used for another round of behavior. No animals died as a result of the experiment.

The mouse's behavior will be catalogued and the same test was repeated with the other mice. Once all the tests are completed, each mouse will be assigned a random number, so as to prevent any bias when viewing the tissue under a microscope.

Tissue Collection:

The mentor will collect the tissue 35 minutes after pup exposure. It will be fresh frozen, meaning it will not stored in any preservative solutions and instead will be placed in OCT, which prevents the tissue from freezing completely and becoming brittle, and then will be placed on dry ice.

The mentor will take coronal sections of the PVN that are 25 microns thick using a Cryostat. The sections will then be mounted onto slides and stored at -80°C.

PLEASE NOTE: All animal handling, including treatment and sacrifice, will be performed by the mentor.

Role of the Student Researcher:

RNAscope Treatment:

The necessary solutions for the RNAscope treatment will be prepared prior to starting the experiment. The RNAscope treatment kit, to be provided by ACDBio, allows researchers to investigate the location of specific cells by tracking the location of specific mRNA, and therefore identify the different types of cells based on their expression profiles. The unique probes bind to the target mRNA with high specificity (which will help distinguish the different sub-populations) and will fluoresce under the microscope. This will allow us to determine which cell subpopulations exist in the PVN and how they differ depending on behavior.

The first step in pretreatment involves fixing the tissue using paraformaldehyde (PFA) and then dehydrating it using ethanol. The tissue will then be baked for an hour to insure it is completely dehydrated and fixed. Next, a Protease, an enzyme that would break down any extraneous proteins, will be applied. This step requires precise timing, more precise than the others, because if the Protease is left too long on the tissue, the enzyme could start to digest the tissue as well.

After the pretreatment, the probes will be hybridized to their respective mRNA strands; this is done with all 9 probes at once, mixed with a probe diluent. Next, three amplifiers will applied separately and then the solution containing the first three fluorophores. Because the microscope can only view so many channels at once, the imaging will be divided into three rounds.

Ultimately, when all the solutions pertaining to the RNAscope treatment are applied, the slides will be stained with DAPI, a fluorescent stain that binds to the Adenine and Thymine rich areas in DNA, found within the cells' nucleus. This allowed the analysis software to identify if a signal is actually coming from a cell (will overlap with DAPI) or if it is just noise or autofluorescence. Finally, mounting medium and a cover slip will be applied and the slides will be stored in the fridge.

C-fos:

In order to assess the activation of the CRH and pDyn sub-populations of AVP neurons in the experiments, the expression of c-fos will be examined as well. C-fos is a protein that rapidly increases in concentration after a cell is activated. The mRNA concentrations increase first, while the protein concentration increases 90 minutes later. Thus, c-fos mRNA will be used as an indicator that a cell was activated during pup-exposure. When a cell tests positive for two or more markers, it is called co-localization.

Imaging:

The images will be taken with an Axio Imager 4.2, which allows for editing of, through a software, the exposure, color, and order that the channels appear in. The sections with the

strongest signals will be chosen so that analysis of the sections is possible. Between eight to ten tiles will be acquired to cover each section; the same area will be targeted on each section

Analysis:

Once the images are acquired, all the channels will be enhanced using the same Axio Image software. This will make it possible to visualize each individual channel and allow the analysis software to process the data. The channels will be aligned using ACDBio Registration software and organized in image stacks using the java-based software Fiji. The MatLab NucleiBot software will then used to quantify the number of cells containing that particular mRNA. A Fisher exact test will be used for statistical analysis (with a threshold of significance of 0.05).

Hazardous Chemicals, Activities, Devices: Paraformaldehyde (PFA)

Research:

Paraformaldehyde (PFA) will be used by both mentor and student to fix the tissue and prevent tissue degradation when applying the RNA HiPlex treatment. The US Department of Human Health and Services have labelled formaldehyde (PFA is a polymerized form of formaldehyde) as a human carcinogen, upon repeated exposure. Some smaller side effects include allergic reactions of the skin, eyes, and respiratory tract.

Risk Assessment:

The lab will undergo review by the members of the Environmental Health and Safety Department at Albert Einstein School of Medicine.

Supervision and Safety Precautions:

The mentor will supervise the student researcher at all times when working with potentially hazardous materials, like PFA, under the fume hood. The student researcher and the mentor will wear standard gloves, closed-toed shoes, and goggles to prevent as much contact

with the PFA. As the PFA is in liquid form, there is no danger of inhalation, so a mask will not be necessary.

Methods of Disposal:

The PFA will be placed in a bottle specifically marked biohazardous materials. When the bottles are full, they will be picked up by the Environment Health and Safety Department to be properly disposed of.

References:

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