The AVP Sub-populations Involved in Infant-directed Aggression

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Abstract:

There are many factors that are involved in or could be the cause of infant-directed aggression. This study examined the different PVN AVP subpopulations that are involved in infant-directed aggression, focusing on pDyn and CRH, as they are both heavily involved in the brain's processing of stress. Three groups of age-matched mice were tested: virgin males, virgin females, and fathers. They would undergo a behavior test where they are exposed to a pup to determine if they are infanticidal or parental. The tissue was then collected and processed using an RNAscope HiPlex treatment that marks expressed mRNA strands, causing them to fluoresce under a microscope. Once the RNAscope treatment was finalized, the sections of the PVN were viewed under a microscope and images were captured for analysis. The analysis included using multiple computer software programs to count the number of cells that expressed the desired markers as well as determine which cells were activated during behavior. The results showed a trend for increased concentrations of AVP/CRH cells in infanticidal VM, possibly indicating that VM mice will view a pup as a threat or stressor, perhaps causing their adverse reaction.

Abbreviations/Key Terms: infant-directed aggression; sub-population; virgin males, VM; virgin females, VF; paraventricular nucleus, PVN; arginine vasopressin, AVP; prodynorphin, pDyn; corticotropin releasing hormone, CRH; c-fos; mRNA; co-localization

Table of Contents:

Introduction:

Parenting is considered an incredibly transformative process: you go from caring only for yourself, to having a whole other helpless being depend on you. “It is up to you as a parent to model and teach your child how life should be lived” (Rich, 2017). It can also be incredibly stressful, especially for new parents; this responsibility is completely foreign to them. This can lead to neglect or, rarely, infant-directed aggression. However, there is little understanding as to what happens in our biology to cause such an adverse reaction or what prevents it from happening in the first place.

Parenting is an evolutionary adaptation that has increased the chances of an offspring’s survival. As opposed to having hundreds, if not thousands, of offspring as many insects and fish do, having a few offspring and nurturing them until they are capable enough to care for and defend themselves increases that offspring’s chances of survival and therefore the propagation of that individual’s genes and the species as a whole. However this is no easy task; offspring are another competitor for resources, resources that may already be scarce or in need for the rest of the population (H. Thomspon, 2014). This can lead to infanticide in mammilian species, frequently by males and, in some cases, females too. Males will often kill another’s offspring in order to ensure their genes are being passed down (like a paternity test); the other male’s offspring acts a threat or stressor to them. The male will kill the other male’s offspring in order to prevent the female from lactating and restart her menstrual cycle. Once this happens, the male can reproduce with the female and this will result in a pause in infanticidal behavior, because the pups that are a result, are his own. There has been little research exploring this transition from aggressive males to parental fathers and what neurological changes may occur in the brain to make this happen.

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). These projections allow it to play important roles in autonomic functions, growth, reproduction, and stress control. For the purposes of this paper, only infant-directed behaviors will be expanded

upon. When processing stress, the PVN collects input from surrounding structures (such as the thalamus and medulla), integrates them, and sends an appropriate stress response. In particular, the PVN is involved in the hypothalamic-pituitary-adrenal axis (HPA) (Schwarzer 2010). In times of stress, the 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 suggests that the PVN may be involved in managing stressful situations, such as raising a child. Additionally, the PVN projects to other areas of the brain, especially within the Hypothalamus, and these projections are thought to play a role in social behaviors, including parenting and other infant-directed behaviors. 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. However, the PVN does not exclusively modulate infant-directed aggression as it plays important roles in normal physiological functions.

Arginine vasopressin (AVP), a neuropeptide synthesized in the PVN, is involved in social behaviors such as pair bonding, social recognition, aggression, and some paternal behaviors (Brunnlieb et al., 2016). Its effects on social behavior are found predominantly in males, though AVP is linked to female social behaviors. In mice, the cells in the PeFA that are activated during infanticide receive dense projections from AVP neurons in the PVN (Autry et al. 2019). However, there are different molecularly-defined AVP sub-populations from which these projections originate. This paper will elaborate on the role of Prodynorphin (pDyn) and corticotropin releasing hormone (CRH) in infant-directed aggression.

Prodynorphin (pDyn), one of the three classic opioid precursors, has been found in high concentrations in the PVN of rodents, with similar expression patterns in humans. This implies that pDyn may mediate some of the functions that the PVN is involved in (growth, reproduction, stress control); mRNA coding for the Kappa Opioid Receptor (KOP), which are the receptors activated by dynorphin, have been found in the ME and posterior pituitary, suggesting that PVN pDyn cells project to these areas (Schwarzer 2010). Therefore, the KOP are in a good position to

modulate the HPA. Additionally, when following activation in the HPA in response to stress, corticotropin releasing hormone (CRH) is released, inducing adrenocorticotropic hormone (ACTH) release in the pituitary, which increases cortisol or corticosterone serum levels. Cortisol and corticosterone serum levels prepare the body for a fight or flight response (McLeod 2010). This sequence of events is disrupted in dynorphin-deficient mice as expression of CRH mRNA is greatly reduced, which in turn decreases corticosterone serum levels.

CRH will also be examined as it plays a key role in the stress response and pDyn has an effect on how much is released during times of stress. CRH, a hormone produced by the PVN, is the central driver of the HPA. As previously stated, it causes the release of ACTH, which will trigger the adrenal glands to release cortisol (Society of Endocrinology, 2017). Furthermore, CRH has been linked to infant-directed aggression and neglect. Injection of CRH into virgin female rats, that are naturally parental, caused neglect and an increased rate of pup-killings (Pedersen 1991). This suggests that CRH is involved in infant-directed aggression, even more strongly so than pDyn.

Purpose/Hypothesis:

There have been few studies that have compared the neurophysiology of instinctively infanticidal to those of parental fathers. Additionally, the role of AVP in infant-directed behavior is controversial as it is linked to both aggression and parenting; however, when interacting with a pup, the behaviors are mutually exclusive. This suggests that certain sub-populations of AVP neurons are active during parenting, while others are active during infanticide. For this research project, three different groups of mice were used to model infant directed behaviors: virgin females (VF), virgin males (VM), and fathers. Virgin females are naturally parental to strange pups, meaning they will exhibit parenting behaviors such as nesting, grooming, or retrieval (bring the pups back to the nest). In contrast, virgin male mice are known to instinctively attack pups upon exposure. However, once they become fathers, this instinct goes away, which begs the question: what causes this transition?

This paper will examine the sub-population of AVP cells that exist in the paraventricular nucleus of the hypothalamus (PVN), specifically the roles of pDyn and CRH. 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.

Methods/Materials:

Choice of Organism:

The animals that were used belonged to the genus and species Mus Musculus and were separated into three groups, all age matched: VM, VF, and fathers. Rats could have been used as an alternative, but Mus Musculus allows for more genetic manipulation and though genetic manipulation was not involved in this study, using a consistent species makes it easier to guide potential future projects. Though mice's emotion processing and control is incredibly simplistic compared to that of humans, they serve as excellent model organisms as mice share similar genetic and physiological characteristics to humans (Spencer, 2002). Additionally, the neuropeptides and hormones that are examined in this paper have similar expression distribution in humans and mice, making them an ideal model organism.

Behavior Assays:

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

I did not touch the mice.

A behavioral test that would determine if the mouse was infanticidal, parental, or neglectful was conducted as follows: a mouse would be selected and their cage would be placed in a room with red light, so as not to disrupt their reverse light cycle. The cage was surrounded

by three cameras to record behavior. The mouse was left in the cage for 5 minutes so it could get accustomed to its new environment.

Following these 5 minutes, a pup was placed in the cage; the recording was started at the same time. The mouse had 10 minutes to interact with the pup and, unless the mouse was infanticidal, each ten minute trial was run to completion. If the mouse was infanticidal, the trial would be stopped after the mouse attacked the pup and the infanticidal behavior was established. If the pup was injured, the pup would be euthanized to prevent more suffering; if not, it could be used for another round of behavior.

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

Tissue Collection:

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

Coronal sections of the PVN that were 25 microns thick were taken using a Cryostat. The sections were then mounted onto slides and stored at -80°C.

RNAscope Treatment:

The necessary solutions for the RNAscope treatment were prepared prior to starting the experiment. The RNAscope treatment kit, 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 was baked for an hour to insure it was

completely dehydrated and fixed. Next, a Protease, an enzyme that would break down any extraneous proteins, was 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 were hybridized to their respective mRNA strands; this is done with all 9 probes at once, mixed with a probe diluent. Next, three amplifiers were applied separately and then the solution containing the first three fluorophores. Because the microscope can only view so many channels at once, the imaging had to be divided into three rounds.

Ultimately, when all the solutions pertaining to the RNAscope treatment were applied, the slides were 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 were applied and the slides were 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 was 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 was 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 were taken with an Axio Imager 4.2, which allowed for editing of, through a software, the exposure, color, and order that the channels appear in. The sections with the strongest signals were chosen so that analysis of the sections was possible. Between eight to ten tiles were acquired to cover each section; the same area was targeted on each section

Analysis:

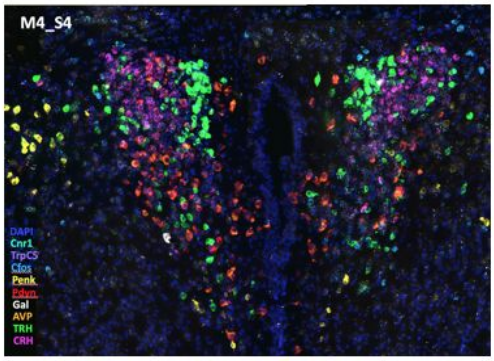
Once the images were acquired, all the channels were enhanced using the same Axio Image software. This made it possible to visualize each individual channel and allowed the analysis software to process the data. The channels were then aligned using ACDBio Registration software and organized in image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

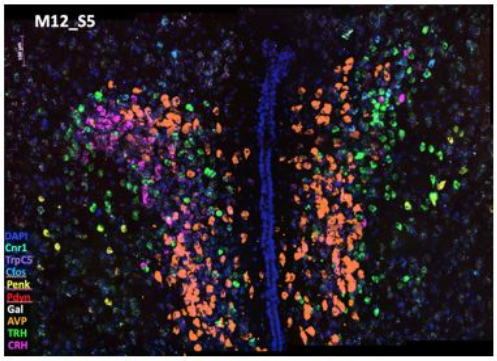
Results:

Distribution of mRNA probes in PVN:

The markers were viewed successfully under the microscope. In addition the target markers of CRH and pDyn, other markers that are known to populate the PVN area were also examined; they may be related to the parental neurons that are activated in VF and fathers. This, however, is not in the scope of the paper.

Figure 1 A-C represents images produced by the Axio Imager, one from each category of mice. Each fluorescent color represents different mRNA for different markers; c-fos is in light blue/cyan, pDyn in red, AVP in orange, and CRH in magenta. The dark blue spots are DAPI, a compound that stains the nuclei of cells.





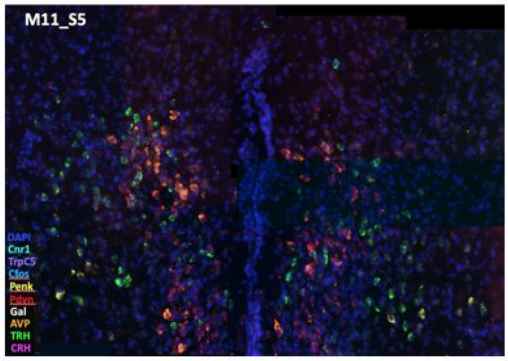


Figure 1 A-C: Representative images showing the distribution of AVP, pDyn, CRH and other common markers across the PVN of Figure A: Mouse 4, Section 4, (VF), Figure B: Mouse 12, Section 5 (VM), and Figure C: Mouse 11, Section 5 (fathers) (left to right).

Co-localization of AVP with CRH and pDyn:

First, the number of AVP positive cells and their subsequent populations were examined, the total cell count was 659 AVP cells in VF, 648 AVP cells in VM, and 792 AVP cells in fathers. Fig. 2A showed that fathers have significantly less AVP/cfos cells than VM and VF ($p < 0.0001$); VM and VF had similar proportion of AVP/cfos cells ($p = 0.4136$). A similar trend was found in AVP/pDyn cells (fathers to VM and to VF: $p < 0.0001$; VM to VF: $p = .2643$) (Fig. 2B). Additionally, Figure 2C showed both fathers and VF had significantly less AVP/CRH neurons when compared to VM ($p < 0.0087$).

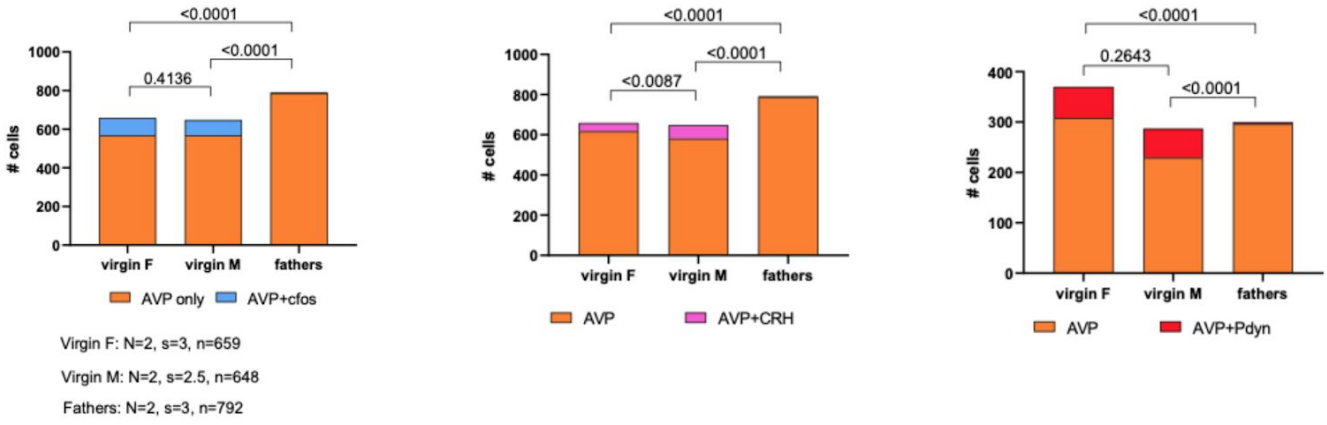


Figure 2 A-C: Graphs depicting the number of AVP positive cells and their co-localization with c-fos, CRH, and pDyn. It is to be read as a fraction or pie-chart. Figure 2A shows the co-localization between AVP cells and c-fos, which is a representation of how many cells were activated during behavior. Figure 2B shows the co-localization between AVP cells and CRH. Figure 2C shows the co-localization between AVP and pDyn. The bars above show the results of the significance tests when two groups are compared (threshold of significance: 0.05).

Co-localization of AVP/CRH and AVP/pDyn cells with neuronal activation marker c-fos:

Next, the number of AVP cells that were active during behavior were counted. Figure 3A showed that fathers had less AVP/CRH/cfos cells than VM and VF, though the difference was not significant (fathers to VM: $p = 0.3$; fathers to VF: $p = 0.58$; VM to VF: $p = 0.65$). Furthermore, fathers had significantly less AVP/pDyn/cfos cells than both VM and VF ($p < 0.0001$) (Fig. 3B). Like in the previous step of analysis, VM and VF had similar proportions of

AVP/pDyn/cfos cells (p = 0.2643). Despite the lack of significance, there was a trend towards a higher proportion of AVP/CRH/cfos neurons in infanticidal VM compared to the parental groups.

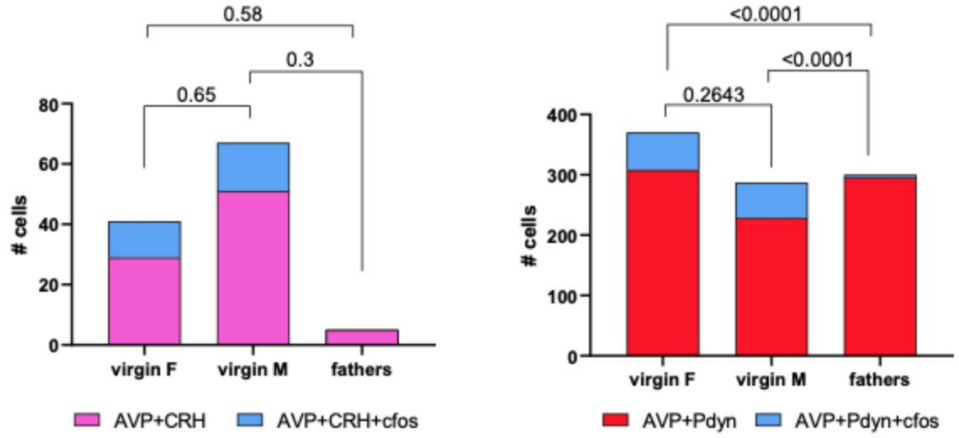


Figure 3 A-B: Graphs depicting proportion of AVP cells that co-localize with AVP sub-populations that were active during behavior (also co-localize with c-fos). Figure 3A showed the proportion of AVP/CRH neurons that were active during behavior. Figure 3B showed the proportion of AVP/pDyn cells that were active during behavior. The bars, again, showed the results of the significance test when the two corresponding groups were compared (significance $p < 0.05$)

Discussion:

The hypothesis that VM that are naturally infanticidal would express higher levels of AVP/CRH and AVP/pDyn was partially supported. There was a significant decrease in the amount of CRH that co-localizes with AVP neurons in fathers and VF when compared to VM ($p < 0.0087$). This could suggest that the distinct infanticidal behavior that is found in VM is a result of an increased AVP/CRH cell count. Or that the parental behavior typically exhibited by fathers and VF is a result of a decreased AVP/CRH cell count. This coincides with the results from Pedersen (1991), which found with increased CRH levels, VF rats (that are naturally parental) turned neglectful or aggressive. Additionally, the proportion of AVP/pDyn cells was significantly smaller in fathers than it is in VM, suggesting the same conclusion ($p < 0.0001$).

However, VM and VF had similar levels of AVP/pDyn cells, suggesting perhaps that pDyn has different effects on males and females ($p = 0.2643$).

When looking at AVP/CRH/cfos and AVP/pDyn/cfos cells, fathers did have less than VM, and in this case VF as well, however there was not a significant difference in the quantity of AVP/CRH/cfos cells (CRH $p = 0.3$). Additionally, there was no significant difference in AVP/pDyn/cfos cells between VM and VF. Therefore, no certain conclusions can be reached in regards to populations of AVP/CRH and AVP/pDyn cells being activated in distinct manners across the three groups during pup exposure. However, there was a trend towards a higher proportion of AVP/CRH neurons in virgin males compared to the parental groups, suggesting they might be more susceptible to stress and more likely to perceive a pup as a stressor. This could, in principle, facilitate the pup-directed aggression.

Significance:

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, Thompson et. al (2004) found that when male test subjects were administered AVP, they had more emotional responses to neutral images, resulting in them perceiving the image as a threat.

Furthermore, most of the hormones that were examined in this paper are found in the same areas of the brain in humans and mediate similar responses (Schwarzer, 2010). If these neurochemical changes allow a VM mouse to transition from instinctively infanticidal to exhibiting parental behaviors towards pups, perhaps this same transition happens in humans, where they transition from caring only for themselves to caring for another individual. Any interruption or error in this transition could lead to unnatural parenting or maladaptive behaviors such as postpartum depression, a condition in which mothers may feel anxiety or find difficulty bonding with the baby (NIMH, 2019). If the trend that higher levels of CRH expression causes

infant directed aggression or neglect proves true, it could indicate these mothers view their child as a stressor and may be the cause of their anxiety and difficulty to bond.

Evaluation:

The scope and application of this study could have been affected by the number of subjects studied. A small number of organisms was selected as we were cognizant that these are living organisms and did not want to use their tissue unnecessarily for a pilot study, but it was large enough to have a high cell count. The data will continue to be collected and analyzed to confirm the initial findings.

Additionally, when sectioning, because the mouse brain is so small, it was difficult to determine if the correct section was reached, meaning sometimes the section may not have been in the optimal location for collecting the desired data. There were also a few extraneous variables that occured when imaging the slides such as damaged tissue, weak signals, or air bubbles over the PVN. This also limited the sections that could be examined, decreasing the available data.

Future direction:

AVP and pDyn are not the only AVP sub-populations that exist in the PVN. Oxytocin, a hormone known to affect social behavior in mammals, is also found in high concentrations in the PVN. The role of oxytocin and other sub-populations can be assessed in the future. Moreover, the quantification refers to number of AVP cells, not the amount of AVP mRNA within AVP positive cells. Consequently, in the future, the AVP mRNA will be quantified using more quantitative measuring techniques such as quantitative Polymerase Chain Reaction, a process which monitors the amplification of a targeted DNA molecule during the Polymerase Chain Reaction.

Conclusion:

Though all the results were not statistically significant, the trend that emerged was CRH and pDyn positive neurons were more active in VM and VF than fathers. This suggests that the trend for increased activation of AVP/CRH and AVP/pDyn neurons may mediate a VM's

response to pup-exposure, perhaps suggesting their susceptibility to stressors and the reason behind their adverse reaction. In contrast, there was little difference in between CRH and pDyn neuron activation in VM and VF, despite the drastic difference in behavior, suggesting these neurons play different roles in males and females. The same neurons could have different projection targets in males and females, or c-fos may not capture the neural activity underlying said behavior (c-fos is only captures short term activation). The potential speculations that resulted from this study, in regard to human behavior, were its implications in understanding maladaptive behaviors such as postpartum depression.

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