The Neurological Underpinnings of "Good" and "Bad" Mother Rats

Running Title: "Neuroscience of Motherhood"

Applicant: Name

Farmville, VA 23909

Faculty Mentor: R. Adam Franssen, Ph.D.
Chichester Science Center, Room 304
Department of Biological and Environmental Sciences
Longwood University
Farmville, VA 23909
Phone: 434-395-2199
Email: franssenra@longwood.edu

Discipline: Biology; Behavioral Neuroscience

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R. Adam Franssen, Ph.D. (Faculty Sponsor) Date

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I. Statement of Guiding Hypotheses

One of the major transitional events in life is becoming a parent. In rats, researchers (including Dr. Franssen and his colleagues) have identified a range of neurologic benefits enjoyed by mothers compared to their non-mother counterparts. Advantages include improved spatial and non-spatial memory abilities, improved recovery from traumatic brain injury, and superior foraging abilities. In my three years working in Dr. Franssen's lab, one of our major findings is that mother rats discriminate between their own pups and pups from another mother by utilizing sophisticated cognitive processing areas of the brain, including the frontal cortex. Moreover, we found that a mother rats will care for all of the pups in a mixed litter (i.e., only half her own) as if they were all her own. This finding surprised neuroscientists that attended our presentations at the Society for Neuroscience and SYNAPSE annual meetings. At those meetings, clinical and research neuroscientists asked us to determine at what ratio mothers will begin to consider a mixed group of pups completely alien. Last year, we determined fairly convincingly that a mixed litter had to contain at least 25% of the mother's own pups for her to retrieve and care for them.

During our analysis of the data from the pup ratio experiment, we discovered something odd. Intriguingly, not all mothers responded in the same way to our conditions. Though the largest number of mother rats retrieved their own pups faster than alien pups, a second group of mothers ("good moms") would retrieve pups from any litter – regardless of own/alien ratio – without hesitation. Perhaps even more surprisingly, a third group of mothers ("bad moms") would completely ignore pups from any litter, even if all of the pups were hers. Taken together, these data suggest that there are underlying neurological differences among mother rats. For this Senior Honor Research program, I aim to investigate the neurobiological underpinnings that drive the differences in mother rats.

II. Research Significance

One of the major research projects in Dr. Franssen's lab is the investigation into the abilities of mother rats to distinguish their own pups from pups from another mother. Prior work had identified that mother rats could indeed identify their own pups (e.g., Beach and Jaynes, 1956) from alien pups. Other researchers found that mother identified their young through a combination of olfactory (e.g., Ostermeyer and Elwood, 1983; Levy et al., 2004) and acoustic signaling (e.g. Brunelli et al., 1995; Nagasawa et al., 2012) that triggers different responses in mothers and non-mothers (Geissler et al., 2013). Our lab has shown that when mother rats are presented with a litter of pups – either 8 of her own pups; 8 alien pups; or 4 of her own mixed with 4 alien pups – discrimination between groups is not only a result of olfactory and acoustic factors, but also relies significantly on the frontal cortex, a region of the brain that we use for higher decision making processes (Fig. 1). Further, we found that mother rats treated a mixed group of pups in a 50-50 ratio as if all of the pups were her own (Fig. 2). Presentation of this finding at regional and national conferences revealed a broad interest among neuroscientists.

When presented with this information, neuroscientists were fascinated. It turns out that many labs, studying everything from addiction (e.g., Frankfurt et al., 2011; Haydari et al., 2014) to hormone expression (e.g., Lonstein et al., 2014) use mother rats for their studies. In these experiments, *researchers do not necessarily use a rat's own pups*! Thus, the implication is that using different ratios of pups could lead to different findings in these studies. Indeed, neuroscientists from a number of sub-disciplines requested that we determine at what ratio (7-1, 6-2, 5-3) do mother rats STOP treating the entire litter is if they were her own rats. Our work to date suggests that the cut-off point is approximately 2 OWN pups to 6 ALIEN pups (~25%; Fig. 3). During our analysis of the data, we found something else of interest – not all rat mothers performed equally in every group (see "Statement" above; Fig. 4).

For this study, we hope to determine the neurological bases for differences in performance of mothers in behavioral trials. Previous studies have shown that mutations in the FosB alleles can lead a mother to stop caring for her pups (e.g., Brown et al., 1996). Although some of our mothers only failed to care for pups during the experiments and care for pups in their home cage, this may be worth exploring. Additional, research has demonstrated that oxytocin (e.g. Sabihi, 2014) and estrogen (review by Bridges, 2015) play a role in maternal behavior. There are undoubtedly dozens of other candidate hormones to explore, but this is certainly a good place to start. We will examine expression in the pre-frontal

cortex – which we have seen plays a role in decision making (Unroe et al., in prep), the hippocampus – learning and memory (e.g., Nguyen et al., 2015), and the amygdala – emotional response (e.g., Bridges, 2015).

As described above, the findings of this project could potentially have a broad range of impacts. In addition to contributing to ongoing studies of the cognitive abilities of mother rats in the Franssen lab, we anticipate that other researchers could use this information to help determine the methodology of their experiments – even if they are not strictly studying parental behavior.

III. Proposed Methodology

Animals

A total of thirty (30) 65-75 day old, pregnant Sprague-Dawley rats (Taconic Biosciences, US) were singly-housed in plastic cages with ALPHA-Dri® bedding (Innovive, San Diego, CA). Rats were provided access to food (Teklad 2018, Harlan Laboratories, US) and tap water ad libitum on a 12/12 light cycle under standard housing conditions. Mothers were allowed time to give birth and care for her pups. The age of pup during testing ranged between 6-9 days old.

Maternal Behavioral

All thirty mother rats were tested in each of six groups, consisting of different ratios of OWN vs ALIEN pups. The OWN:ALIEN ratio groups used in this experiment were 8:0, 4:4, 3:5, 2:6, 1:7, and 0:8. Mothers were taken out of her home cage and placed in a new plastic cage with water. The mother was taken to a testing room and given 20 minutes to acclimate to her new cage environment. While mothers were acclimating and testing, pups were kept in their home cage and placed under a heat lamp to keep pup body temperature from dropping. During the acclimation period, pups were taken from their home cages and marked with an odorless marker. Pups were marked with either and X or II, depending on the day of testing, to indicate whether the pup was an OWN pup or ALIEN pup. Marked pups were placed in a Pyrex "pup cup" and kept under a heat lamp to keep the poikilothermic pups warm. After the 20 minute acclimation period, the "pup cup" was taken into the testing room and placed into the testing cage with the mother rat. Mother rats were given 20 minutes to retrieve and interact with the introduced pups. After the 20 minute testing time, the testing cage was removed and returned to the animal storage room. The mother was returned to her home cage and pups were returned to their respective cages. The Pyrex cups were sprayed with 70% ethanol, wiped with a paper towel, and dried in order to use the cup again in another trial without the presence of odor from pups of a previous trial.

Behavioral tests were controlled for time of day, age of pup, and source of alien pups. All alien pups came from a mother that was not being tested that day, as well as from a mother whose pups were not already used for the same test mother in a previous trial on that day. All animal procedures were approved by Longwood University's Institutional Animal Care and Use Committee.

Analysis of Maternal Behavior

The mother's behavior was recorded for 20 minutes to observe interactions with the pups, including latency to retrieve 1st pup, 4th pup, and 8th pup; time spent interacting with pups, including grooming, sniffing, nursing, and nesting; time spent self-grooming; and time spent not interacting with pups, including sleeping, drinking water, and other non-interaction activities (exploring cage, sitting, laying, etc.). In some cases, a mother would perform both an interaction activity as well as a non-interacting activity (e.g., some mothers would self-groom themselves at the same time that they were nursing the pups). In this instance, the non-interaction activity (self-grooming, sleeping) was counted as the primary activity. Based on these criteria, mothers were grouped as "good" or "bad" mothers.

Neural Tissue Collection

Fixed brain tissue for immunocytochemical analysis was obtained via transcardial perfusion. Rats were individually placed into an airtight chamber with 1 mL of Halothane gas (Sigma-Aldrich, Co; St Louis, MO) until respiratory rate slowed and animals were nonresponsive. Rats were then transcardially perfused with 100mL phosphate-buffered saline solution (PBS) followed by 100mL 4% paraformaldehyde solution. Brains were extracted and post-fixed in 4% paraformaldehyde overnight at 4°C, then transferred to a 10% Sucrose solution for 24 hours at 4°C, and were then stored in 20% Sucrose solution at 4°C until sectioning (at least 24 hours).

Brain sections were sectioned via cryostat (Microm HM525) at 40µm, then stained for neural activity using oxytocin, estrogen, and FosB antibodies (Santa Cruz Biotechnology; Dallas, TX). Based on previously published methodologies, immunohistochemistry for all markers was performed for visualization with DAB (Vector Laboratories, Burlingame, CA; Franssen et al., 2011). Tissues were incubated overnight in rabbit anti-c-fos primary antibody (1:10,000 ImmunoStar, Inc.; Hudson, WI) in phosphate buffered saline (PBS), washed, and incubated for 60 minutes in goat anti-rabbit biotinylated secondary antibody in PBS (1:1,500

Both proteins were visualized using Vector Avidin-Biotin Complex and 3–3′ diaminobenzidine tetrahydrochloride (ABC and DAB kits; Vector, Burlingame, CA). Stained cells were considered to be expressing the protein studied and analyzed using neuron counts under bright field microscopy.

Neural Data Collection & Analysis

Cells immunoreactive for estrogen, oxytocin, and FosB will be counted using a light microscope and ImageJ (https://imagej.nih.gov/ij/) software using the threshold function. Neurons were counted under double-blind conditions and compared using SPSS (IBM, Armonk, NY) after counting was completed. Significance was determined using Repeated Measures, Linear Mixed Models analysis.

IV. Resources and Locations

a. Key Resources
   i. Rat housing facility, food, and water
   ii. Rat behavioral mazes and equipment
   iii. Camera equipment and video analysis software
   iv. Perfusion pump (collection of brains)
   v. Cryostat (sectioning of brains)
   vi. Immunohistochemistry reagents & equipment (e.g., pipettes)
   vii. Slides and coverslips
   viii. Microscopes
   ix. Data analysis software

b. Locations
   i. All of the bench resources required for this experiment are located in Dr. Franssen's research laboratory Chichester 209/11.
   ii. Software programs such as Excel and SPSS are available on lab (Chichester 209), office (Chichester 304), and personal computers

V. Timeline for Completion

Summer 2015- Spring 2016

- Learn laboratory techniques/skills, including:
  a. Cryo-sectioning of brain tissue
  b. Immunohistochemical staining of brain tissue & creating microscope slides
  c. Data collection of both behavioral and neural data & Data analysis using SPSS

- Collection of data for 12 rats
  a. Conduct and quantify behavioral data
  b. Quantify mothers as "Good or Bad"
  c. Sacrifice and collection of brain tissue

Summer 2016

- Collection of data for 18 additional rats
  a. Conduct and quantify behavioral data
  b. Sacrifice and collection of brain tissue

NOTE: The above work will be conducted by PRISM student Teresa Fruchterman. Her project is to complete the project determining at which ratio mothers will treat an entire mixed litter of pups as her own. She will and Dr. Franssen will harvest the brains for my use in the fall.

Fall 2016

- Finish analysis of behavioral data
    a. Combine additional 18 rats with prior 12 rats to quantify behavioral data
    b. Quantify all 30 mothers as "Good or Bad" using statistical methods
        i. SPSS statistical software
        ii. Compare groups using Linear Mixed Models, Repeated Measures design

- Cryo-section brains
- Immunohistochemically stain neural tissue for: Oxytocin, Estrogen, & FosB
- Begin quantification of stained neural tissue
- Grant writing for antibodies
- Grant writing for conference travel
- Initial draft of SHR paper, including analysis of behavioral data submitted to Dr. Franssen by finals week

Spring 2017

- Continued neuro-quantification
- Statistical data analysis
- Second draft of SHR paper, submitted to Dr. Franssen by Spring Break
- Presentation of preliminary data at the SYNAPSE annual meeting in Clinton, SC
- (will serve as draft of oral defense)
- Completion of SHR paper
- Oral defense for SHR committee

VI. Anticipated Committee Members

R. Adam Franssen, Ph.D.	Associate Professor of Biology, Longwood University
Catherine L. Franssen, Ph.D.	Assistant Professor of Psychology, Longwood University
Erin D. Clabough, Ph.D.	Assistant Professor of Biology, Hampden-Sydney College

VII. Approval by the Department Chair (sent via email)

VIII. LITERATURE CITED

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IX. FIGURES

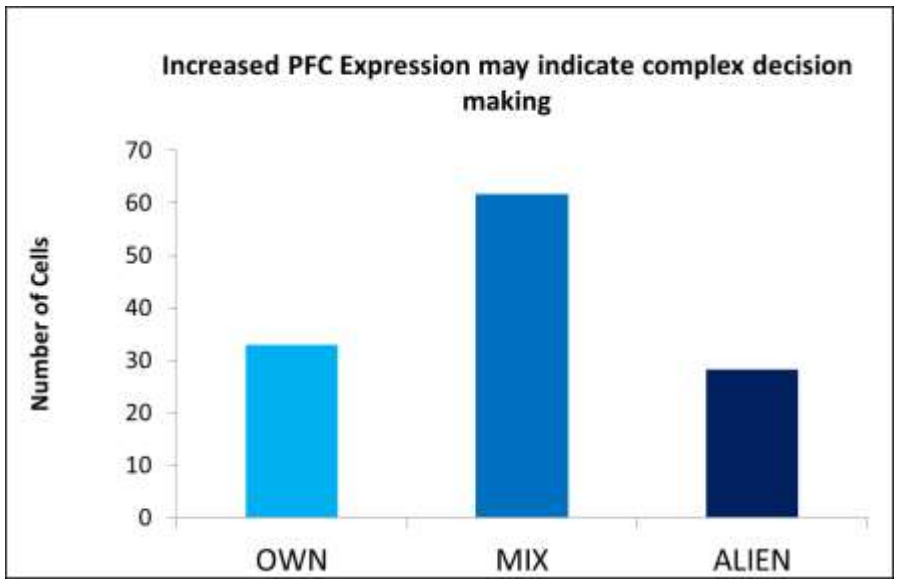

Figure 1. Preliminary data suggests that mother rats use complex decision making centers of the brain to determine whether to retrieve a mixed-litter of pups.

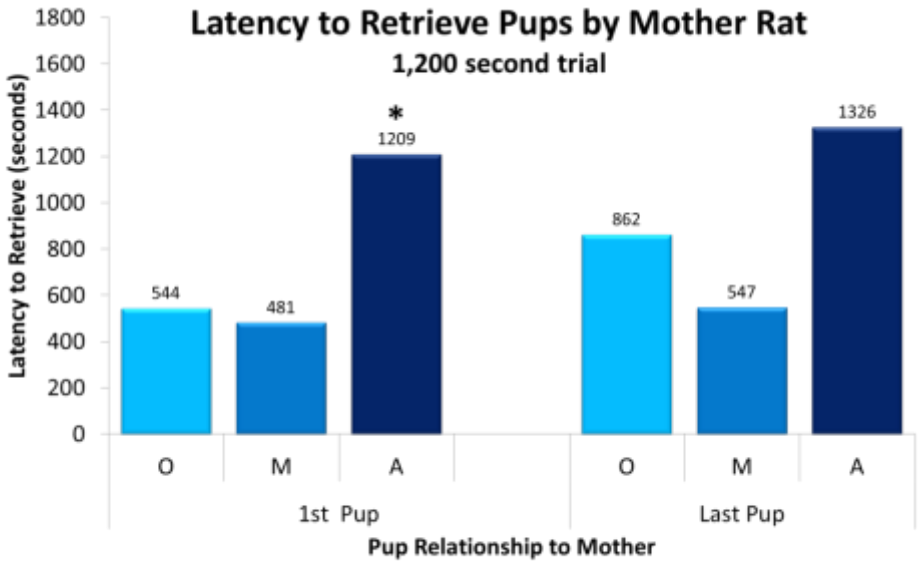

Figure 2. Mother rats will retrieve pups in a mixed-litter as quickly as if the entire litter consisted of only her pups, which is significantly faster than retrieval of a litter of only alien pups ($p<0.05$).

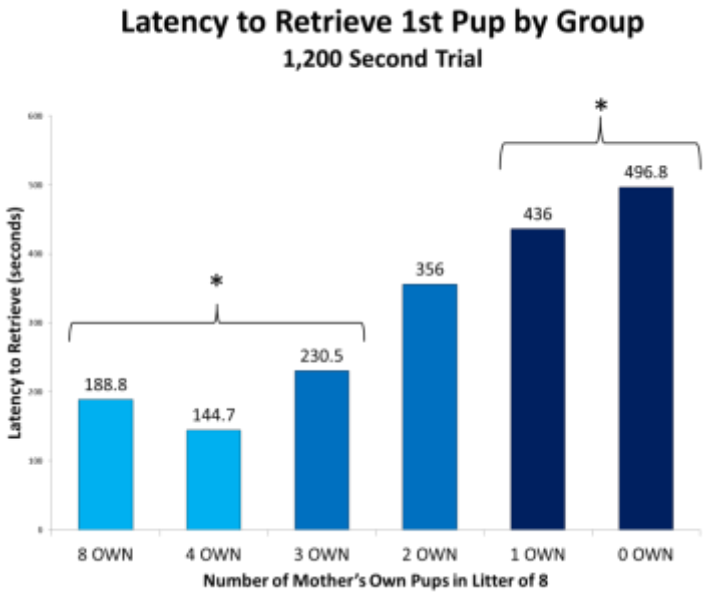

Figure 3. Mother rats will retrieve pups in a mixed-litter as quickly as if the entire litter consisted of only her pups, as long as there are at least 25% of her own pups in the litter. Mothers would retrieve pups in litters with 8, 4, or 3 of her own pups significantly faster than retrieval of a litter containing 1 or 0 of her own pups. Litters with 2 own pups were not significantly different than either the 3 own or 1 own groups ($p<0.05$).

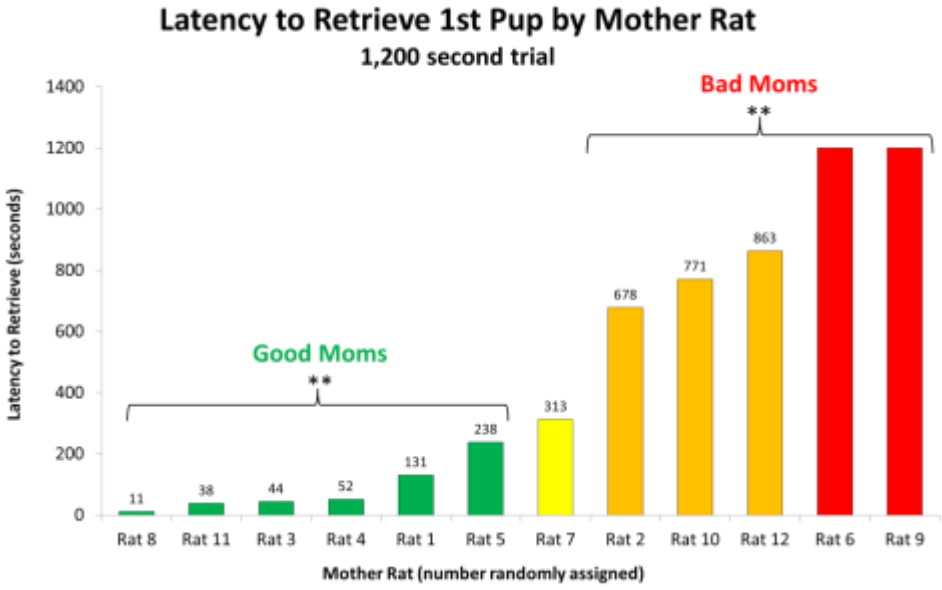

Figure 4. Evidence of "good" and "bad" mother rats. Some rats (shown in green; Good Moms), would quickly retrieve pups regardless of group. Other rats (shown in orange and red; Bad Moms), would retrieve slowly regardless of group. Rat 7 is shown in yellow; her performance was not significantly faster than Rat 2's, nor was it significantly slower than Rat 8's. Note that significance remains even if the rats in red (which never retrieved; Rat 6 & Rat 9) are excluded from the analysis ($p<0.01$).