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# Estrogen Receptor Levels Higher in “Bad” Maternal Rats than in “Good” Maternal Rats

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## Introduction

### Maternal Care

In mammalian species, parental care is critical for the survival as well as the mental and physical well-being of offspring (Dulac, 2014). This is particularly true in humans; researchers have long been interested in what the effects are of differences in parental upbringing, including the absence of parents (e.g., Cabrera et al., 2000) and differences in disciplinary parenting styles (e.g., Baumrind, 1967). Though tempting to think of all parents as "good" parents in both the human and animal worlds, that clearly is not the case; sadly, there are "bad" parents as well. For instance, cross-sectional studies in humans on parent-child relationships have shown that adverse and traumatic childhood experiences including neglect, abuse, or parental loss correlate to mood and anxiety disorders later in life (Bodensteiner, 2014). Moreover, such individuals have tendencies to form antisocial personalities and impulsive aggression. There are quantifiable physical effects of poor parenting as well - girls lacking proper parent-child relationships experience a variety of physiological and sociological changes vs. their "normal" counterparts, including 'decreased age at menarche, earlier onset of sexual behavior, and increased number of sexual partners.' These traits often have both physiological and psychological repercussions (Bodensteiner, 2014). In sum, early life experience, though relatively brief, are critical for molding the future of an individual's success and overall fitness to survive the demands of life (Anacker, 2013). Thus, it is especially important to understand factors that can lead to lower levels of parental care ("bad" parenting) than expected from "good" or "average" parental care. These factors may be sociological, psychological, environmental, or genetic and their effects in humans are not trivial. For example, postpartum depression affects more than 10% of mothers in the United States, potentially resulting in poor care for offspring that then lead to negative consequences described earlier (Dulac, 2014). Given the complexity of human interactions and conditions, researchers can attempt to examine the factors that affect maternal behaviors in a model rat system - allowing us to control for more variables - in hopes of better understanding the factors that affect maternal behaviors in humans.

Pup Retrieval, Good and Bad Moms

Recent findings in our lab suggest that mothers more quickly retrieved their own pups vs alien (Figure 1). In this case, we use children at a park as an example. If there is a child in distress, such as when he/she falls off the swing, then a parent is going to retrieve the child faster if the child is theirs. If given enough time with no parent in sight, another parent may retrieve the child and make sure he/she is okay. In this anecdotal case, as well as research in our lab, suggests that parents will eventually retrieve a child, or pup, in distress, even if the pup is not the offspring of the original parent (Figure 1). In addition to this retrieval behavior that we see, our lab has also discovered that rats make use of the medial prefrontal cortex (mPFC) to make complex decisions to care for another pup or not (Figure 2).

A recent study in our lab has demonstrated that within a population of mother rats, there are some mothers that retrieve incredibly quickly, while some mothers take much longer to retrieve or do not retrieve at all (Unroe et al., in prep; Figure 3). Interestingly, this striking dichotomy of maternal behavior was statistically significant, even when non-retrieval mothers were excluded from the data set. Therefore, it is important to determine what neurological factors are driving these different maternal behavior in maternal rats. These factors are most likely the hormones of pregnancy.

There are a number of hormones, including estrogen, prolactin, and progesterone, that have been implicated in the regulation of maternal behavior. Not surprisingly, these hormones are part of the dramatic changes that take place in a pregnant female (e.g., Kinsley and Lambert, 2006; 2008; Franssen et al., 2012). Estrogen has been shown to be a key regulator of the onset of maternal behavior (Bridges, 2015) and a recent study suggests that, without estrogen, mother rats no longer express maternal behavior (Murakami, 2016). In this study, the ovary of the rat was removed, preventing endogenous estrogen from being produced. These rats were shown to not demonstrate any maternal behavior toward their pups. To further support the hypothesis that estrogen in require for mothers to perform, estrogen was given to the ovariectomized mothers, and maternal behavior returned to normal levels (Murakami, 2016). Therefore, we indirectly measured estrogen in this study by staining for the alpha subunit of the estrogen receptor (ERα).

The regions of the brain whose roles have been elucidated in the regulation of maternal behavior are the hippocampus, associated with learning and memory (Nguyen et al., 2015); the prefrontal cortex, which is the site for decision making (Winn, 2001), the medial

preoptic area, which is implied in the onset and maintenance of maternal behavior (Winn, 2001); and the amygdala, which regulates emotional response (e.g., Bridges, 2015). Therefore, these are the regions which will be analyzed in this study because of their implications as regions that regulate maternal behavior.

Although maternal behavior is used in a variety of studies, there are few studies that focus on the dichotomy of the quality of rat mothers, as seen in our "good," fast retrieving mothers and their "bad," or slow retrieving counterparts. Here, we investigate whether estrogen plays a role in the determination of whether a mother rat will retrieve and care for her pups quickly, which would make her a "good" mother.

## Methods

### Animals

Thirty 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 2014S, Harlan Laboratories,US) and water ad libitum. Animals were kept on a 12-12 light dark cycle. After delivery of litters, maternal rats were housed with pups for approximately one week to ensure survival and health of the pups. The age of pup during testing ranged between 6-10 days old. All animal procedures were approved by Longwood University's Institutional Animal Care and Use Committee.

### Behavioral Protocol

All thirty mother rats were tested in each group, consisting of different ratios of OWN (pups from testing mother) vs ALIEN (pups from another mother) pups. The OWN:ALIEN ratio groups used in this experiment were 8:0, 4:4, 3:5, 2:6, 1:7, and 0:8. Each mother rat was moved from her home cage to the test cage and allowed to acclimate for 20 minutes. 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 cup and kept under a heat lamp. After the 20 minute acclimation period, test litters were placed into the testing cage with the mother rat. Mother rats were then 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 was sprayed with 70% ethanol, wiped with a paper towel, and dried to allow the use of the cup again in another trial (Figure 4).

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 rat in a previous trial on that day.

Behavioral Analysis

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, which can serve as an anxiety measure; and time spent not interacting with pups, including sleeping drinking water, and other non-interaction activities (exploring cage, sitting, laying, etc.) (Figure 5). 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 Preparation and Staining

After all tests were conducted, 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 via tail pinch. Then, rats were 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 tissue was kept refrigerated at 4°C at all times.

Brains were cryosectioned (Microm HM525) at 40µm, then stained for neural activation of the alpha subunit of the estrogen receptors (ERα). Immunohistochemistry for ERα, oxytocin, and fosB as performed for visualization with DAB (Vector Laboratories, Burlingame, CA) as previously described (Franssen et al., 2017). Tissues were incubated for 6 hours in rabbit anti-ERα primary antibody (1:1,000). ImmunoStar, Inc.; Hudson, WI) in phosphate buffered saline (PBS), washed, and incubated for 10 minutes in goat anti-rabbit

biotinylated secondary antibody in PBS (1:1,500). ERα were visualized using Vector Avidin-Biotin Complex and 3–3′ diaminobenzidine tetrahydrochloride (ABC and DAB kits; Vector, Burlingame, CA). Once stained, brain tissue was mounted on a microscope slide using Permount.

Neuroquantification

After brain tissue was fixed on a microscope slide, the slide was placed under a microscope for imaging to determine the number of ERα or oxytocin present in each region. Using a microscope camera, images of anterior medial amygdala (MeAD), the hippocampus (CA1), the medial prefrontal cortex (mPFC), and the medial pre-optic area (MPOA) were obtained (Figure 6). Then, a 481x400 pixel box was drawn in order to establish a consistent parameter for neuron counting to occur. Lastly, the image was upload to the NIH ImageJ program to accurately count the number of neurons in the indicated area of the brain. For images that were stained too lightly for ImageJ to quantify, images were hand counted.

Behavioral

Mother rats were described as "Good", "Average", or "Bad, based on analysis of their behavioral data. Specifically, we grouped rats in two ways: 1) latency to retrieve first pup and 2) mothers that retrieved, groomed, and nursed pups. Those data were compiled and a Chi-Squared analysis was used to determine into which category of motherhood. We then compared the brains of animals in each group.

For each section of brain, an ANOVA was used to determine if there were statistical differences among the groups, followed by post-hoc T-tests significant behavioral differences between groups for both latency to retrieve data and mother-pup interaction data.

**Results**

First, the number of ERα neurons were counted in the CA1 region of the hippocampus (Figure 7A). These data indicates that the average number of ERα neurons in the CA1 of good, average, and bad mothers, were 12.04 ± 1.09, 19.49 ± 0.89, and 18.57 ± 0.72 neurons, respectively (mean ± SEM). A One-Way ANOVA and post hoc tests revealed that there was a significant difference between good moms and both average and bad mother rats ($p<.01$),but there was no difference between average and bad mothers ($p<0.42$). These data suggest that there is a higher expression of estrogen receptors in the brains of slow retrieving mothers (Figure 7A).

Next, the number of ERα neurons in the MPOA was analyzed (Figure 7B). It was determined that the average number of ERα neurons in the MPOA of good, average, and bad mothers were 19.33 ± 1.23, 24.95 ±0.85, and 28.73 ± 1.51 neurons, respectively. These data indicate that there is a significant difference between good and bad mothers ($p<.01$), good and average mothers, ($p<.01$), as well as bad and average mothers ($p=0.043$). These data indicate that there are more estrogen receptors in the medial preoptic area in the mothers that take longer to retrieve their pups (Figure 7B).

Third, the number of ERα neurons in the CG1 region of the prefrontal cortex was analyzed (Figure 7C). Our data indicates that the average number of ERα neurons in the CG1 of good, average, and bad mothers were 21.75 ± 1.85, 20.38 ± 0.62, and 23.24 ± 1.07 neurons, respectively. These data indicate that there is higher estrogen receptor in bad mothers compared to average mothers ($p<.0.01$), but that there is no difference between estrogen receptors in good and average ($p=0.373$) as well as good and bad mothers ($p=0.50$) in the prefrontal cortex (Figure 7C).

Lastly, we analyzed the MeAD region of the mother rat brains (Figure 7D). Our data indicates that the average number of ERα neurons in the MeAD of good, average, and bad mothers were 15 ± 1.15, 21.23 ±0.85, and 25.23 ± 1.27 neurons, respectively. These data suggest that there is a significant difference between good and bad mothers ($p<.01$), good mothers and average mothers ($p<.01$), as well as average mothers and good mothers ($p<.01$). Our data suggest that there is more estrogen receptor expression in the medial amygdala of mother rats that are "bad," or take longer to retrieve their pups (Figure 7D).

*In Progress*

In addition to the ERα, our lab is currently working on imaging and counting the oxytocin stained tissue. The lab will also begin the immunocytochemistry for fosB on the remaining brain tissue. The same regions mentioned above will be counted in the same manner to determine the number of oxytocin and fosB immunoreactive.

**Discussion**

In our initial study, we made the striking discovery that not all mother rats behave in the same way in response to introduction of a litter of pups (regardless of the pup group composition; Figure 3). We used latency to retrieve the first pup in a litter to establish three separate groups of maternal care: "good," average, and "bad" and demonstrate that those

behavioral groups are significantly different from one another (Figure 8). Here, we further investigated the neurobiological, factors underlying those behavioral differences.

The results of the current study suggest that "bad" mother rats possess a higher number of estrogen receptors in brain regions relevant to maternal behavior than "good" mothers (Figures 7). This result was surprising in that we originally anticipated that there would be an increase in receptors in "Good" mothers compared with "Average" or "Bad" mothers. We hypothesize that the increase in the number of these receptors in these slow retrieving mothers due to upregulation of estrogen receptors as a result of a deficiency of the estrogen hormone in these rats. Receptor upregulation can occur when a neuron does not receive enough input signals from the presynaptic neuron (Figure 9; Winn, 2001). Thus, it appears that mothers with more estrogen in their system (i.e., "Good Moms") have fewer estrogen receptors, whereas those mothers that are estrogen deficient (i.e., "Bad Moms") would have more receptors in an attempt to find estrogen. To test this hypothesis, future studies can determine if slow retrieving mothers have less endogenous estrogen by testing for the hormone directly, as opposed to the receptors, which were examined in this study.

Here, our data indicate the estrogen plays an important role in maternal behavior. However, our lab is interested in looking at other candidate molecules that are associated with maternal behavior. Other hormones, including oxytocin, have been implicated in social bonding, especially in mate bonding and maternal bonding, to support reproductive function. Specifically, oxytocin has been shown to be necessary for social bonding between and mother and offspring (e.g., Feldman et al., 2007; Matthiesien et al., 2001; Galbally et al., 2011). For instance, when oxytocin is pharmacologically blocked, then mothers rats no longer express maternal behavior (van Leengoed, 1987). Conversely, if virgin female sheep are infused with oxytocin, then these females express maternal behavior toward lambs, which typically does not occur in virgin female sheep (Kendrick, 2004).

In addition to estrogen and oxytocin, other factors have been shown to affect maternal behavior. For instance, studies have shown that mutations in the FosB alleles can lead a mother to stop caring for her pups (Brown et al., 1996). Both of these will be investigated to determine the role they play in the making a mother rat a "good" mom or "bad" mom (Brown, 1996; Sabihi, 2014). Further study could also include prolactin and progesterone, which may be contributing to the maternal behavior pathway as well (Sheehan, 2002).

Our findings also have implications in a clinical trial setting. Understanding maternal behavior is important because other studies measure maternal behavior as a response variable to experimental treatments. First, clinical trials are routinely conducted to determine the effect of a particular drug or treatment on the brain and those studies begin in lab animals like rats (Figure 10). By better understanding drug interactions in rats, we can make predictions about how the drug might affect the human brain (Figure 11). For instance, several studies on addiction (e.g., Frankfurt et al., 2011; Haydari et al., 2014) and hormone expression (e.g., Lonstein et al., 2014) use maternal behavior as a behavioral measure. In addition, translational pre-clinical trials utilize maternal behavior. Examples include: Vitamin D present in rat pups (O'Loan, 2007), hyperactivity in rats separated from pups (Aisa, 2007), teratogenic effects of maternal antidepressant (Forcelli, 2007), and contraceptive usage on maternal rats (Liu, 2010). Second, basic understanding of behavior-brain interactions provides researchers with the best possible chance of potentially find biological mechanisms to rescue bad behavior (such as environmental enrichment; e.g., Johnson et al., 2013).

Based on our results, it may be more important than previously understood to include as high a sample size as possible to account for the differences between "good" and "bad" mothers (Figure 3). The results of studies in our lab are important to guide the methods of other studies using maternal behavior as a measure.

Overall, our study suggests that estrogen plays a role in maternal behavior, as our data indicate differences in ERα immunoreactive neurons in "good" mothers versus "bad" mothers. The quality of motherhood, and overall parenting, is important in the development of animals and humans (Dulac, 2014). Further, the quality of care that is given to offspring can also affect the future generations. For example, one study suggests that mothers that are "high licking and grooming" – "good" mothers in our case – have offspring that are also "high licking and grooming" parents. The same is true for "low licking and grooming" rats ("bad" mothers) (Weaver 2004). Therefore, it is important for us to understand what is necessary to possess positive maternal behavior. Further research in our lab hopes to understand what molecules play a role in the regulation of maternal behavior, which can have a significant impact of the offspring and further generations.

**Acknowledgements**

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## Figures

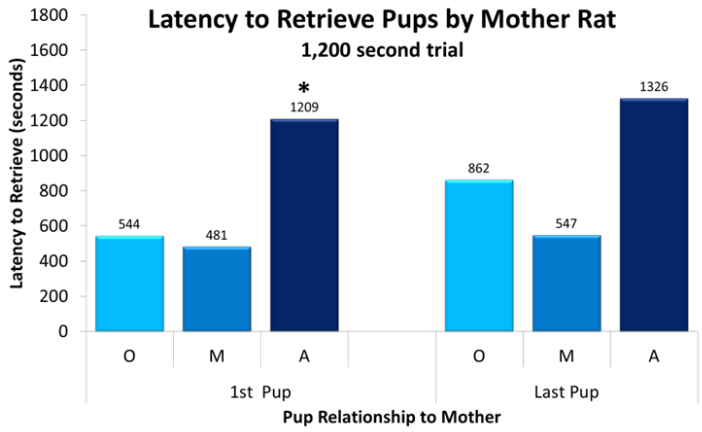


**Figure 1**. Mother rats will retrieve pups in a mixed-litter as quickly as if the entire litter consisted of only her pups, which is significantly faster than retrieval of a litter of only alien pups ($p<0.05$). OWN (O) represents mothers that were given all their own pups, while MIX (M) represents mothers given 50% her own pups and 50% pups from other mothers. ALIEN (A) mothers were given all pups from other mothers.

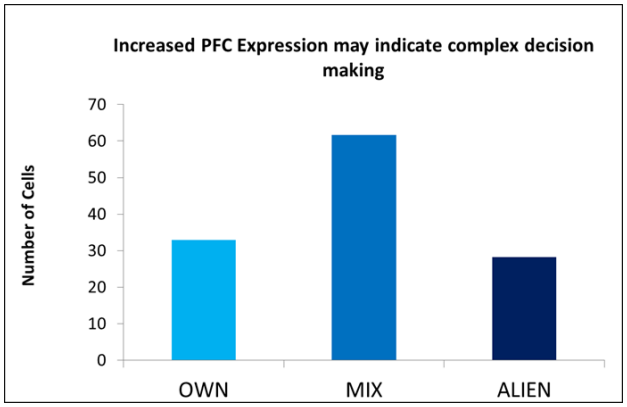


**Figure 2**. Preliminary data suggests that mother rats use complex decision making centers of the brain to determine whether to retrieve a mixed-litter of pups. OWN represents mothers that were given all their own pups, while MIX represents mothers given 50% her own pups and 50% pups from other mothers. ALIEN mothers were given all pups from other mothers.

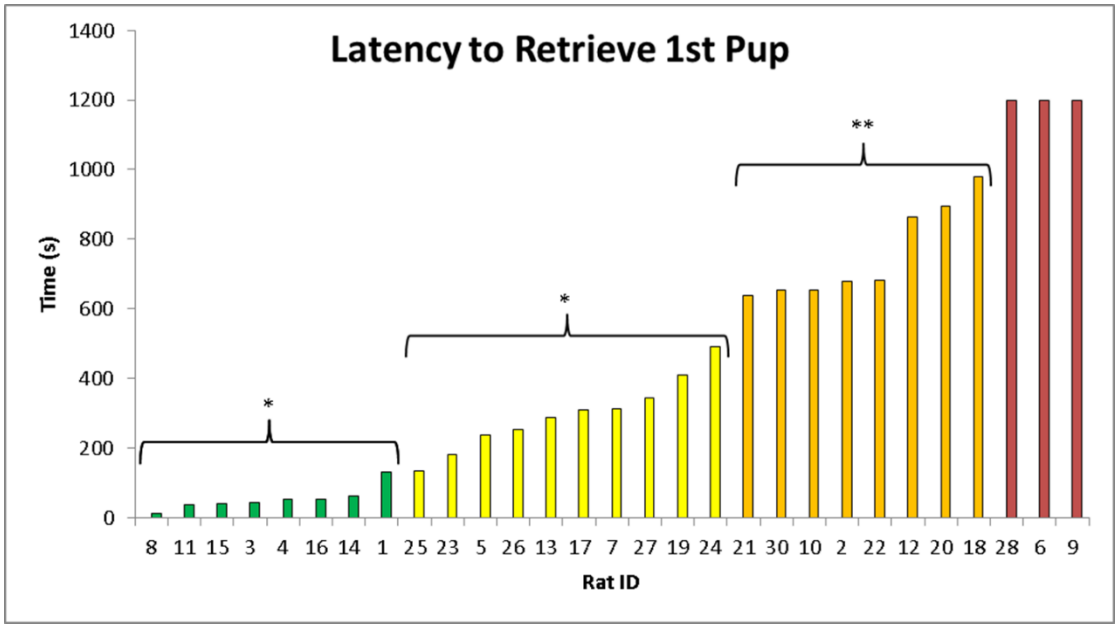


**Figure 3**. Evidence of "good" and "bad" mother rats. Some rats (shown in green; Good Moms), would quickly retrieve pups regardless of group. Average mother rats, shown in yellow, differ significantly in behavior compared to both good and bad mothers. Other rats (shown in orange and red; Bad Moms), would retrieve slowly regardless of group. Mothers in red did not retrieve pups in the course of our 20 minute study. Note: Bad mothers are still significantly different from average and good mothers when the non-retrieval rats are excluded from the data set.

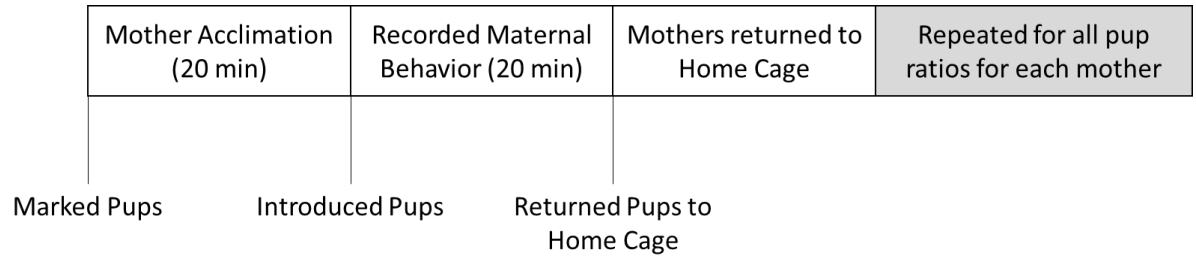


**Figure 4**. Visual representation of behavioral protocol.

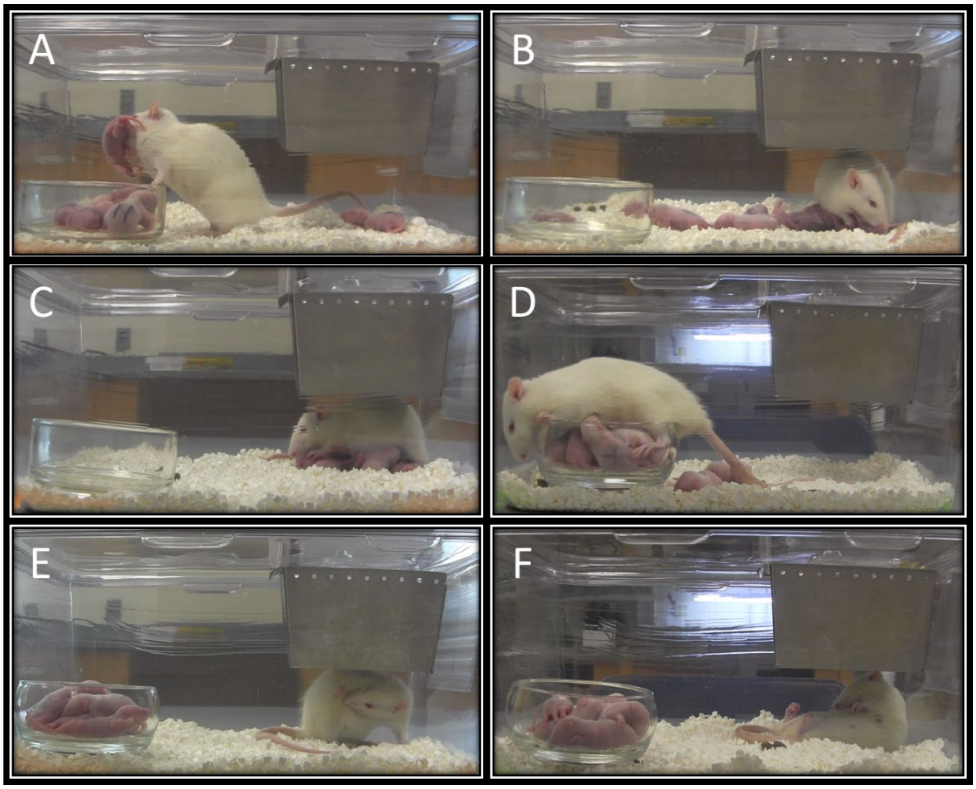


**Figure 5**. Rat mothers were measured in terms of latency to retrieve and number of pups retrieved (A), grooming (B), and nursing pups (C&D). Surprisingly though, some mothers were quick to care for pups, others were not. Rat mothers were also scored on the amount of time exploring the cage and spent self-grooming (E), and sleeping (F). The more time spent on these behaviors, the less time spent on pup care.

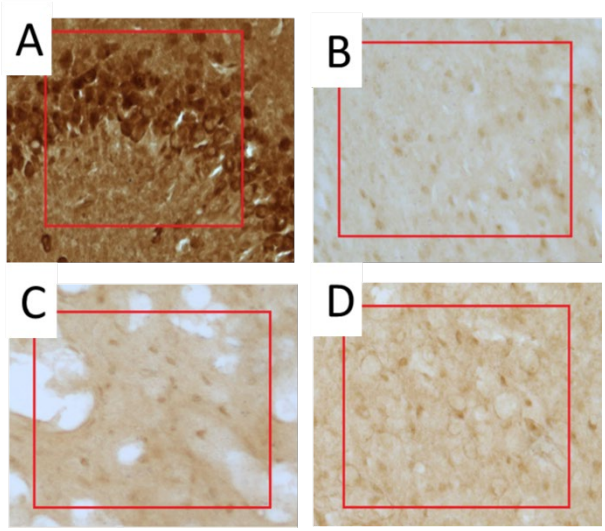


**Figure 6**. Representative images of each brain region that were used in this study, which are A) hippocampus – learning and memory, B) medical preoptic area – onset of maternal behavior, C) PFC – decision making, and D) MeAD – maternal anxiety.

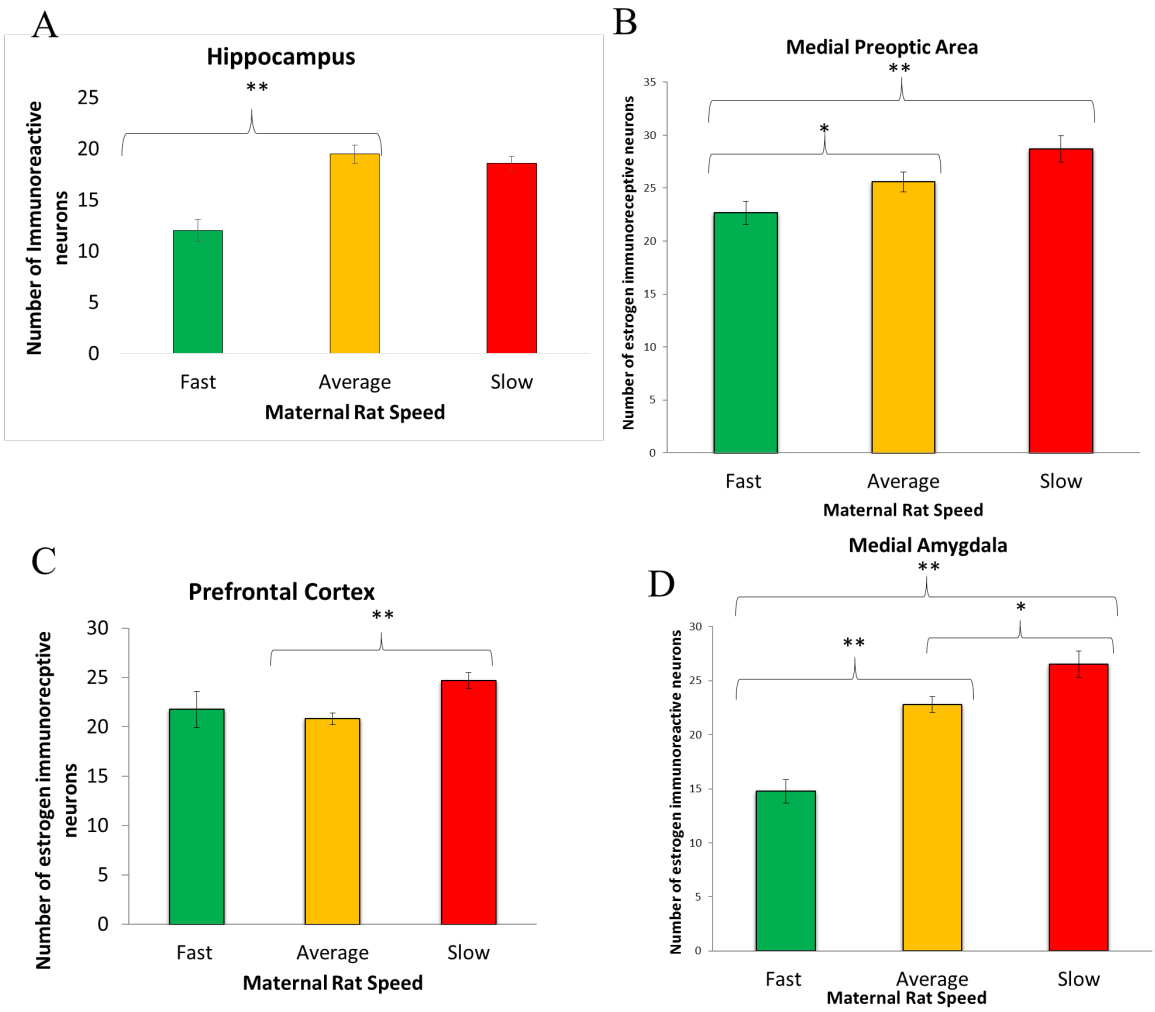


**Figure 7.** The number of ERα neurons in each brain region for each type of mother. Here, "good," or fast retrieving, mothers are shown in green, "average" retrieving mothers are shown in yellow, and slow retrieving "bad" mother rats are shown in red for the hippocampus (A), medial preoptic area (B), the prefrontal cortex (C), and the medial amygdala (D). * signifies a significance of $p<0.05$, while ** signifies $p<0.01$.

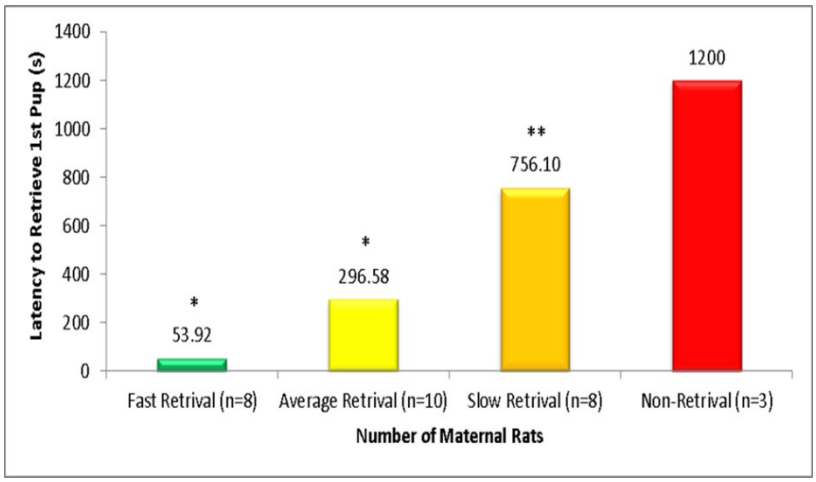


**Figure 8**. Evidence that there is a statistically significant difference between fast retrieving "good" mothers, average mothers, and slow retrieving "bad" mothers.

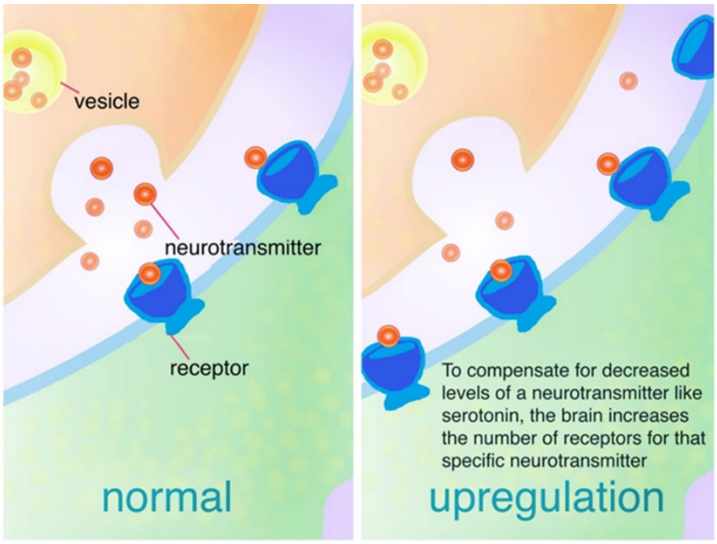


**Figure 9**. Visual representation of the concept of upregulation. In the case of this study, we hypothesize that "bad" mothers have an increased number of estrogen receptors in response to low amounts of the endogenous estrogen hormone. (Modified from Reynolds, 1999)

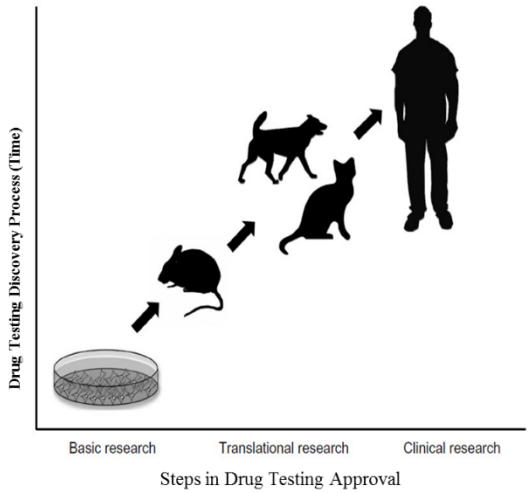


**Figure 10**. Visual representation of the stages of drug trials and drug discovery. These translation research studies often utilize behavior, sometimes maternal behavior, to determine if a novel drug treatment will alter behavior. (Cekanova, 2017)

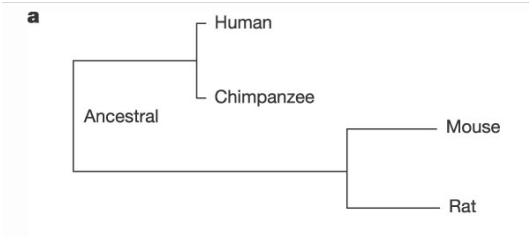


**Figure 11**. Phylogenetic tree which demonstrates the close relationship between humans and our rat model organism. (Modified from Waterson, 2005)